NIMD

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NIMD FORUM 2002

—The study of fetal methylmercury exposure and children development—

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Contents

Preface

I appreciate for the attendance of honored guests, distinguished and participants to this meeting.

It is my great pleasure to be with you here and address you, on behalf of National Institute for Minamata disease, at the opening of NIMD forum 2002.

First of all, I would like to thank all of you, especially Prof. Myers and Prof. Davidson who have traveled a long way from the US for this forum.

Our Institute holds an international conference every year on mercury pollution and health effects. This year we planned to have a forum on the study of fetal methylmercury exposure and children development.

As you know, it has been over 45 years since the first case of Minamata disease was reported in Japan. Since then, a lot of efforts have been put to solve the problem. Concerning the relief of the patients, political decision was made in 1995 to solve the issue ultimately. Effort has also been done to clean up the polluted marine environment. Nowadays, we believe that no situation and condition exists for causing Minamata disease.

Although we do not see any evidence of mercury pollution in Japan, there are still environmental pollutions reported in other countries, and also, a new subject for discussion is coming up. The problem of developmental effects of low dose methylmercury exposure has been a subject of debate in these days.

Our Institute has conducted the experiments with animals on this theme. From this year, Ministry of the Environment and National Institute for Minamata disease started an epidemiological research, which is, to our regret, rather behind the foreign epidemiological studies. Our Institute needs to strengthen information exchange with the experts in the world to improve the quality of the research, and to prepare for international discussion on the subject.

I do hope that this forum will be valuable opportunity for the purpose.

Nov. 17, 2002

Dr. Ryo Nomura
Director General
National Institute for Minamata Disease

Welcome Address

I appreciate Dr. Ryo Nomura, the director general of NIMD and his excellent staffs for setting NIMD Forum 2002 in Tokyo and for giving me an opportunity to say a few words at the beginning of this Forum.

On behalf of Ministry of the Environment, I would like to thank all of you, especially Prof. Myers and Prof. Davidson from the University of Rochester Medical Center, for your participation to the Forum.

Today's topic, human health effects of fetal methylmercury exposure, is one of the greatest concerns among the world now. FDA and EPA have issued advisories on fish to women and children last year, and UNEP Chemicals is undertaking a process to develop a global assessment of mercury and its compounds to be presented to the UNEP Governing Council in 2003.

Lifestyle and diet of Japanese people have changed from the previous decade, but we are still mass consumers of fish in the world. According to National Survey on Nutrition, the average of the fish consumption per day is about 100g. To investigate the effect of fetal low-dose methylmercury exposure, Japanese Government and NIMD started a cohort study in Miyagi Prefecture, in the north of Japan from this year. It is appropriate that this study has been chosen for today's topic.

I hope this Forum would bring us the most meaningful opportunity and promote understanding of scientific assessment on the design of cohort studies for fetal methylmercury exposure.

Thank you very much for your attention.

Dr. Kazuko Kamiya
Director
Special Environmental Diseases Office
Ministry of the Environment.

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University of Rochester School of Medicine and Dentistry

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University of Rochester School of Medicine and Dentistry

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Studying Neurotoxic Exposures in Children: Methylmercury as a Prototype

Philip W. Davidson and Gary J. Myers

University of Rochester School of Medicine and Dentistry

Studies of neurotoxic exposures in humans may be conducted for numerous reasons. The most common are for scientific or for health outcome purposes. The designs of such studies are quite different. Clinical studies for health outcome reasons do not involve hypotheses and are concerned with a site-specific exposure that is suspected to cause health effects. The sample is almost always confined to the group of individuals who were exposed and the statistical analyses are designed to confirm or disprove a dose-response or dose-effect association.

Scientific studies on the other hand are typically more complex to design and conduct. They test a hypothesis and require attention to numerous details to be certain the outcomes are relevant:

- The Hypothesis
- Exposure Effects
- Experimental Design
- Sampling the Population
- Exposure
- Measurement Issues
- Covariates
- Analysis Issues

This paper will review each of these issues using methyl mercury as a prototype. The emphasis will be on neurodevelopmental outcomes in infants and children.

The Hypothesis

Theory

The formulation of the hypothesis is the first issue to be considered in designing any study of associations between neurotoxic exposures and neurodevelopmental outcomes. The hypothesis will dictate not only the experimental design, but also will directly determine sample size and composition and many aspects of the statistical analysis. Is the hypothesis exploratory or confirmatory? Exploratory studies are designed to provide evidence for an association between an exposure and its effects on child development. There may be little direct evidence for the association at the dosage or dosage range in question, and the specificity and sensitivity of the effect may be unclear or unknown. Such hypotheses must be tested with sufficient sample size and power to detect an association and should use a liberal statistical rejection rule that is biased toward providing protection against a Type II error (failing to reject a null hypothesis when it is actually false). Confirmatory studies in contrast are designed to test the details of an association that has already been demonstrated through preliminary exploratory studies. The sample required

for a confirmatory study will need to cover the exposure range suspected to demonstrate the expected effect and its size and power will need to provide biased protection against rejection of a true null hypothesis. Thus, the statistical rejection rule will be conservative. The hypothesis also should take into account the expected relationship between exposure and effect. Whether the expected association is linear or non-linear will determine the type of analysis plan required to test the hypothesis.

Example

The Seychelles Child Development Study (SCDS) was confirmatory. The study was designed to test the hypothesis that levels of MeHg exposure reached by fish consumption might adversely affect child development. This hypothesis came from data in Iraq on 83 mothers with prenatal exposure from consumption of MeHg treated seed grain (Marsh et al., 1987). Some of these children were cases of poisoning. A dose-response curve, shown in Figure 1, determined that fetal exposure to MeHg measured in maternal hair growing during pregnancy of around 10 ppm might adversely affect development (Cox et al., 1989). It was known that this level of prenatal exposure could be achieved by fish consumption. However, exposure in Iraq was not from fish consumption. Additionally, there were very few subjects with exposure under 50 ppm in maternal hair with adverse neurodevelopmental effects and the presence of some subjects with very high levels of exposure (over 600 ppm) might have skewed the data.

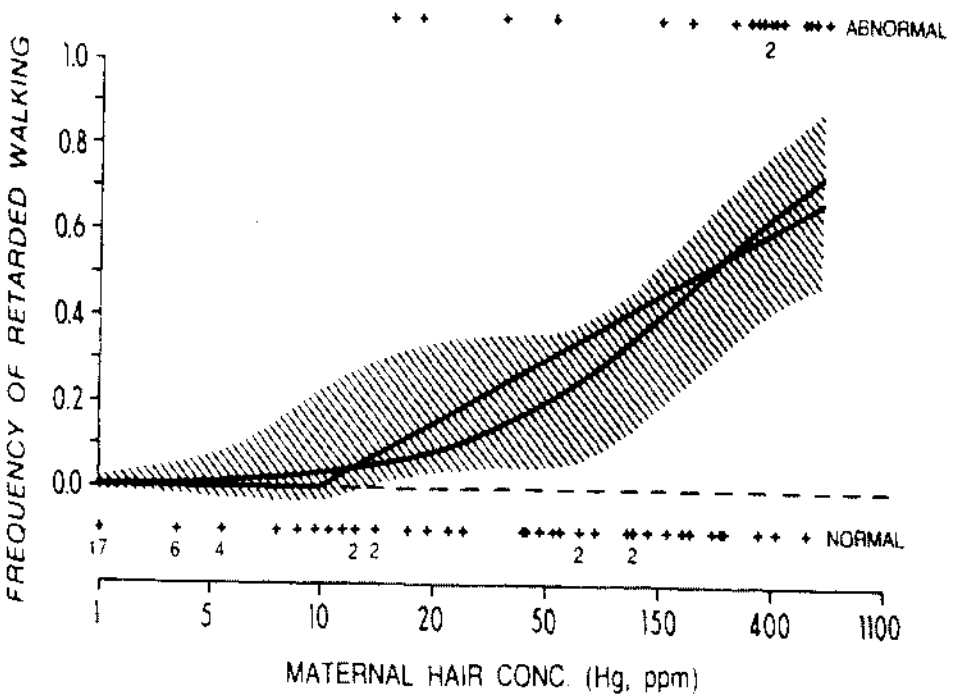

Figure 1. Hockey Stick Dose-Response Plot from the Iraq Study (Cox, et al., 1989).

There are millions of people around the world who consume fish as their primary source of protein. Consequently, testing the hypothesis directly seemed eminently feasible.

An appropriate population with daily fish consumption was found in the Republic of Seychelles and the SCDS was begun (Marsh et al., 1995). The hypothesis being tested by the SCDS was that exposure to MeHg from fish consumption during pregnancy (prenatal exposure) is associated with subtle adverse neurodevelopmental outcomes in children.

Exposure Effects

Theory

What are the expected effects on neurodevelopment? Neurotoxicants can have devastating global effects on neurodevelopment, such as mental retardation, seizures, or cerebral palsy. However, these effects more often follow high dose exposures to toxins such as methyl mercury. Lower doses would be expected to have more subtle effects. These effects might affect one or many domains of development, such as sensory deficits, motor coordination problems, or specific learning disabilities. Likewise, the timing of the exposure might lead to very different effects. For example, the fetal brain is believed to be more sensitive to methyl mercury exposure than the postnatal brain, The neuropathological effects of prenatal exposure are global in nature while postnatal effects are not well described, but are believed to be intermediate between those of fetal and adult exposure. Postnatal exposure might affect sensory and motor systems but not global functions such as intelligence. Measuring specific developmental effects require different endpoint than measuring global effects. The timing of the expected dose-effect during the developmental period will determine whether assessment at one or several ages will be required. Child developmental test procedures differ from one age to another. Exploratory studies typically are designed to have fewer controls while confirmatory studies require more extensive control.

Example

The SCDS was designed to determine if prenatal exposure adversely affects a child's neurodevelopment. Neuropathological studies from Minamata (Harada, 1968; Choi et al., 1978) indicated that prenatal exposure disrupted neuronal migration and cortical development in a global manner. We therefore expected global effects. Despite this, the test battery was designed to examine a variety of neurodevelopmental domains.

Experimental Design

Theory

Exploratory studies often employ cohort designs, while group designs may be more useful for confirmatory studies. In a cohort design, children are typically enrolled during a time-limited enrollment period and then tested for exposure and outcome, either at one particular age, or at several ages. A group design typically involves enrollment based upon target ranges of dose, and of timing, magnitude and type of expected effect. In the case of methyl mercury, confirmatory studies are difficult to undertake since the threshold for exposure effects at lower dosages is not known yet. But if one hypothesized for example that the actual threshold were 30 ppm in maternal hair, then a confirmatory study using a group design might limit membership in the reference group to < 10 ppm and in the

exposure group to over 40 ppm. Such a design might also employ more groups. Group designs do not permit enough precision to detect the nature of an association across a dosage range. Such an association is better tested using a cohort design, which allows enrollment of a large number of subjects who represent a wide exposure range.

Designs can be cross-sectional or longitudinal. If exposure to a neurotoxicant is expected to cause a static effect on development, and the timing and earliest age at which the effect is detectable is known, then a cross sectional design may be useful. This is rarely the case. The longitudinal design is more useful when timing and stability of the exposure effect over time are not known, when the influence of other factors, such as exposure to other toxins is present, or when factors that might mitigate exposure effects are unclear. The longitudinal design is generally more powerful. Cross-sectional designs are less useful in testing the neurodevelopmental effects of methyl mercury, since too little is known about the nature and timing of exposure effects, especially at lower dosages. On the other hand, longitudinal studies are much more expensive, and it is more difficult to maintain the cohort and control the study.

Example

The SCDS utilized a longitudinal cohort design. Finding a suitable population for a study of methyl mercury is challenging. Populations with exposure in the postulated toxic range of 10 ppm (measured in maternal hair) are difficult to find. We selected the Seychelles Islands primarily because the average citizen including women of childbearing age consumes fish daily. When the main cohort was enrolled during 1989 and 1990, the average mother consumed fish with 12 meals each week and the mean maternal hair MeHg level during pregnancy was approximately 6 ppm (Shamlaye et al., 1995). Despite the daily consumption of fish by nearly all mothers, there was a wide range of prenatal exposure (1-27 ppm) among them providing a large control group. We have looked for a population with higher exposure from fish consumption, but have been unable to find one with sufficient subjects for a study.

Although the design of the SCDS was longitudinal, the mean age of the children examined in Iraq was 30 months and a dose-response curve was found in that population (Cox et al., 1989). Consequently, we expected to find neurodevelopmental effects during the first three years of life. However, experimental data suggested that late effects might occur, so the study was designed to follow the children to age five. Each mother-child pair was examined 4 times during the first five years of life.

Sampling the Population

Enrollment

Theory: There are generally two methods to achieve enrollment. The investigator may define a specific start point and end point and then enroll all eligible subjects who are available during the enrollment window. Alternatively, the enrollment period may be unlimited and enrollment may continue until a sufficient number of subjects have been identified. The first approach is referred to as closed enrollment and the latter, as open enrollment. Closed enrollment will produce a cohort more quickly and permit testing within a narrow age window in a relatively short space of time; open enrollment will require more time and will allow testing over a wider window. Cohort effects, defined as events

unrelated to the experiment that may act as confounders, are more likely with closed enrollment.

Example: Enrollment in the SCDS main study occurred during a specific age window. Children were enrolled during their sixth month of life (the target age was 6.5 months of age +/- 2 weeks). A pediatric neurologist did the examinations, so enrollment was limited by the presence of the examiner in the Seychelles when the child reached that target. The target for the number of subjects was approximately 750 or half of the approximately 1500 children born in Seychelles during the year enrollment occurred (Myers et al., 1995).

Inclusion and Exclusion Criteria

Theory: Defining inclusion and exclusion criteria *a priori* is an important tool to control for confounders (factors that alter either the exposure or the child's performance on endpoint measures). Typically inclusion criteria are specific rules for eligibility, such as age, exposure, and developmental status at the time at the time of enrollment. Exclusion criteria would include disorders or environmental events that affect child development independent of exposure to methyl mercury, such as perinatal trauma, prenatal infections, maternal substance abuse, maternal illness during pregnancy, known causes of mental retardation, or postnatal trauma to or infection of the brain.

Example: In the SCDS, the main study inclusion criteria included being Seychellois and reaching the age of 6 months during enrollment. Exclusion criteria were defined *a priori* and are shown in Figure 2.

