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Original Article

Factors Affecting Hand Tremor and Postural Sway in Children

Toyoto IWATA[1], Kunihiko NAKAI[2], Mineshi SAKAMOTO[3], Miwako DAKEISHI[1], Hiroshi SATOH[2] and Katsuyuki MURATA[1]

[1]*Department of Environmental Health Sciences, Akita University School of Medicine, Akita, Japan*
[2]*Department of Environmental Health Sciences, Tohoku University Graduate School of Medicine, Miyagi, Japan*
[3]*Department of Epidemiology, National Institute for Minamata Disease, Kumamoto, Japan*

Abstract

Objectives: It is crucial to consider covariates relevant for outcome variables in developing dose-effect relations of environmental hazardous toxins. The aim of this study was to clarify the covariates affecting hand tremor and postural sway in children.

Methods: Hand tremor and postural sway, as well as hair mercury concentrations, were measured