Figure 2. Exclusion criteria used in the SCDS.

Maintaining the Cohort

Theory: In long-term longitudinal studies, loss to follow-up may occur. Strategies to reduce or eliminate large-scale loss to follow-up may include providing stipends to participants, or making special efforts to communicate with cohort members during lulls in the study. Replacement rules may be needed if the subject drop out is too great to assure sufficient power at the end of a longitudinal study.

Example: In the Seychelles the loss to follow up has been small. The island is small and tracking families is relatively easy. In addition, the population is very cooperative and generally interested in having their children evaluated. Our strategy of having a senior nurse who manages the project also contributed to retention. The project manager is able to stay in touch with families informally, as well as on the occasions of evaluations. Overall loss to follow up in the SCDS has been less than 8% over nearly 13 years as shown in Figure 3.

Maintaining the Cohort

	Age at Testing (months)				
	6.5	19	29	66	107
Enrolled	779	740	738	736	735
Exclusions	39	2	1	2	18
Final Cohort	740	738	737	734	717

Figure3. Children Participating in the SCDS Cohort at Each Age of Testing after Exclusions.

Tracking Non-Enrolled Subjects

Theory: Ascertainment bias may occur when subjects are voluntarily recruited from a larger population pool. To measure the bias, it may be important to determine the characteristics of subjects who elected not to participate in the study.

Example: The SCDS was able to obtain information from the Ministry of Health and children's health records on those eligible children who did not participate in the study. There were no significant differences between those participating and those who did not (Shamlaye et al., 1995).

Measurement Issues

Measuring Exposure

Theory: Neurodevelopmental effects may be related to a large number of exposure variables. These include the actual dose (effects may be seen only after some threshold is exceeded), the type of exposure (e.g., chronic versus bolus doses), timing and duration of exposure (e.g., prenatal versus postnatal exposure and delayed toxicity), presence of multiple, additive exposures, and the exposure to mixtures of several neurotoxic compounds. These factors must all be taken into account when exposure is measured.

Example: In the SCDS we elected to measure prenatal exposure in maternal hair growing during pregnancy. It is relatively easy to collect, can recapitulate exposure during the entire pregnancy and the entry of MeHg into hair appears to be similar to its entry into the brain (Clarkson, 2002). Therefore it appears to reflect the brain exposure better than other biomarkers such as cord blood. In the Seychelles, PCBs are below detectable levels, lead levels are generally low (Davidson, 1998) and pesticide and other toxins are consistently low.

Measurement of Neurodevelopmental Outcomes

Theory: The hypothesis will drive the selection of endpoint measures. Different measures will be appropriate depending upon whether global cognitive or domain-specific subtle effects are predicted. Measurement of global deficits and some specific deficits can usually be accomplished with standardized intelligence tests. Experimental tasks may be required for domains where standardized tests are not available or applicable. Likewise, different tests are applicable to different ages. The older the child, the more complex the measures can be. Some modification of tests and tasks may be necessary when testing takes place in locales outside the test's standardization parameters, e.g., using a test constructed for administration in English in a locale where English is not the primary language. When modification is required, field testing will typically be required to establish the performance characteristics of the translated or modified version and normative scaling may no longer be applicable. Raw or un-scaled scores will be applicable in such cases.

Example: In the SCDS our hypothesis suggested that neurological and global cognitive tests would be most likely to be affected. Exposure in Iraq was associated with developmental and neurological endpoints. Data from Japan suggested that prenatal exposure affected the brain globally. The testing design for the SCDS Main Study is shown in Figure 4.

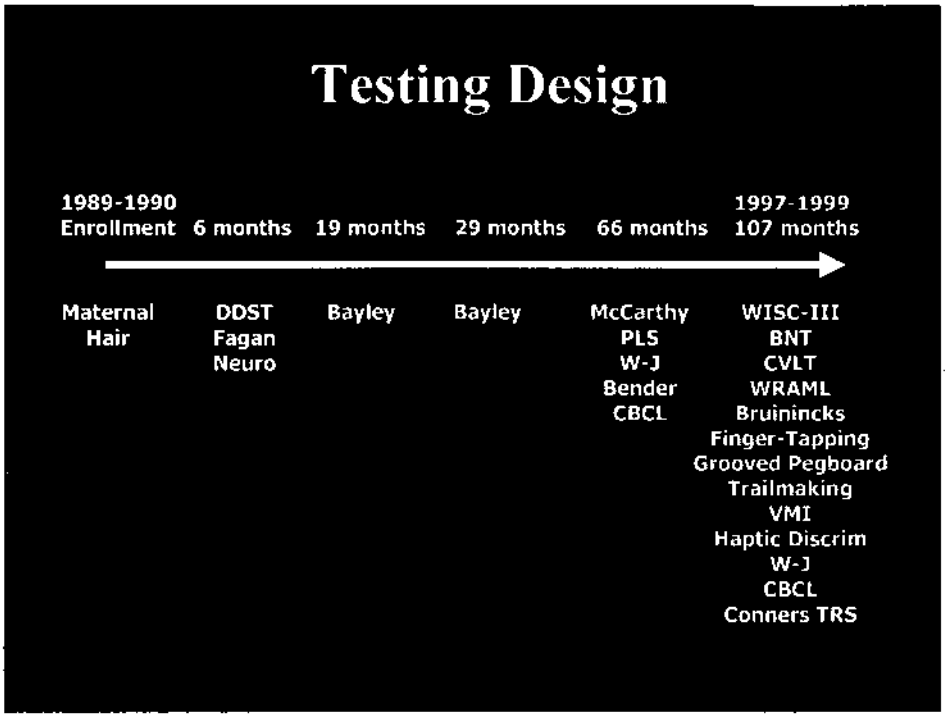

Figure 4. The Testing Design for the Main Study.

Sensitivity and Specificity

Theory: The degree to which a test will detect a true positive effect is called its sensitivity. The degree to which it detects false positives is its specificity. Sensitivity is depicted in the cartoon in Figure 5. The biologic function in this cartoon is measured by a test of its behavioral effect. A test with ideal sensitivity detects the behavioral phenotype across the full range of expression. Typically, we do not achieve this level of sensitivity so we miss some portion of the phenotypic effect, usually at the low end of the range of expression. Specificity is depicted in Figure 6. One test or task may separate the actual phenotype from others, while another task may not. Both of these measurement characteristics must be (but often are not) taken into account as tests are selected for use in toxicological studies.

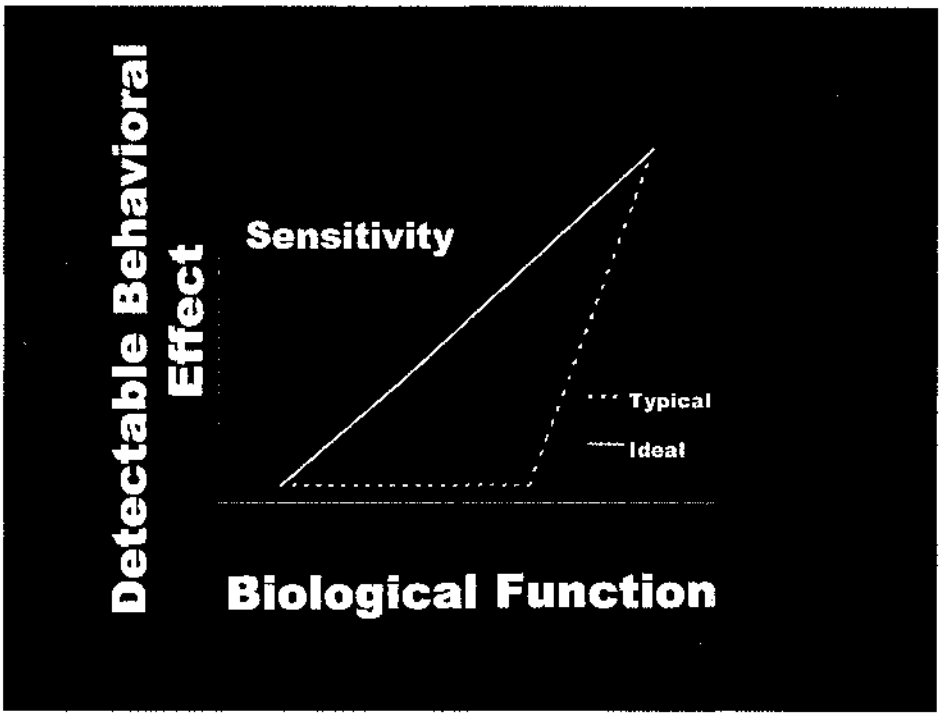

Figure 5. Sensitivity.

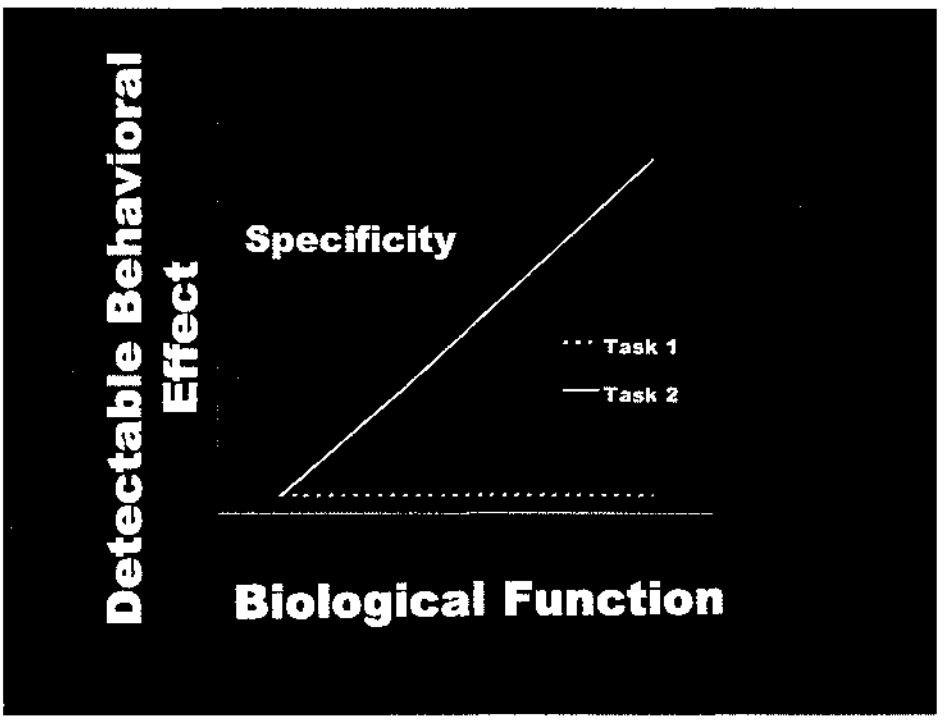

Figure 6. Specificity.

Example: Most Seychellois speak some English, but the language in daily use is Creole. Consequently, the tests such as the Bayley Scales of Infant Development and the Wechsler Intelligence Scale for Children that utilize language were translated into Creole and administered in Creole. Each test was first piloted on subjects who were not part of the Main cohort and the data analyzed to determine if the test was functioning appropriately in Seychelles (Davidson et al., 1994). For analysis raw scores were used for most tests.

Training and Reliability for Test Administration

Theory: Administration of any measure of a child's abilities by a tester carries with it the possibility of variability in administration. This may be minimized with professional training in psychological test administration, and with experience administering the specific tests included in the protocol. However, using trained and experienced testers may not always be possible. In addition, local testers may have better rapport with subjects and be more facile with language issues. In such cases, training in both psychological testing and in administering the protocol tests should be provided to the testers. Differences in tester performance may occur when more than one tester is used or when the same tester administers tests over a long time. In such cases, it is advisable to report inter-tester reliability. Two methods should be used: First, for a small but representative subset of cohort children, the same child can be tested by a primary tester while being observed silently by a second tester. The independently recorded scores of both testers are then correlated. Another complementary procedure, sometimes called *Gold Standard* reliability, involves the first tester being observed by an expert tester and the independently recorded scores of the two are correlated.

Example: In the SCDS, the design called for multiple testers, since there were too many children per week to be tested by only one examiner. We chose professionals with experience in evaluating children and provided practicum training in administration of the specific tests included in the battery. Prior to completion of their training, each tester was required to demonstrate 100% agreement with the psychologists on the study team who were experts in administration of the test battery.

To assure that testing fidelity, both between testers and within testers was maintained over the course of each examination period, we used both gold standard and inter-tester reliability. In addition, a small number of protocols were checked for scoring reliability.

Blinding

Theory: Both testers and investigators should be blinded to the individual exposure levels of cohort children. Failure to blind these individuals may lead to biases in the resulting test results. If the cohort is being followed longitudinally, parents and other caregivers must also be blinded to the exposure levels to prevent cohort effects.

Example: The SCDS has been careful to blind every participant on the clinical side from knowing individual Hg values. This includes the families, the Ministry of Health and anyone in Seychelles and the clinical investigators who travel to Seychelles. The Ministry of Health placed confidence in the study team and agreed that if adverse effects were identified, the project would notify them immediately. Consequently only group information regarding Mg values has been available in Seychelles and to the clinical investigators.

Covariates

Theory

Factors that modify the exposure, or independently influence child development are co-variates or confounders. Factors that modify the association between exposure and child development are known as effect modifiers. The cartoon in Figure 7, modified from Gordis (1996, p. 71) depicts how these factors can influence the outcome of epidemiological studies. Figure 8 illustrates how covariates might affect the outcome of developmental neurotoxicological studies of methylmercury. Exposure modifiers might include other chemicals such as selenium or micronutrients in fish that may affect the amount of methyl mercury actually reaching the brain, or genetic or metabolic factors. Factors that affect development may occur during the prenatal, perinatal, or postnatal period, and include maternal IQ, socioeconomic status (SES), stimulation at home, maternal or child medical or mental illnesses or preexisting conditions that independently influence brain function or behavior such as exposure to toxins other than MeHg, sensory loss, or factors that might affect the child's test performance, such as experience with computers (if they are required in testing). Effect modifiers may influence both the independent and the dependent variable, such as social factors that might differentially alter fish consumption depending upon the SES or the maternal IQ of the mother. Some confounders may be controlled by the natural environment (e.g., all children enter and leave school at the same ages, all children are immunized), while others may have to be controlled for by experimental design modifications or by co-varying them in the statistical analysis. Yet other confounders may be uncontrolled, unknown, or immeasurable, creating unavoidable error variance.

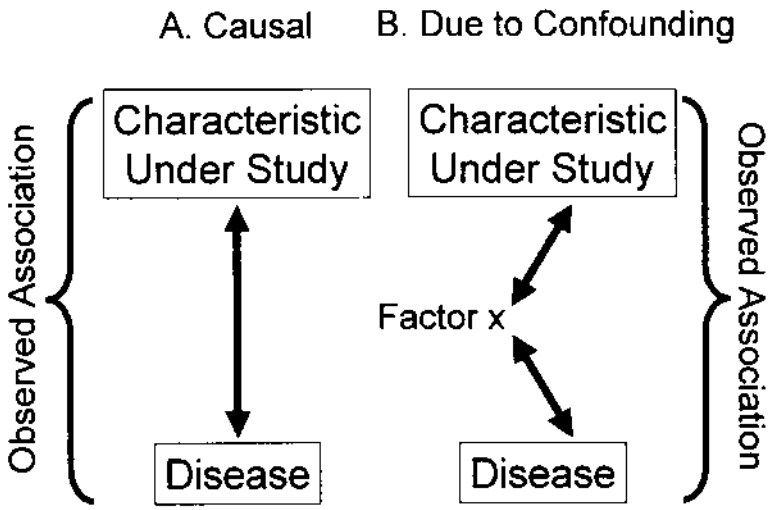

Figure 7. Covariate effects. Modified from Gordis (1996, p. 71).

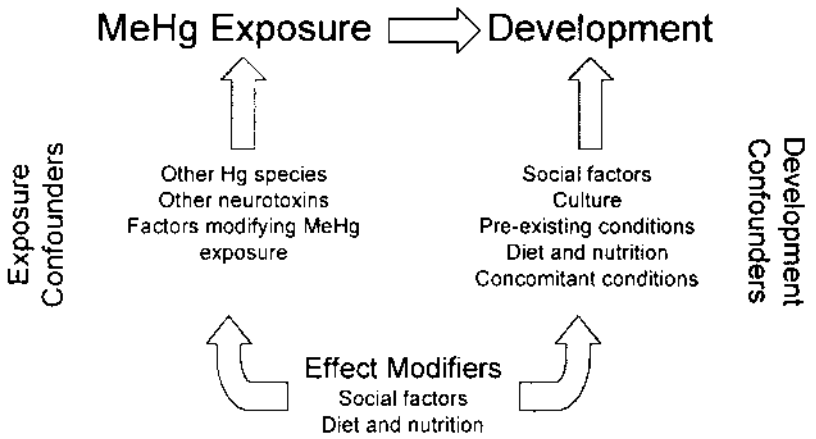

Figure 8. Illustration of the Impact of Covariates on the Association between a Predictor and an Outcome in Methylmercury Studies.

Example

In the SCDS covariates that affect the mother, environment and child were all considered. These are presented in Figures 9 and 10.

Figure 9. Maternal and environmental covariates measured in the SCDS.

Figure 10. Child covariates measured in the SCDS.

Analysis Issues

General Principles

Theory: Analysis plans for most developmental neurotoxicology studies will require multivariate statistical procedures, because most designs involve the need to account statistically for the influence of factors other than the independent and dependent variables. This means that the investigator must decide upon the constituents for each statistical model, known as parameters. Specification of parameters should be done *a priori*, i.e., there is a rational theoretical basis for their inclusion independent of how they are operating in the experiment at hand. The alternative is *a posteriori* parameters. These are included in the model based upon how they operate in the particular experiment at hand, typically assessed by a direct statistical test. Statistical consultants may differ on their preference for one approach versus the other, and each approach has pluses and minuses.

The theoretical framework of the study and the hypotheses should drive decisions about modeling strategies and setting the α level as noted earlier.

Example: In the SCDS the analysis plan was developed prior to the Main study. The primary analysis consisted of multivariate linear or logistic regression with exclusion of outliers and examination for influential points. Each model also included a gender by mercury interaction because of earlier reports that males are more sensitive to the adverse effects of methyl mercury (McKeowen-Eyssen, 1989 Marsh et al., 1987). Because the study is confirmatory, we have used a two-tailed p value of <0.05 as the level of significance.

Linear Versus Non-linear Models

Theory: Most human behaviors are non-linear functions. However, there is a much greater range of statistical theory and procedures that addresses linear modeling compared to non-linear modeling. Thus, most developmental neurotoxicological studies employ linear modeling for the primary analysis. Never-the-less, non-linear modeling may be indicated theoretically. If so, linear procedures should also be employed. .

Example: The SCDS has used linear models for the primary analysis, but has also done secondary analyses with non-linear models. We examined the evaluations at 5.5 years of age with generalized additive models (Axtell et al., 1998).

Primary Versus Secondary Analyses

Theory: Models that involve multiple comparisons can generate significant *p* values by chance alone and some of these values will be spurious by chance alone. Some investigators address this issue by confining their primary analyses only to those tests that are essential to test the primary hypothesis. Other, less well-founded hypothetical associations may be tested in separate, secondary analysis. In such cases, the primary analysis remains the principal finding of the study, irrespective of the outcome of secondary analyses. Secondary analyses may be performed to clarify primary results or to generate new hypotheses. The assumption of independence of measures made by most statistical procedures would be violated if a secondary analysis is used to replace the primary analysis results.

Example: In the SCDS numerous secondary analyses have taken place (Myers et al., 1997; Palumbo et al., 2000;Axtell et al., 2000). However, to date these have all supported the accuracy of the primary analysis results.

Statistical Outliers

Theory: All multivariate statistics test the hypothesis that the actual data conform to the shape of a theoretical association between the independent and dependent variable, once data points have been adjusted for the effects of confounders. Some specific data points may not fit the theoretical line, either because they do not reflect the assumed relationship between independent and dependent variable, or they are spurious by chance alone. These points are known as statistical outliers (points that lie outside the model line by some predetermined quantifiable magnitude, usually > 3 SD). Some statistical procedures automatically adjust for such points but many do not. The greater the number of outliers the less likely the model will be significant. For significant models with a small number of outliers, the nature of the outliers is of some importance. Exactly what did each score reflect and how did it influence the model is of theoretical interest and should be investigated.

Example: In the SCDS outliers have been examined routinely and the results, as well as the figures are reported with and without their inclusion. To date the inclusion or exclusion of outliers has not change the significance of any association.

Concluding Remarks

In this paper we have tried to outline critical issues related to the conceptualization, conduct, and analysis of data from large-scale epidemiological studies in developmental neurotoxicology. We have used the SCDS as an example of how one research team planned and designed a longitudinal epidemiological study. Defining criteria for inclusion/exclusion, covariates to be measured and having an a priori analysis plan are important aspects of the study. Epidemiological studies require extensive planning and attention to detail. However, many issues are beyond the control of the investigator. This makes it even more important to define and control those aspects that the investigator can influence.

One important message should be that such studies are very complex and require a substantial degree of detailed thought prior to collecting the first data point from the first child. Failure to carry out such careful planning will nearly always lead to design or analysis flaws that will undermine the validity of the study. The second message should be that epidemiological studies, because of their size and complexity, require substantial fiscal and human resources. They also require long periods of time to complete. It is therefore important to choose one's target questions carefully and comprehensively. The third message is that epidemiological studies may not provide confirmatory data, and cannot prove a negative. It is very important to strive for convergence of results among many studies before basing public policy decisions on such studies.

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Current Hair Mercury Levels in Japanese: Survey in Five Districts

Akira Yasutake*, Miyuki Matsumoto, Masako Yamaguchi and Noriyuki Hachiya

National Institute for Minamata Disease, 4058-18 Hama, Minamata, Kumamoto 867-0008, Japan

Abstract

To understand the current Japanese hair mercury levels we planed the survey of hair mercury among general populations of different regions in Japan. Present paper, as the first report of the survey, summarized results obtained in five districts, Minamata, Kumamoto, Tottori, Wakayama and Chiba. Hair samples were collected at beauty saloons, barbershops and primary schools in each district with questionnaires on age, sex, amount and species of fish usually consumed, hair-dyed and artificial hair waving "*permanent wave*". Total mercury levels of 3686 hair samples collected were analyzed by an oxygen combustion-gold amalgamation method. The geometric mean of total mercury concentration was significantly higher in male than in female, i.e., 2.55 μg/g and 1.43 μg/g, respectively. The sex difference was also observed on hair samples without the artificial waving, i.e., 2.64 μg/g and 1.64 μg/g, respectively. The geometric mean in each district varied from 2.23 to 4.79 μg/g for males and from 1.23 to 2.50 μg/g for females. The average hair mercury levels were highest in Chiba among five districts both in males and females. A multiple regression analysis revealed a significant correlation of the mercury level with age, sex, amount of daily fish consumption, tuna and bonito as usually consumed fish, artificial waving and Chiba as residential area. In the laboratory experiment, we found that treatment of hair samples with a lotion for artificial waving caused a 30%-reduction in the mercury content. Furthermore, longitudinal hair analysis showed marked difference in the concentration between hair root and tip of the hair taken from artificially waved females; higher values were observed at the hair root. These results suggested that artificial waving significantly remove hair mercury and that hair analysis at the hair root should be necessary to estimate an accurate methylmercury exposure for waved persons.

Introduction

Human hair is well known as an excellent marker for methylmercury (MeHg) exposure, and its mercury levels have been frequently analyzed in cases of accidental mercury pollution, such as in Minamata and Niigata in Japan, and in Iraq, to estimate human exposure. WHO (1990) has reported, based on the data in Minamata, Niigata and Iraq, that no health effect was observed with the hair mercury levels below 50 μg/g for adults. However, since fetus is most vulnerable to MeHg toxicity, the report also suggested that the levels of pregnant women should be kept less than 10 μg/g. The United States Environmental Protection Agency (EPA) postulated 0.1 μg/kg-body weight/day as a reference dose (RfD) of methylmercury, an ingestion dose limit that is considered to cause no adverse health effect to human, including sensitive subpopulations (1997). Since the EPA's RfD had been based on a benchmark analysis on data from the acute exposed population in Iraq accident (Cox et al. 1989), the United States National Research Council (NRC) reevaluated the RfD level as a scientifically justified level. NRC pointed out that its elucidation should be based on the recent data obtained from the cohort study currently conducted in Faroe Islands, Denmark (2000). The EPA's RfD level corresponds to a hair mercury concentration 1.0 μg/g. However, the hair mercury levels of most general population are expected to be higher than 1.0 μg/g in high fish-consuming countries such as Japan. Accordingly, it would not be adequate to employ the EPA's critical level as it is in Japan. Although it may also be necessary to establish the revised critical level in Japan, very limited information, such as the current exposure level of mercury among general population, is available.

We planed to analyze hair mercury levels of general populations in Japan over varying ages. Since amount and species of fish consumed were expected to be different among districts in Japan, we selected five sampling areas considering the geological characters. The analytical results may be useful as a basal data for determination of the critical hair mercury level in Japan.

Method

Hair sampling

Hair samples were collected at beauty saloons and barbershops in Minamata, Kumamoto, Tottori, Wakayama and Chiba with questionnaires on fish consumption (amount and species), age, sex, hair-dyed and artificial hair waving "*permanent wave*" during 2000 to 2002. The hair samples were also collected at primary school in each district to supply samples from children. The age of participants was distributed from

0 to 95, and 92.4% ranged between ages 5 and 74. This study had been approved by review of the institutional ethical board.

Mercury analysis

For mercury analysis, the hair samples collected, 0.1 to 1 g from each person, were washed with detergent well, and rinsed two times with acetone to dry. The dried hair was cut to small pieces (< 2 mm) with scissors. Aliquots of samples (15 to 20 mg) were dissolved in 2N NaOH with heating at 60 °C for 1 hr. Ten or twenty μl of the solution was used to analyze total mercury levels by oxygen combustion-gold amalgamation method using an atomic absorption detector MD-1 (Nippon Instrument, Co., Ltd., Japan). 2.5 nM mercuric chloride (0.5 μg Hg/ml) in 0.5 M L-cysteine/0.2% BSA solution was used as external standard.

Effects of artificial hair waving

Effect of artificial waving lotions was examined using hair samples collected from 4 female Minamata citizens who had no artificial hair waving. The hair samples were treated up to 3 times with Volutis Elastine N (Nippon Loreal, Co. Ltd., Tokyo), a waving lotion commonly used in beauty saloons and barbershops, according to a procedure recommended by the manufacture at 2 to 3-days intervals. After each treatment, mercury levels in the aliquots (c.a. 1 mg) of the hair samples were determined directly (without dissolving in 2N NaOH) using the analyzer above. To examine further an effect of the artificial waving on hair mercury level, 10 pieces of whole hair strands were collected from 136 adult women (≥20 years old) in Minamata City with a questionnaire on artificial hair waving. The hair samples thus obtained were adjusted at the roots and cut in 1-cm sections. Mercury levels at each section (0.5 to 1 mg) were analyzed as above.

Statistical analysis

Because hair mercury concentrations were distributed in a lognormal profile, Student's t-test was performed on the mercury content data after logarithmic conversion. Multiple regression analysis was conducted by using SPSS statistical package (SPSS Japan Inc., Tokyo). The amount of daily intake of total fish and shellfish was estimated from the serving frequency of any fish or shellfish and the amount of fish and shellfish consumed in each serving.

Results

Distributions, range and geometric mean of the mercury level of 3686 hair samples collected in five districts are summarized in Fig 1 and Table 1. Because the mercury concentrations were apparently distributed in a lognormal manner as shown in Fig.1, geometric mean was used as a representative for hair mercury levels instead of arithmetic mean. Hair mercury concentrations were distributed at higher range in total male population (n=2020) with a geometric mean of 2.55 μg/g than in total female population (n=1666) with 1.43 μg/g. If the analysis was restricted within data from participants without artificial hair waiving, a geometric mean was 1.64 μg/g for females or 2.64 μg/g for males. On the other hand, 1.24 μg/g and 1.97 μg/g were obtained from artificially waved females and males, respectively. The sex difference in the geometric mean was statistically significant on each of the three comparisons ($P<0.001$).

Age-dependent alteration of the mercury levels showed a similar feature through five districts as shown in Fig. 2. The male levels increase in an age-dependent manner up to 50's or 60's. However, further aged generations showed somewhat lower levels. On the other hand, the age-dependent alteration in female levels was not so marked as in males, especially in certain populations such as Minamata and Kumamoto (Fig. 2a, b). Interestingly, the female levels showed a transient reduction at 20's, which was not shown in the males. The sexual difference became apparent at ages older than twenty.

The average mercury level of the hair sample varied from 2.23 to 4.79 μg/g for males and from 1.23 to 2.50 μg/g for females among the different sampling sites (Table 1). The geometric mean was significantly higher in Chiba, 4.79 μg/g in males and 2.30 μg/g in females, than in others ($P<0.001$). The age dependent distribution profile obtained in Chiba, as in Fig. 2e, was slightly different from those of other four sampling sites representing a relatively higher exposure level to methylmercury in most age classes of Chiba. Table 2 indicated the results of multiple regression analysis. The analysis revealed that the mercury content was significantly correlated with several covariates such as sex, age, the amount of daily intake of total fish/shellfish, tendencies of eating certain fish including tuna or bonito, and artificial waving ($P<0.001$). A residence site Chiba was still one of the significant determinants for hair mercury level even after the adjustment of these main variables.

Table 3 indicates the cumulative frequency of individual hair mercury content among the total population. About the half of the population possessed the hair mercury contents that exceed 2 μg/g, and about 1/10 of males and less than 4% of females exceeded 5 μg/g. On the other hand, only 17% of the total population, 28% of

females and 8 % of males, had hair mercury levels below the EPA-recommended 1 μg/g. On the subsample of reproductive aged females (15 to 49 years of age), 66% of the participants exceeded the EPA-recommended level, and approximately 2% was above 5 μg/g. It was also found that the hair levels of small portions of the subject population, 0.4% females for example, were more than 10 μg/g, which is a critical hair mercury level with possible adverse effects on developing fetus *in utero* (NRC, 2000).

The lower levels in the adult females might be partly due to artificial hair waving. A frequency of the hair waving above 20's in each district was about five folds higher in females (44.6 to 68.7%) than males (8.4 to 17.1%). Yamamoto and Suzuki (1978) demonstrated that thioglycolate in the artificial waving lotion effectively removed hair mercury. To make sure we treated hair samples from non-artificial hair waved women up to 3 times with waving lotions that were commonly used in Japanese beauty saloons. More than 30% of the hair mercury was removed by single treatment of the lotions (Fig. 3). Repeated treatments further removed the hair mercury. Removal of a portion of hair mercury was evident also from the longitudinal hair analysis of whole hair samples from female Minamata citizens. The typical features for waved and non-waved hairs were shown in Fig. 4. The levels at the root of the artificially waved hair were significantly higher than those at the tip ($P<0.001$) (Table 4). On the other hand, the difference was not significant between the two sides of the non-waved hairs.

Discussion

Present study has been conducted to obtain information on hair mercury levels in general population of Japan as reference data for estimating standard exposure levels of methylmercury mainly through the dietary fish/shellfish consumption. The survey is still continued to expand the subject population and the present paper showed preliminary results on data obtained in the first five sampling sites. Average hair mercury levels were estimated to be 1.43 and 2.55 μg/g, as geometric means, for female and male populations, respectively. These levels are relatively higher than the levels estimated from blood or toenail mercury concentrations recently observed in several western countries (CDC 2003, Guallar et al. 2002, Sanzo et al 2001). It can be considered that the difference mainly originated from the different food consumption habit among populations. The implications of the present results will be discussed in detail elsewhere from a viewpoint of risk management of the low level methylmercury

exposures.

The participants of this survey were volunteers and did not associate with any physical complaints, and the hair mercury levels obtained could be considered as a general population level in Japan. However, there might be some limitations on interpretation of the results. Because the survey was conducted without random sampling, selection bias might exist. Alternatively, present results indicated that the hair mercury level varied among population of different districts. Chiba, for example, was a significant factor that increased the hair mercury level even after adjustment of other main confounders such as age, sex, amount of fish/shellfish consumption and the tendency to consume certain fish species. It is not clear what kinds of characteristics are associated with the regional difference observed. It might be plausible that there were any dietary habits failed to be surmised by the questioner used in this study. In any cases, it should be pointed out, based on the present results, that the selection of sampling site is one of the most important factors in the stratification for sample collection on population survey aiming at the evaluation of general exposure level for methylmercury.

The second issue is the effects of the artificial waiving on hair mercury concentration. It may result in sometimes more serious problem for hair mercury assessment on general population, because the individual hair mercury content might be reduced by as much as 50% after the artificial treatment. In the present survey, 25 % reductions were observed on geometric mean of the mercury level, if the hair sample had been collected from participants with the waving treatment in both sexes. Thus, analysis of the hair root would be preferable to estimate exact mercury exposure level for individuals.

The present hair mercury data would reflect a part of the food habit of current Japanese. Although it was suggested that a large part of Japanese population is exposed to methylmercury at doses over the EPA/NRC recommended level through the dairy intake of fish/shellfish, this does not necessarily imply that they are exposed to the doses with a substantial hazard to the fetus. However, very little portion (0.4%) of females at the reproductive ages, that showed hair mercury levels above 10 μg/g, may have to change amount or species of fish consumed in their daily life on pregnancy to avoid possible adverse effects on developing fetus. The results of present study should be helpful to establish a healthy diet with appropriate consumption of fish and shellfish, which are nutritionally outstanding foods containing valuable nutrients such as n-3 polyunsaturated fatty acids.

Acknowledgement

The authors thank Ms. M. Ogata, Ms. N. Ogata, Ms. A. Hyodo and Ms. Y. Miyamato for their technical assistance in processing the hair samples and mercury analysis.

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Table 1. Geometric mean and range of the hair mercury content in five geological populations

	Sex	N	Hair mercury content (μg/g)		
			Geometric mean	Min	Max
Minamata	F	594	1.23	0.09	7.33
	M	344	2.39	0.22	10.56
	Total	938	1.76	0.09	10.56
Kumamoto	F	327	1.33	0.14	6.20
	M	388	2.23	0.20	19.18
	Total	715	1.57	0.14	19.18
Tottori	F	209	1.40	0.26	12.52
	M	616	2.32	0.00	10.21
	Total	825	2.04	0.00	12.52
Wakayama	F	303	1.46	0.00	8.09
	M	417	2.32	0.10	20.66
	Total	720	2.04	0.00	20.66
Chiba	F	233	2.30	0.14	25.75
	M	255	4.79	0.26	26.76
	Total	488	3.37	0.14	26.76
Total	F	1666	1.43	0.00	25.75
	M	2020	2.55	0.00	26.76
	Total	3686	1.96	0.00	26.76

Table 2. Factors that determine the hair mercury content as dependent variable by a multiple regression analysis

Independent variables	Standardized partial regression coefficient	Partial correlation coefficient	Multiple correlation coefficient
Sex	0.162	0.170	0.585
Age	0.264	0.352	
Daily amount of total fish/shellfish consumed	0.190	0.245	
Artificial hair waving	-0.185	-0.252	
Tuna : usually consumed fish	0.058	0.167	
Bonito : usually consumed fish	0.063	0.068	
Chiba : residence	0.281	0.242	

$P<0.001$

Table 3. Cumulative frequency of the individual hair mercury content

Sex	Age	Mercury concentration (μg/g)					Total
		≤ 1	≤ 2	≤ 3	≤ 5	≤ 10	
Female	All	464	1161	1473	1612	1659	1666
		(27.9%)	(69.7%)	(88.4%)	(96.8%)	(99.6%)	(100%)
	15-49	200	453	542	577	585	588
		(34.0%)	(77.0%)	(92.2%)	(98.1%)	(99.5%)	(100%)
Male	All	162	724	1206	1716	1975	2020
		(8.0%)	(35.8%)	(59.7%)	(85.0%)	(97.8%)	(100%)
Total	All	626	1885	2679	3328	3634	3686
		(17.0%)	(51.1%)	(72.7%)	(90.3%)	(98.6%)	(100%)

Table 4. Ratio of hair mercury levels at hair tip to root in female Minamata citizen

Artificial waving	No (38)	Yes (98)
Hg Ratio: Tip/Root	0.92 ± 0.21	0.56 ± 0.22

Numbers of hair samples are shown in parentheses

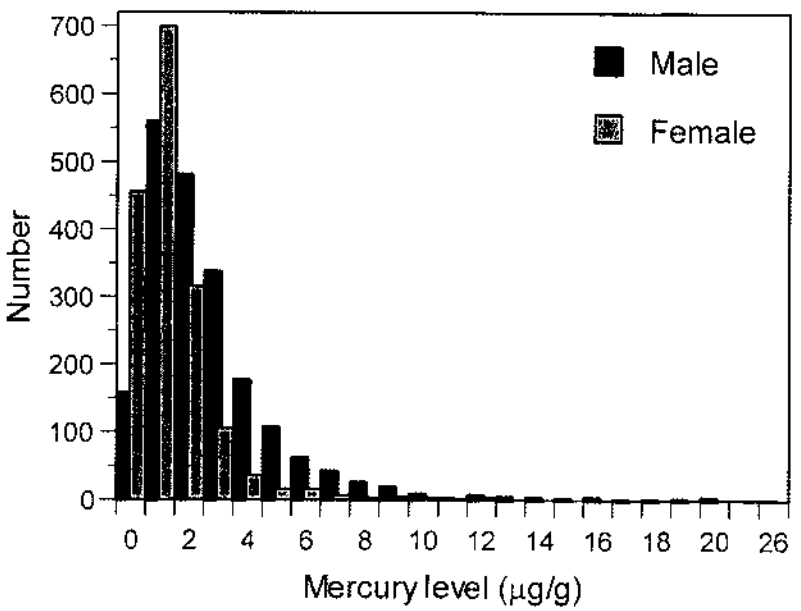
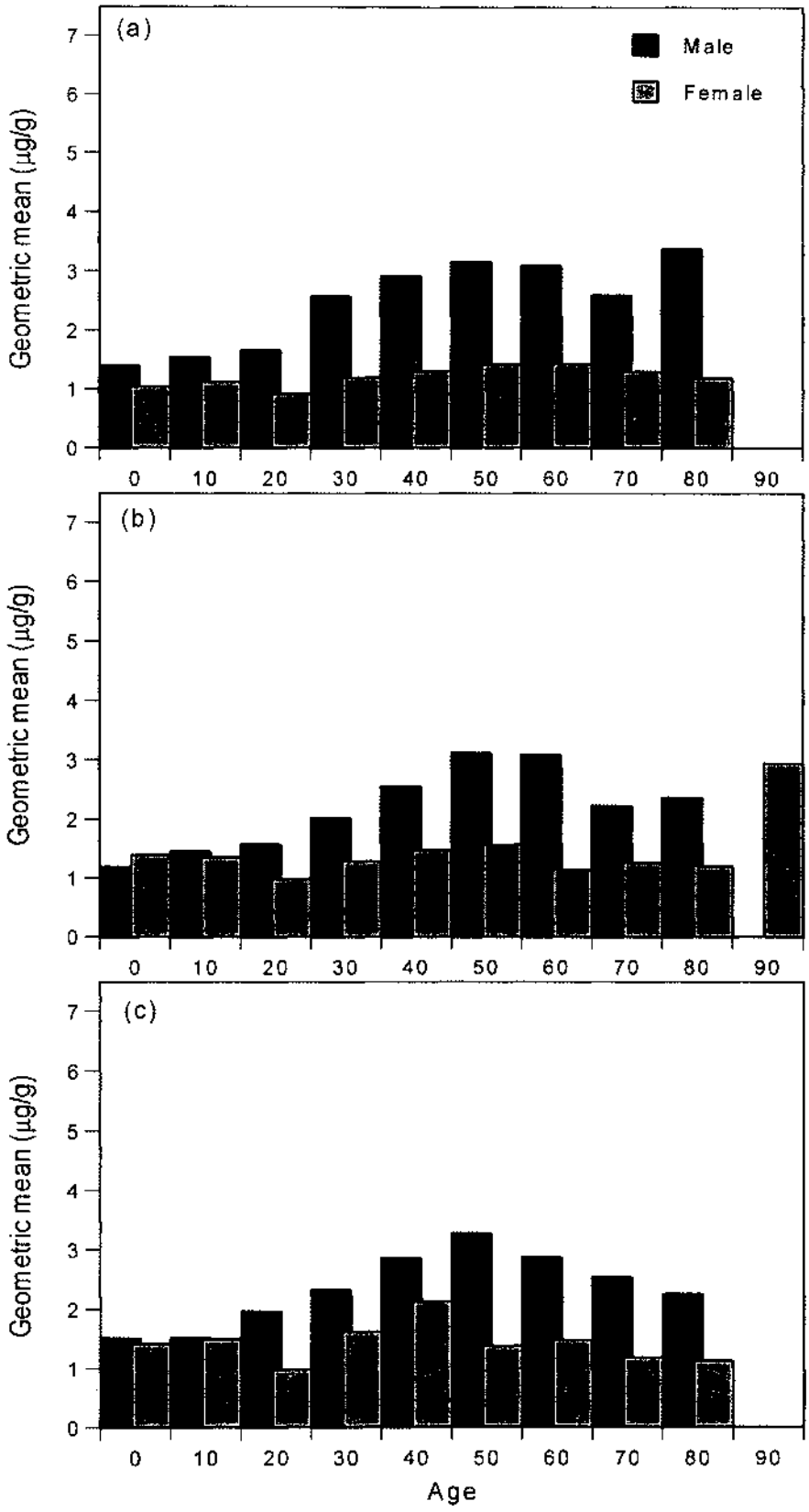


Fig 1. Distribution of the hair mercury content among the total population. Open bar and solid bar indicate female and male population, respectively.


(a)
Male
Female
Geometric mean (μg/g)
0
1
2
3
4
5
6
7
0
10
20
30
40
50
60
70
80
90
(b)
Geometric mean (μg/g)
(c)
Geometric mean (μg/g)
Age

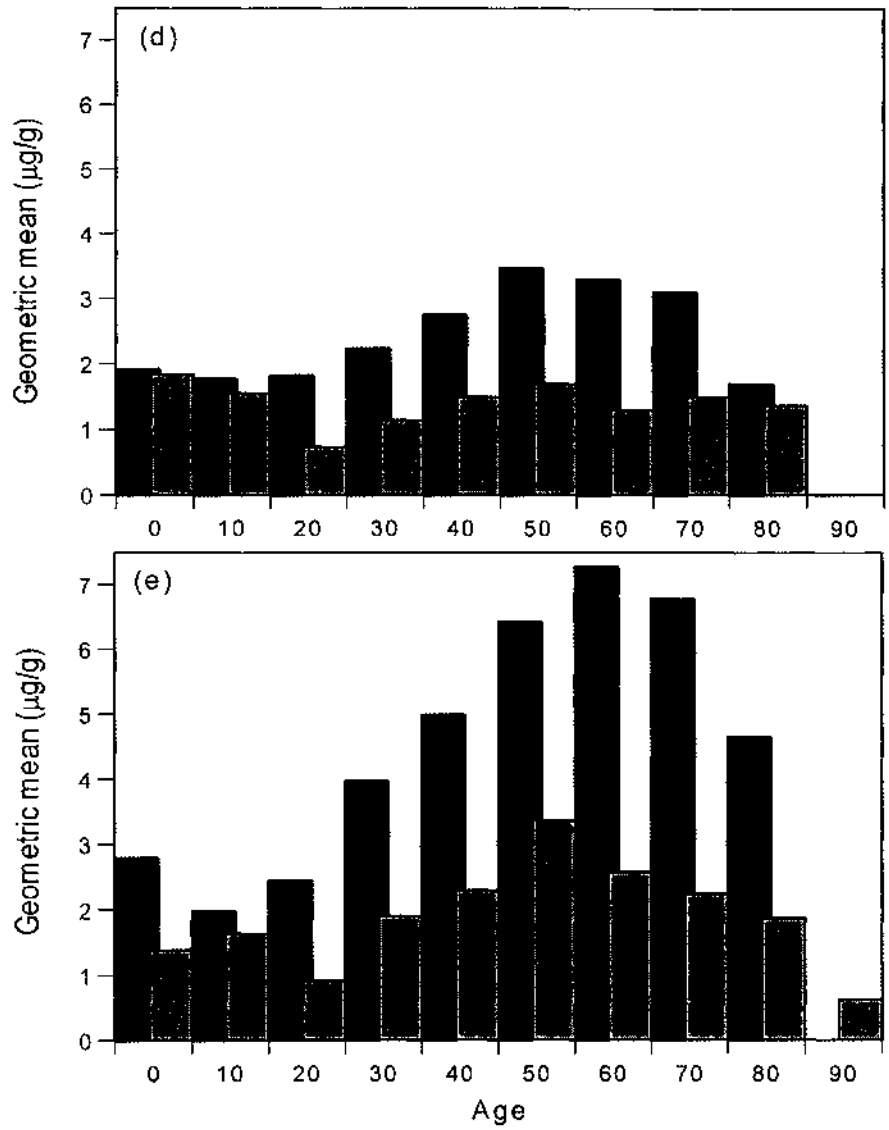


Fig. 2. Age dependent distribution of geometric mean of the hair mercury content in Minamata (a), Kumamoto (b), Tottori (c), Wakayama (d) and Chiba (e). Open bar and solid bar indicate female and male population, respectively.

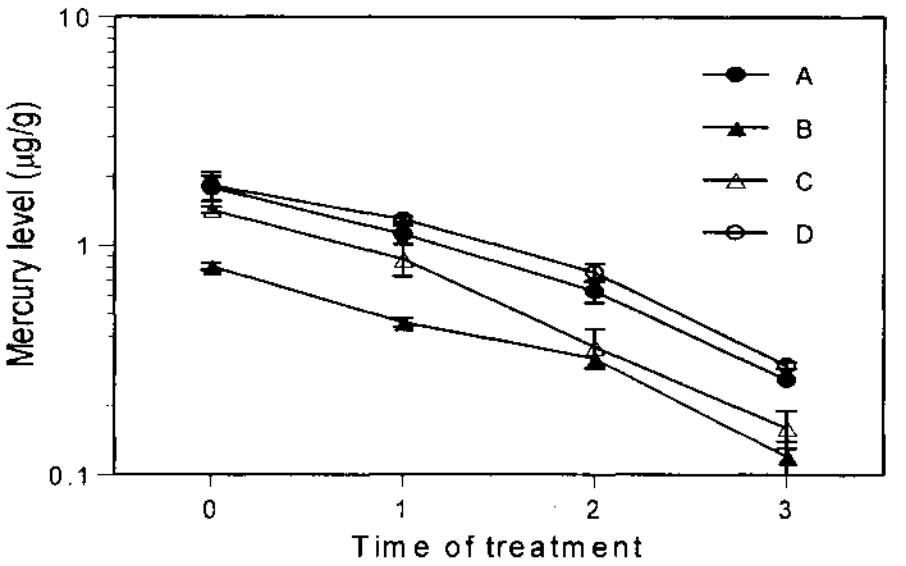


Fig. 3. Effect of artificial waving on hair mercury levels. Non-artificial permanent waved hair samples from 4 women (A to D) were treated with waving lotion up to 3 times. Hair mercury levels were determined after each treatment. Each value represent mean ± SD of 3 measurements.

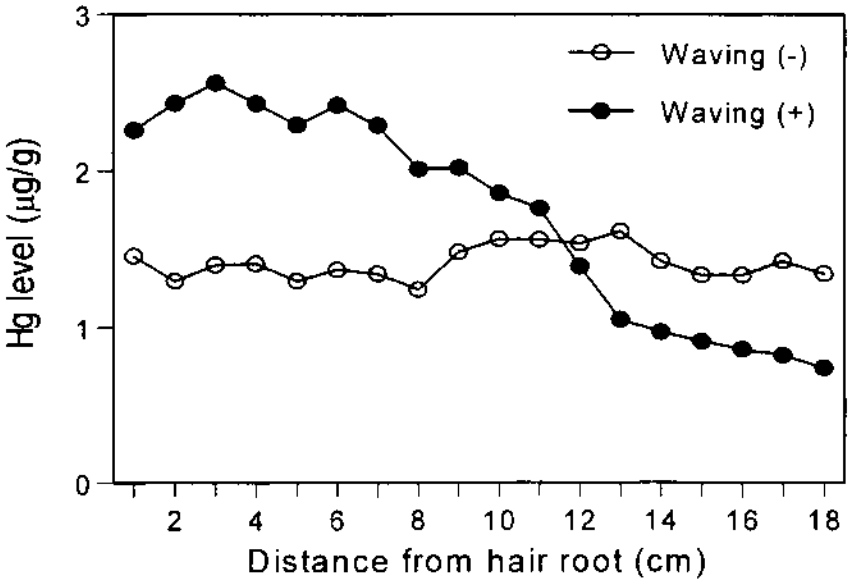


Fig. 4. Whole length analysis of hair mercury levels. Mercury levels of whole hair strands from two women (with and without artificial permanent waving) were analyzed at 1-cm sections.

Neurodevelopmental effects of methylmercury in Japanese children: a cross-sectional study in Akita

Katsuyuki Murata[1], Mineshi Sakamoto[2], Kunihiko Nakai[3], Yoko Iwasaki[4], Miwako Dakeishi[1], Toyoto Iwata[1], and Satoshi Terui[1]

1 Department of Hygiene, Akita University School of Medicine, Akita, Japan
2 Department of Epidemiology, National Institute for Minamata Disease, Minamata, Japan
3 Environmental Health Sciences, Tohoku University Graduate School of Medicine, Sendai, Japan
4 Environmental Health Department, Ministry of Environment, Tokyo, Japan

Abstract

Objective: Prenatal high-level exposure to methylmercury causes diffused neuronal degeneration in the brain including the brainstem. However, the risks associated with exposure from contaminated seafood remain unclear, and may differ in food culture. A cross-sectional study was carried out during the period of July-September in 2002, to clarify neurodevelopmental effects of prenatal methylmercury in Japanese children.

Methods: The participating subjects, from whom informed consent was obtained, were 154 mothers and their 7-year-old children who were born in the period of April 2 nd, 1995 to April 1 st , 1996, residing in two cities and three towns of Akita Prefecture, Japan. As exposure biomarker, hair samples in mothers and children, and umbilical cords were collected, and mercury concentrations were determined. As outcome variables, brainstem auditory evoked potentials (BAEPs), computer-assisted tests (tremor, postural sway, coordination, and reaction time), and corrected QT interval and R-R intervals on ECG were performed in all children. Questionnaires and interview were conducted for all mothers to obtain dietary information (especially, seafood) and information on child's birth and past and present medical history.

Results: The geometric mean of hair mercury were 1.64 (range, 0.45-6.3) μg/g for 154 children (mean 6.92 years old) and 1.73 (range 0.5-5.8) μg/g for the 154 mothers (mean 35.7 years old), respectively. Child's hair mercury concentration was significantly correlated with maternal hair mercury ($r=0.291$, $p<0.001$). Although some outcome variables, such as BAEP latencies, postural sway parameters and eye-hand coordination,

significantly differed in sex, any outcome variables except the eye-hand coordination (the standard deviation of reaction time) were not significantly correlated with maternal hair mercury concentration after adjusting for age and sex.

Conclusions: It is suggested that any effects of prenatal methylmercury exposure on neurodevelopment may not be observed in Japanese children, unless Japanese food culture changes extremely; because the prenatal exposure was thought to be below the allowable level that has been reported to be approximately 10 μg/dl.

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[1]Department of Hygiene, Akita University School of Medicine, Akita, Japan
[2]National Institute for Minamata Disease, Minamata, Japan
[3]Environmental Health Sciences, Tohoku University Graduate School of Medicine, Sendai, Japan
[4]Environmental Health Department, Ministry of Environment, Tokyo, Japan

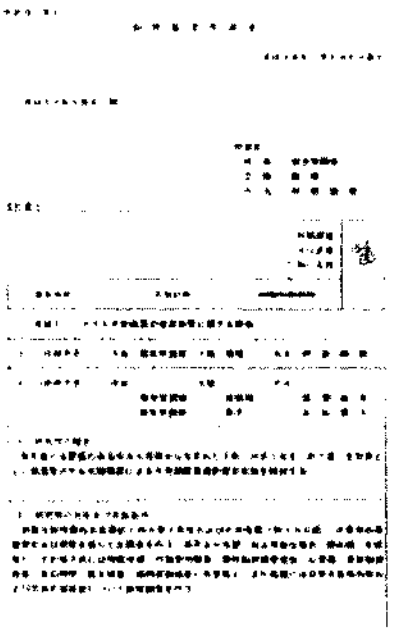
Objectives

Prenatal high-level exposure to methylmercury causes diffused neuronal degeneration in the brain including the brainstem. On the other hand, the risks associated with exposure from contaminated seafood remain unclear, and may differ in food culture.

The cross-sectional study was carried out during the period of July-September in 2002, to clarify the effects of methylmercury on child neurodevelopment in Japan.

General procedures (1)

The study protocol was approved by the ethical review committee at the Akita University School of Medicine.



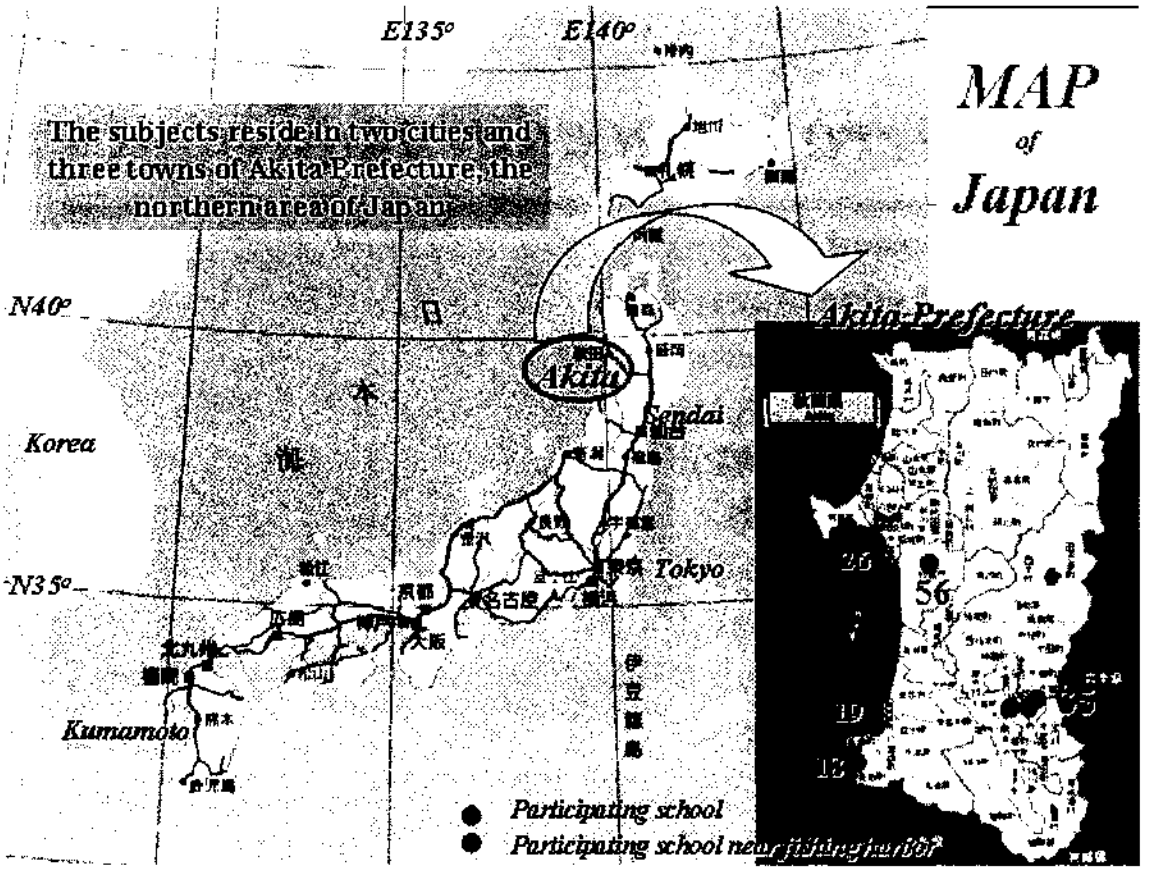
General procedures (2)

The nature of the procedures used in the present study was explained to the parents at eight elementary schools, and mothers and the children were invited for this study.

Study Population (1)

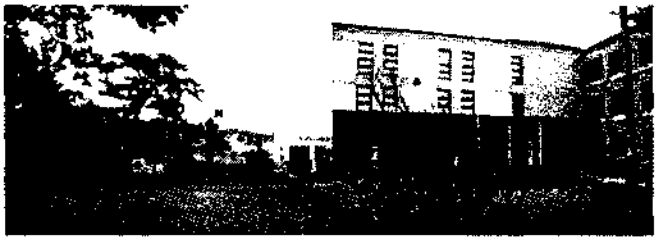
The participating subjects, from whom informed consent was obtained, were 154 mothers and their 7-year-old children who were born in the period of April 2nd, 1995 to April 1st, 1996.

The children were in the first grade of eight elementary schools, and four of the schools were located in near the fishing harbor.



Akita Prefecture

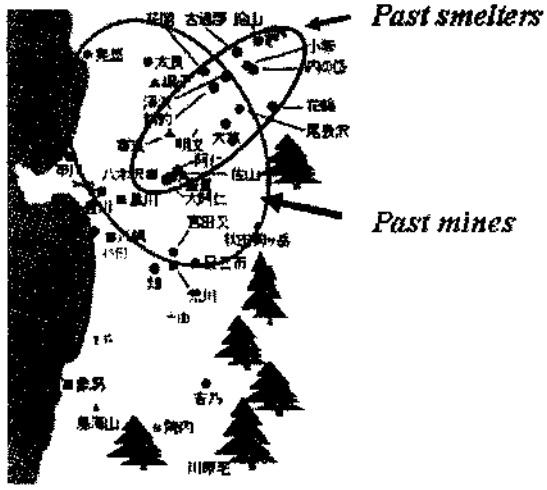
Participating elementary schools

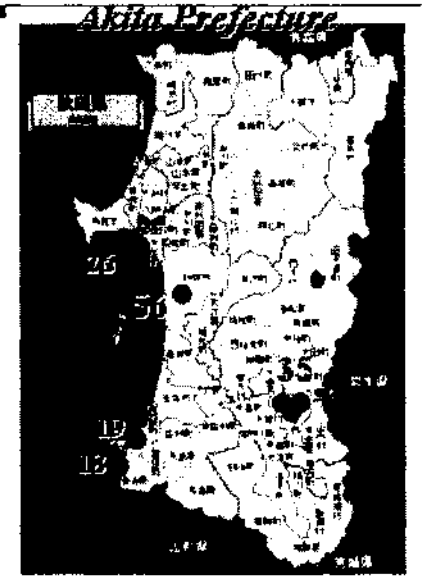


Study Population (2)

There were many mines and smelters in Akita Prefecture twenty years ago; for this reason, it is probable that soil or water has been contaminated by lead, copper, cadmium, *etc.* The study population did not include those who came from such areas.

Past mines and smelters in Akita





● *Participating school*
● *Participating school near fishing harbor*

Mercury exposure assessment

Hair samples were collected from the occipital area of the scalp in all children and mothers. The hair length was generally about 10 cm (range, 1-30 cm), thus representing the average mercury exposure during recent months.

Total mercury was determined by cold vapor atomic absorption spectrophotometry method at the National Institute for Minamata Disease. The method involves samples digestion with HNO_3, $HClO_4$ and H_2SO_4 followed by reduction to Hg^0 by $SnCl_2$.

Exposure biomarkers

○ Child hair at age 7 years

Estimated mercury concentrations at present

○ Maternal hair at present

Estimated mercury concentrations at birth

○ Naturally dried cord tissue

Estimated (total and organic) mercury concentrations at birth

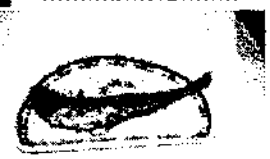
Interview survey with mothers

- ☐ Medical records during pregnancy and delivery *including smoking and drinking habits*
- ☐ Gestation period and birth weight
- ☐ Past & present history of illness in child
- ☐ Dietary habits in mother
- ☐ Frequency and dose of fish intake
- ☐ Hair character (*hair coloring and permanent*)

Survey on detailed fish intake habits

Detailed fish intake habits (frequency and dose of fish intake) were examined by interview method.

"Hata-kata"
(*Uranoscopus japonicus*)



Criteria for clinical tests

- ☐ **Sensitive to toxic exposures**
- ☐ **Reflecting functional domains**
- ☐ **Reasonably specific, with limited potentials for confounding**
- ☐ **Appropriate for age and culture**
- ☐ **Skilled professional examiners**
- ☐ **Computer-assisted objective methods**

Evaluation of outcome variables

- ☐ **Completeness of record**
- ☐ **Psychometric properties:**
  - ■ **no floor/ceiling**
  - ■ **wide range of responses**
- ☐ **Examiner competence**
- ☐ **Association with known predictors**

Used tests for assessment in children

Station A *(about 15 min.)*

- ☐ Tremor test
- ☐ Postural balance test
- ☐ Coordination test
- ☐ Reaction time

Station B *(about 15 min.)*

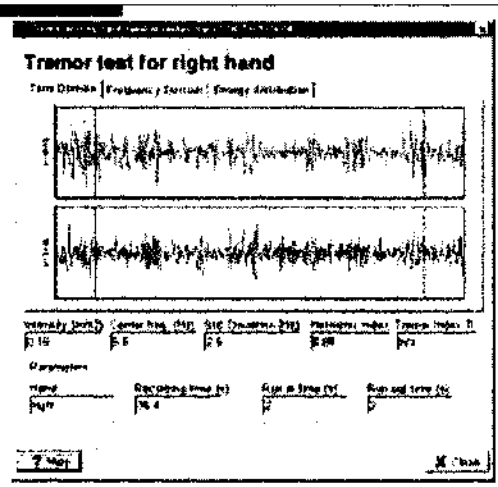
- ☐ Corrected QT interval (QTc) on ECG
- ☐ 300 R-R intervals on ECG
- ☐ Eye-hand coordination test

Station C *(about 15 min.)*

- ☐ Brainstem auditory evoked potentials (BAEPs)

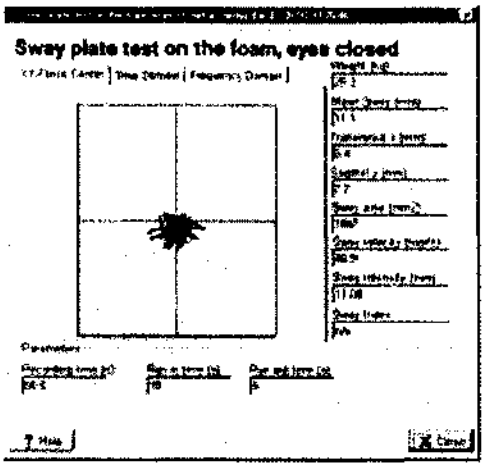
Three children per hour were examined at the upper three examination stations.

Tremor test



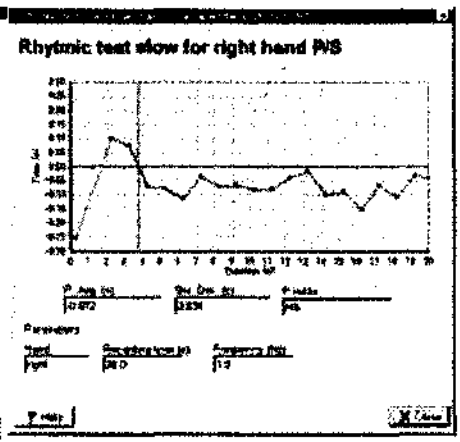
Hand tremor was measured successively for each hand during 16.4 sec. by asking the subjects to hold a light stylus as they would hold an ordinary pen, with the elbow joint bent at a right angle and free of body contact or any obstacles. The stylus was held horizontally, parallel to the abdomen at approximately 10 cm in front of the navel and the index finger was positioned about 1 cm from the tip of the stylus.

Postural sway test



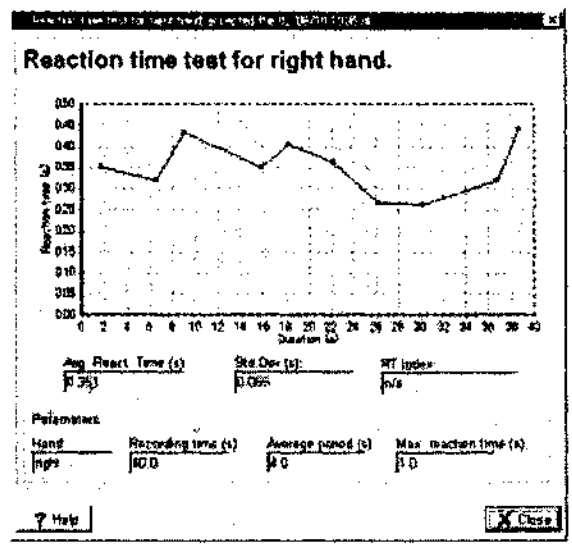
The postural sway was quantitatively measured with flat floor using the computerized posturography. Subjects were asked to stand quietly on the platform without foam under eyes-open and eyes-closed conditions; again, they were asked to stand on the platform with foam in the same manner.

Ear-hand coordination test



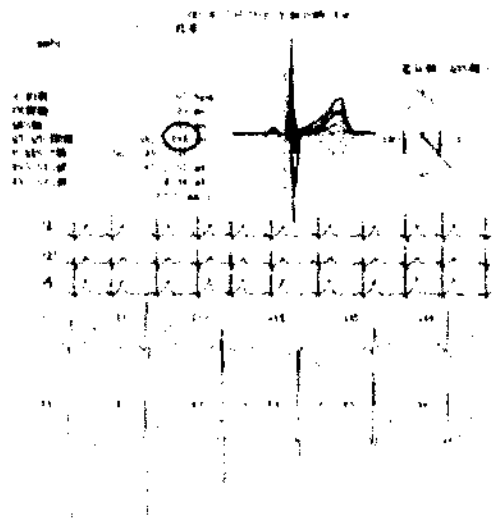
Coordination was examined with the CATSYS System, which is composed of a drum that records hand pronation-supination movements. This test was performed with right and left hands under the following standard condition: hand pronation-supination at a constant slow (1 Hz) and a constant fast (2.5 Hz) metronome beat.

Reaction time test

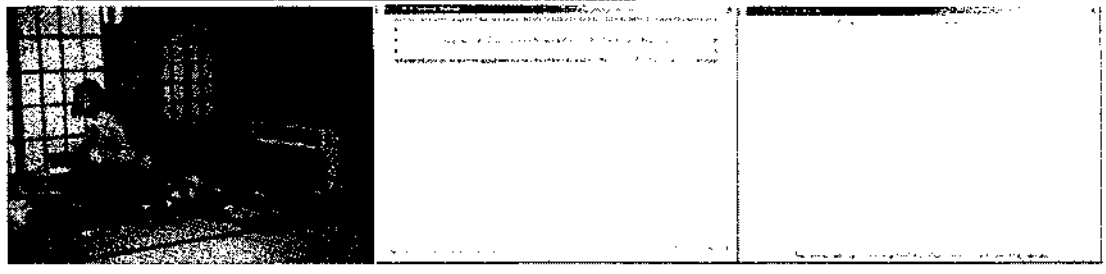


Reaction time to a sound stimulus was measured with right and left hands, by using the CATSYS System and a hand-held-switch.

Corrected QT (QTc) interval on ECG



R-R intervals on ECG



After the subject had lain quietly supine, 300 R-R intervals on ECG were measured in real time and stored on the harddisc; consecutive 100 R-R intervals with the minimal standard deviation were automatically extracted from the obtained data to avoid non-stationarities. The CV_{R-R} was defined as the ratio of the standard deviation of the R-R intervals to their average value ($R\text{-}R_{mean}$ ms). The power spectrum of R-R intervals was computed by autoregressive spectral analysis.

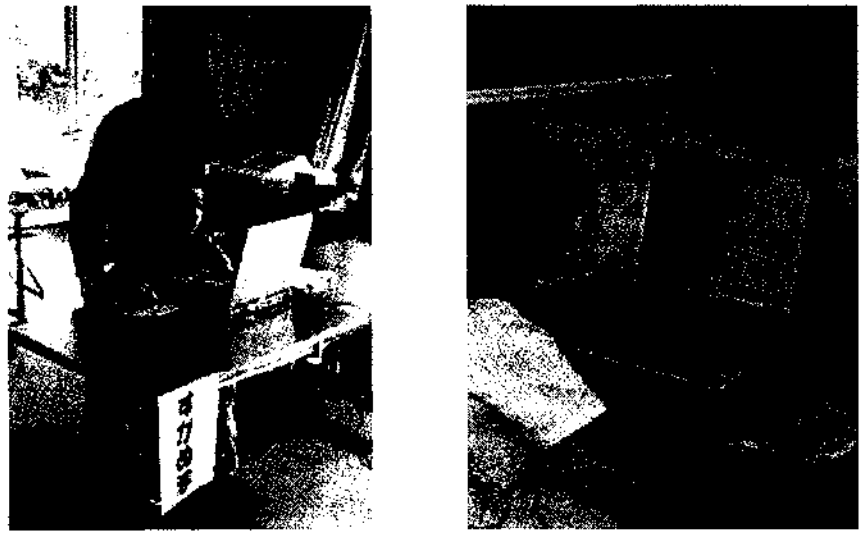
File name: M218 Start point 182 Number of sample 100

437.2 (Total power)
R-R interval Mean: 670.9 ms SD: 21.0 ms CV: 3.13 %

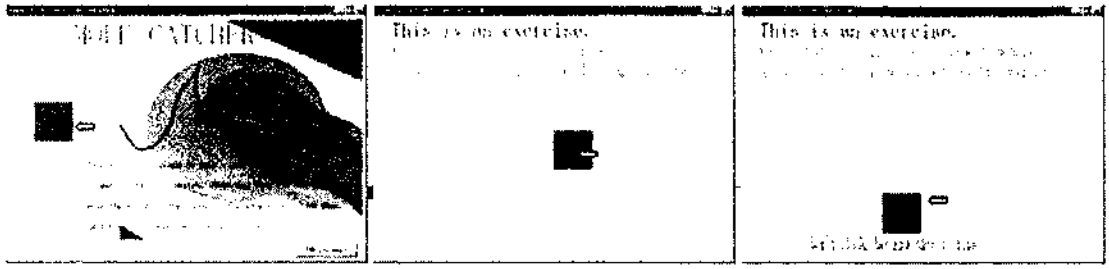
Component Power and the Related Indicators (Heart rate 89)

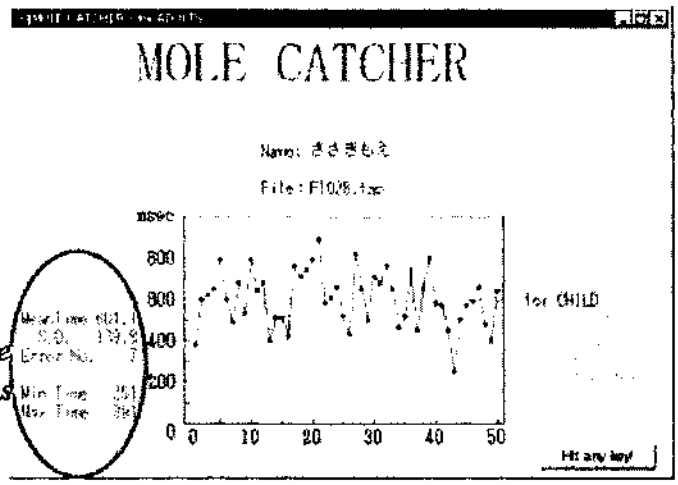
	Power	C-CV	%		
Low frequency:	346.5	2.77	53.2	%LF	55.8
High frequency:	217.0	2.20	33.3	%HF	44.2
Adjusted Total Power:	651.6				
LF/HF ratio	1.597				

Eye-hand coordination test



The program of "Mole Catcher" was made by K Murata to assess eye-hand coordination.





Outcome variables

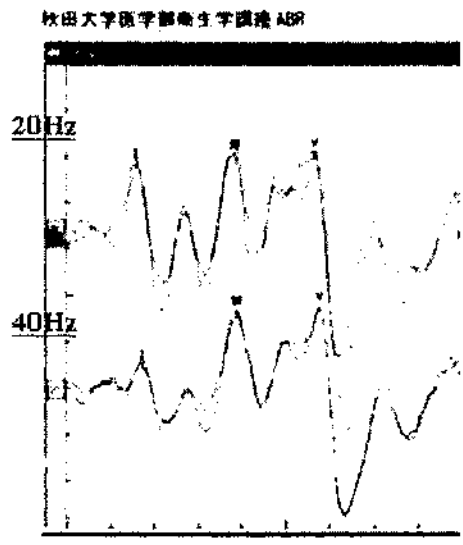
Eye-hand coordination test

The subject holds the mouse in the right hand, sitting in front of the computer. When the subject finds not a blue but red square on the display, he/she moves the arrow of the mouse onto the red square and clicks the left switch on the square as soon as possible. In this test, "Error" indicates that he/she switches-on when the arrow is located outside the red square.

Eye-hand coordination test

Brainstem auditory evoked potentials

Brainstem auditory evoked potentials (BAEPs) were measured with a four-channel electromyography (*Nihon Kohden Neuropack μ*). BAEPs were recorded in subjects lying comfortably. Click signals with an intensity of 65 dB HL were presented to the right ear through electromagnetically shielded earphones at 20 Hz and 40 Hz independently; the other ear was masked with white noise of intensity of 30 dB HL. Evoked potentials were recorded using 3 standard EEG electrodes placed on the vertex, the right mastoid ipsilateral to stimulation and the left mastoid (ground). The responses were averaged 2,000 times after amplification and filtration (bandpass, 200-2,000 Hz), with one replication for each rate. Peaks I, III and V are thought to reflect the volume-conducted electric activity from the acoustic nerve, pons (superior olivary nucleus) and midbrain (inferior colliculi), respectively.



Brainstem auditory evoked potentials

Daily variations (coefficients of variation, CVs) in the BAEP latencies at 20 and 40 Hz, in a 20-year-old student for 14 days, were 3.0% and 3.4% for peak I latencies; 1.4% and 1.6% for III latencies; 0.9% and 1.6% for V latencies, respectively. The bed differed in place where the examination was done.

Statistical Analysis

The dose-effect relations of outcome variables of neurophysiological and neurobehavioral tests to methylmercury exposure were analyzed by using

1) multiple regression analysis

2) analysis of covariance

3) benchmark dose calculation

(if the relations were statistically significant)

Results (1)

Summary of 154 participating subjects in Akita

	Population in Akita in May 2001	Participating subjects	Elementary school*	Participation rates (%)
Urban areas (Cities)	666,137	82 [36 boys 46 girls]	A (-)	47
			B (+)	56
			C (+)	46
Rural areas (Towns & villages)	517,870	72 [40 boys 32 girls]	D (+)	18
			E (+)	26
			F (-)	60
			G (-)	50
			H (-)	52

* (+) shows the school near the fishing harbor

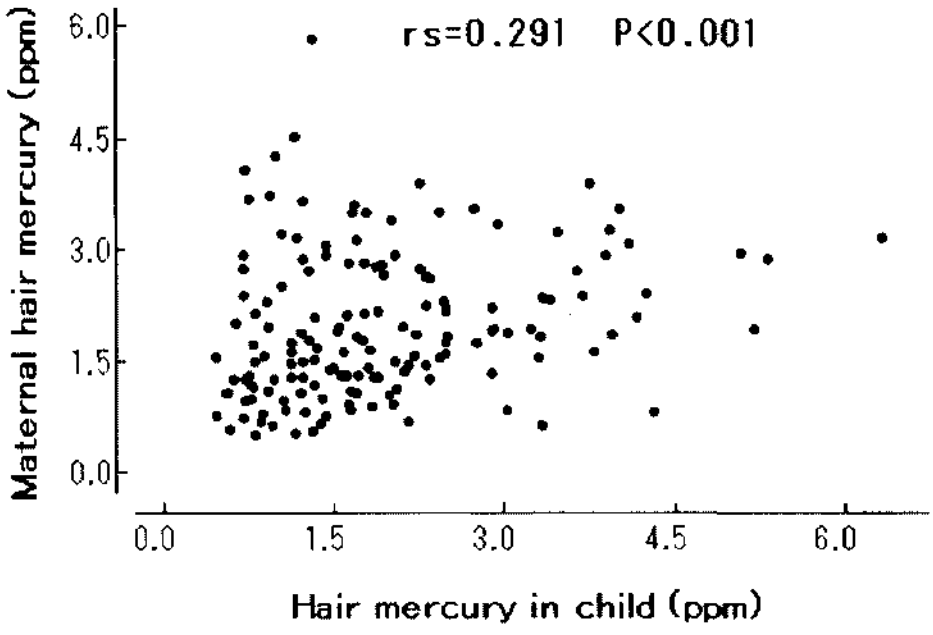
Results (2)

Summary of 154 participating subjects in Akita

	Participating subjects	Elementary schools	Hair mercury in mothers (μg/g) (Mean, range)	Hair mercury in children (μg/g) (Mean, range)
Urban areas (Cities)	82	A (-)	1.92, 0.49~4.08	1.99, 0.45~5.32
		B (+)	1.81, 1.04~3.74	2.00, 0.94~3.31
		C (+)	1.92, 0.69~5.83	1.69, 0.71~5.20
Rural areas (Towns & villages)	72	D (+)	1.81, 0.67~5.83	2.01, 0.62~4.31
		E (+)	2.08, 0.53~3.69	1.91, 0.56~6.32
		F, G, H (-)	2.17, 0.56~4.51	1.90, 0.58~4.10

* (+) shows the school near the fishing harbor

Hair mercury concentrations (ppm) in child and mother, in 2002



rs means the Spearman rank correlation coefficient.

Results (3)

	Mean (or Number, %)	SD	Range
Current age of mothers (years)*	35.7	4.2	25~48
Current age of children (years)*	6.92	0.31	6.34~7.51
Body weight at birth (gram)**	3174	401	2192~4568
Gestation period (weeks)	39.2	1.2	36~42
Smoking during pregnancy	7, 4.6%		
Drinking during pregnancy	19, 12.3%		
Natural delivery (no caesar)	143, 92.9%		
Gestosis (edema, anemia, *etc*)	53, 34.4%		

*154 children and mothers ** 3 children with low birth weight (< 2500 g)

Results (4)

- ☐ **There were no children with phenylketonuria, maple syrup urine disease, homocystinemia, galactosemia, congenital hypothyroidism, neuroblastoma, or adrenal hyperplasia.**
- ☐ **According to present and past history of illness, one child with spinal muscular atrophy will be excluded in this study.**

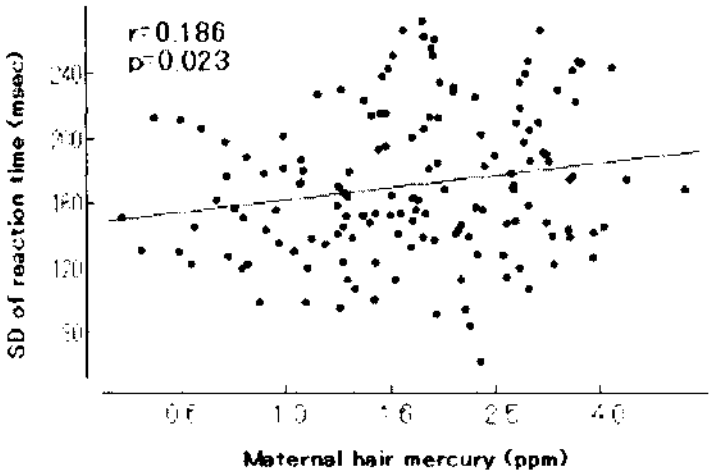
Present & past history of illness in 154 children	Number
Febrile convulsion	12
Otitis media	57
Spinal progressive muscular atrophy	1

Results of postural sway test	BOYS	GIRLS	Statistical significance (P)
	Mean± SD	Mean± SD	
Without foam			
Transversal sway distance (mm) [eyes open]	5.79±1.96	4.73±1.16	<0.001
Sagittal sway distance (mm) [eyes open]	5.84±1.69	4.95±1.69	<0.001
Sway area (mm^2) [eyes open]	916±505	610±291	<0.001
Sway velocity (mm/s) [eyes open]	17.0± 4.8	14.8± 4.2	0.003
Transversal sway distance (mm) [eyes close]	6.48±1.93	5.24±1.41	<0.001
Sagittal sway distance (mm) [eyes close]	6.37±1.78	5.52±1.47	<0.001
Sway area (mm^2) [eyes close]	1296±723	826±439	<0.001
Sway velocity (mm/s) [eyes close]	23.8± 7.5	20.3± 6.6	0.002
With foam			
Transversal sway distance (mm) [eyes open]	6.33±1.70	5.10±1.15	<0.001
Sagittal sway distance (mm) [eyes open]	7.03±2.08	5.91±1.86	<0.001
Sway area (mm^2) [eyes open]	1351±641	910±434	<0.001
Sway velocity (mm/s) [eyes open]	24.3± 6.5	20.2± 6.0	<0.001
Transversal sway distance (mm) [eyes close]	8.18±2.42	6.44±1.57	<0.001
Sagittal sway distance (mm) [eyes close]	8.12±2.84	6.68±1.79	<0.001
Sway area (mm^2) [eyes close]	2352±1656	1450±882	<0.001
Sway velocity (mm/s) [eyes close]	35.0±11.0	28.6± 8.9	<0.001

Other neurobehavioral tests	BOYS	GIRLS	Statistical significance (P)
	Mean± SD	Mean± SD	
Tremor test:			
Intensity (m/s^2), right	0.185±0.074	0.158±0.48	0.01
Center frequency (Hz), right	5.55±0.95	5.77±0.95	>0.05
Intensity (m/s^2), left	0.218±0.100	0.193±0.066	>0.05
Center frequency (Hz), left	5.21±0.97	5.23±0.77	>0.05
Ear-hand coordination test:			
Mean difference in slow rhythm (s), right	-0.066±0.062	-0.067±0.059	>0.05
Mean difference in slow rhythm (s), left	-0.071±0.055	-0.065±0.055	>0.05
Mean difference in fast rhythm (s), right	-0.068±0.048	-0.059±0.058	>0.05
Mean difference in fast rhythm (s), left	-0.070±0.046	-0.057±0.051	>0.05
Reaction time:			
Mean time (s), right	0.361±0.061	0.369±0.052	>0.05
Mean time (s), left	0.381±0.063	0.391±0.064	>0.05
Eye-hand coordination:			
Mean time (ms)	631± 80	674± 59	<0.001
Variance (SD, ms)	172± 46	171± 46	>0.05
Minimal time (ms)	222±137	268±152	>0.05
Maximal time (ms)	936± 83	961± 48	0.023
Error number	5.2±4.2	3.0±3.0	<0.001

Neurophysiological tests	BOYS	GIRLS	Statistical significance (P)
	Mean± SD	Mean± SD	
Brainstem auditory evoked potentials:			
Peak I latency (ms) [20Hz]	1.77±0.16	1.72±0.13	>0.05
Peak III latency (ms) [20Hz]	3.96±0.18	3.82±0.16	<0.001
Peak V latency (ms) [20Hz]	5.77±0.20	5.61±0.20	<0.001
Peak I latency (ms) [40Hz]	1.83±0.18	1.77±0.16	>0.05
Peak III latency (ms) [40Hz]	4.06±0.18	3.91±0.18	<0.001
Peak V latency (ms) [40Hz]	5.91±0.19	5.75±0.21	<0.001
ECG:			
Heart rate (/s)	81.2± 9.3	84.4±11.3	>0.05
QTc interval (ms)	390.4±15.3	390.8±17.1	>0.05
R-R interval analysis:			
CV_{RR} (%)	6.00±2.21	6.01±2.36	>0.05
C-CV_{HF} (%)	3.61±2.07	4.10±2.33	>0.05
C-CV_{LF} (%)	3.85±1.61	4.34±1.94	>0.05
%LF	53.3±12.5	52.7±12.7	>0.05

Relation of eye-hand coordination (SD of reaction time) to maternal hair mercury concentration



Effects of perinatal exposures to methylmercury and environmentally persistent organic pollutants on neurobehavioral development in Japanese children: A protocol for the prospective cohort study

Kunihiko Nakai [1], Mineshi Sakamoto [2], Katsuyuki Murata [3], Tomoko Oka [1], Keita Suzuki [1], Kunihiro Okamura [4], Toru Hosokawa [5], Yasuaki Mori [6], Hiroshi Satoh [1]

Departments of Environmental Health Sciences [1] and Obstetrics [4], Tohoku University Graduate School of Medicine, Sendai; [2] National Institute for Minamata Disease, Minamata; [3] Department of Hygiene, Akita University School of Medicine, Akita; [5] Department of Human Development, Faculty of Education, Tohoku University, Sendai; [6] Miyagi Prefectural Institute of Public Health and Environment, Sendai, Japan.

Abstract

Adverse effects following prenatal exposure to methylmercury (MeHg) were apparent in Minamata and Iraq disasters. Several prospective cohort studies in fish-eating population have been done to elucidate the effects of lower exposure. The primary source of human exposure to MeHg is fish. The fish is also contaminated with several environmentally persistent organic pollutants including polychlorinated biphenyls (PCBs), dioxins, and pesticides. These chemicals are recently shown to affect the neurobehavioral development. In the present report, we present a protocol for a prospective cohort for the examination of perinatal exposures to MeHg and other chemicals on neurobehavioral development in Japanese children.

Key words: Cohort, Development, Dioxin, Methylmercury, Polychlorinated biphenyls.

Introduction

The neurobehavioral effects of prenatal exposure to MeHg have been of great concern to several fish-eating populations in the world. It has been shown that the prenatal MeHg exposure caused the delay of development of cognitive functions in Faroe Islands (Grandjean P et al., 1997), Madeira Islands (Murata K et al., 2002), and New Zealand (Kjellstorm T et al., 1989; Kjellstorm T et al., 1986) . On the other hand, the studies that have been conducted in the Seychelles showed the absence of toxic effects of the prenatal exposure to MeHg (Davidson PW et al., 1998). Although these differences are due in part to the deference in the research strategy, test batteries used in the project, and others, one possible explanation is likely to be the deference in the ecological systems. Japanese people like fish and shellfish eating, and are carrying a unique and peculiar dietary habits. Considering that these Japanese ecological system is different from that at the above areas, a Japanese cohort study to examine the effects of prenatal exposure to MeHg on neurobehavioral development should be absolutely necessary.

Perinatal exposures to environmentally persistent organic pollutants such as PCBs, dioxins, and pesticides have been also indicated to affect the neurobehavioral growth in children (Huisman M et

al., 1995; Jacobson SW et al., 1984; Rogan WJ et al., 1986; Stewart P et al., 2000; Nakai K and Satoh H, 2002). Since it is thought that the intake of PCBs, dioxins and other chemicals are come from the fish-eating in Japan, this suggests that the exposure to MeHg should be considered under the combined effects with the exposures to PCBs, dioxins and other chemicals.

In the present report, we present a protocol for a prospective cohort for the examination of perinatal exposures to MeHg and other environmentally persistent organic pollutants on neurobehavioral development in Japanese children.

Place of the cohort study

Since fish-eating is the major source for the MeHg intake, the people who are living near the seaside and keep the conventional dietary habit using sea-food are the target for the cohort. In addition, Japanese like tuna and its MeHg concentration is high. The area where the consumption of tuna fish is large should be considered to decide the study place. If the sample size of the cohort is expected to be more than 500, the population size of the location should be moderately large. On the other hand, the movement of population is higher in the larger city, and larger cities therefore may not be suitable for a prospective cohort study. Finally, a preliminary study will be recommended for the final decision of place of the cohort study by comparing the mercury concentrations in hair of the pregnant women at the candidate places.

Recruitment

Healthy pregnant mothers are recruited with informed consent. 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 have to be completed without overt signs of serious illness or complications. The study protocol should be approved by the medical ethics committee of the Tohoku University.

Sample collections

The hair sample is collected from the mother after delivery. Most epidemiological studies on MeHg exposure have used mercury concentrations in hair to estimate the body burden (WHO, 1990). Since hair growth rates are independent of gender or racial differences (Cernichiari E et al., 1995), by assuming a constant rate of hair growth equal to 1.1 cm per month (Cox C 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 are cut next to the scalp, in the nape area, with stainless steel scissors. The samples are placed into the plastic bag and kept in a desiccator until analysis. Since hair mercury concentration can be reduced after hair permanent, a questionnaire regarding hair cosmetic treatment including bleaching, permanent and coloring is necessary.

Blood samples are collected from the mother after delivery. One of the target substance to determine in the maternal blood is polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid and arachidonic acid, that are mainly taken through fish-eating. This marker might be a surrogate index to

assess the intake of fish. In addition, PUFAs are known to play an essential role in the brain development (Birch EE et al., 2000). PUFAs in the maternal blood will be important information to consider the neurobehavioral development. Since PUFA is directly affected by the daily food intake, fasting venous blood sample is collected using EDTA as the anti-coagulant in the early morning to avoid the effect of food intake. PUFAs in plasma will be assayed within 4 weeks after blood collection to avoid decomposition (Otto SJ et al., 1997).

Special remarks are necessary for the determination of PCBs and dioxins. Since most commercially available plastic and glass materials are possibly contaminated with a significant amount of the above organic pollutants, all materials used for sample collection and storage should be certified to be clean before use. For the blood collection, a vacuum system certified to be without contamination is prepared. Blood is collected in the vacuum system heparin tube (26 mL) , and centrifuged within 4 h for 20 min at 3,000 rpm; plasma and whole blood are stored at -80 until analysis.

A blood sample (more than 50 mL) of the umbilical cord is taken into a bottle using heparin as the anti-coagulant after the delivery. This bottle should be previously cleaned by heating in a chemically clean chamber to exclude the possible contamination of PCBs and dioxins.

The tissues from placenta and cord are collected after the delivery. The placenta is considered useful for monitoring several pollutants because the placenta serves as the point of contact between maternal and fetal circulation, and it functions as a barrier against toxic substances. Since the placenta is a large organ which is a heterogeneous mix of placenta cells and decidual matter tainted with maternal and fetal blood, a representative samples of placenta is obtained as follows: the placenta is divided into 20-30 pieces, and they are randomly separated into 4 groups and pooled. Each bottle contains 50-100 g tissue. A representative sample should be prepared from the homogenized material (Iyengar GV and Rapp A, 2001). The entire cord is stored in a cleaned glass tube without preparation.

The mothers will be finally asked to give us a sample of breast milk (more than 50 mL) a month after the delivery. The cleaned bottle is used for the shipping of breast milk.

Questionnaire

Several questionnaires are taken after the delivery. To assess the fish-intake and the general nutrition status of the mother, a questionnaire of food intake frequent (FFQ) with 122 single foods and recipes (Date C et al., 1996) and some additional items regarding seafood is performed. This is a standardized FFQ to enable to assess not only major nutrient intake but also several essential nutrients including retinol and folic acids in Japanese people. Another questionnaire is performed to ask the following questions: parent's educational background, parent's occupation, income, smoking habit including passive smoking, hair treatment, dental treatment, and others.

Neurobehavioral assessment

All testers who perform neurobehavioral assessments should not be informed the results of exposure information including alcoholic/smoking habits, hair mercury content, FFQ, and others.

Brazelton Neonatal Behavioral Assessment Score (NBAS) is performed when the infant is at 3

days old. For this purpose, the testers had been trained in the training center in Japan, Nagasaki University School of Medicine (Prof, Akiyama).

The cognitive activities of the children at 7 months old are evaluated with Bayley Scales for Infant Development, second edition (BSID), Kyoto Scale of Psychological Development (KSPD) and Fagan Test of Infant Intelligence (FTII). BSID and FTII are major tests in the test batteries that have been used in several prospective cohort studies (Nakai K and Satoh H, 2002).

BSID is an established neurobehavioral tool. This 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 child's level of cognitive functioning (memory, learning, and problem solving), language development (expressive/receptive language, vocalization), and personal/social development. The motor scale assesses fine and gross motor functioning. Since there is no Japanese age-norms are available, raw-scores are used for analysis. To make a Japanese standardized protocol of BSID, we translate the original manual to make a Japanese version. To examine its reliability, it will be recommended to check the protocol with the 'Gold Standard' of any established research center, for example, at the University of Rochester School of Medicine (Davidson PW et al., 1995).

KSPD is a Japanese standard developmental test (Maehara T et al., 2002), and therefore, the developmental performance of the children is expressed as the developmental age (DA) for each and total behavior area(s). The developmental quotient (DQ), a score calculated by dividing the estimated DA by the chronological age and then multiplying 100, can be obtained. The result of KSPD will be compared with that of BSID.

FTII is a non-invasive test of information processing which may be applied to infants up to one year of age (Fagan JF and Detterman DK, 1992). Intelligent activity in the infant can be indexed by visual information processing, and is thought to be predictive of later intellectual performance. This result will be compared with that of another intelligence test such as Kaufman Assessment Battery for Children when the children are older than 42 months old.

BSID and KSPD are also used for the assessment of neurobehavioral development when the children are at 18 months old. The test battery for the elder children is not yet decided at present.

Chemical determinations

The total mercury analysis is carried out by cold vapor atomic absorption according to the method of Magos and Clarkson with some modifications. In brief, without washing the hair samples (Boischio AA and Cernichiari E, 1998), each sample, weighing approximately 20 mg, is acid digested by adding 0.5 mL of HNO_3, 0.5 mL of $HClO_4$ and 2 mL of H_2SO_4 at 180°C for 30 min. The resultant ionic mercury is then reduced mercury vapor by adding 0.5 mL of 10% tin chloride in a flameless atomic absorption monitor (HG-201, Sanso Co. Ltd., Tokyo). Analytical accuracy is ensured by analyzing the Human Hair Reference Material NIES CRM No. 13 from the National Institute of Environmental Studies (Lot #650, Tsukuba, Japan). In fish-eating populations, the total mercury concentration is mostly MeHg. Indeed, a few samples were determined to know the exact MeHg concentration by the method of Akagi and Nishimura (Akagi H and Nishimura H, 1991). MeHg in hair was 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 Minamata Diseases. The content of MeHg was confirmed to be more than 95% of the total mercury content.

The exact assay method to determine PCBs is not yet determined. Most references in which PCBs were analyzed use the sum of several major PCB congeners including 138, 153, and 180 as the marker of PCB exposure. However, the PCB congener which is responsible for the neurotoxic effect is not yet identified; there is no rational reason that the sum of major PCB congeners in the samples associates with the health hazard of PCB toxicisities. Indeed, the main hypothesis that PCB affects neurodevelopment involves disruption of thyroid hormone homeostasis (Porterfield SP and Source, 2000), and the candidate PCB congeners which may disturb the homeostasis may include several minor congeners and their OH-metabolites (Chauhan KR et al., 2000; Cheek AO et al., 1999). These findings suggest that all PCB congeners possibly detectable in the biological samples should be assayed with a very high sensitive assay method.

We recommend a reporter gene assay for the determination of the toxic potency of dioxins and related chemicals. CALUX assay (Chemical Activated LUciferase gene eXpression assay) which has been developed by Xenobiotic Detection Systems (North Carolina, USA) uses a patented recombinant mouse cell line that contains the luciferase reporter gene under control of dioxin responsive elements (Denison M et al., 1998). This assay has several advantages including high sensitivity, easy pretreatment, and rapid determination, in comparison with a high-resolution gas chromatography/mass spectrometer (HR-GC/MS). This biological assay also needs smaller sample volume, and this is another important advantage for the epidemiological study. We confirmed that the correlation between two assays, CALUX assay and HR-GC/MS, was extremely good when certified environmental reference materials were determined separately (Nakamura T et al., 2002). A whole blood sample more than 20 mL is essential for the determination of PCBs and dioxins.

Other major biochemical analysis of maternal and cord blood samples include plasma selenium and thyroid hormons including TSH, T4 and T3. Selenium is considered to play an essential role in protecting against MeHg toxicisity (Goyer RA, 1997). Monitoring of thyroidal function is necessary to examine the above hypothesis that PCB affects neurodevelopment due to the disruption of thyroid hormone homeostasis.

Potential confounders/covariates

The quality of the home environment is assessed with a questionnaire, the Evaluation of Environmental Stimulation (EES) (Anme T et al., 1998), which have been established in Japan modified after the HOME score (Home Observation for Measurement of the Environment) (Caldwell BM and Bradley RH, 2001). HOME is a validated method for the assessment of the home environment. However, trained observers must visit each home to assess the home environment by HOME. In addition, there is no Japanese version which had adapted to fit the Japanese cultural context. The EES is a questionnaire which directly evaluates the interaction between the child and caregiver, and its effectiveness as a support system. It is shown that the results of EES highly associated with that of HOME.

Parental socioeconomic status (SES) is rated using the Hollingshead Four Factor Index of Social Status (Hollingshead AB, 1975) with several modifications to make the category and prestige of occupation to fit the Japanese economical context.

Maternal intelligence quotient is measured using Raven standard progressive matrices. Only Raven colored progressive matrices have been already introduced into Japan only to the people older than 40 years old. We therefore use the original Raven standard version and analyze it using the raw data.

Other major potential confounders include age at examination (days). gestational age (weeks), alcohol/smoking during pregnancy, Apgar score, neonatal illness/jaundice, spontaneous delivery, parity, chronic diseases, and duration of breastfeeding (months).

Statistical analysis

The exact statistical model and the analytical method are not yet decided.

Comments

This present report describe a protocol for the longitudinal prospective cohort study to examine the effects of perinatal exposures to MeHg and other environmentally persistent organic pollutants on neurobehavioral development. The authors believe that this protocol is not yet perfect, and therefore welcome any suggestions and critical comments to improve this.

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〒867-0008　熊本県水俣市浜 4058 番 18 号

TEL: 0966-63-3111

FAX: 0966-61-1145

4058-18 Hama, Minamata City, Kumamoto 867-0008, Japan

TEL: +81-966-63-3111

FAX: +81-966-61-1145

E-mail address: kokusai@nimd.go.jp

Web Address: http://www.nimd.go.jp

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問い合わせ先：国際・総合研究部

Inquiry: Department of International Affairs and Environmental Sciences

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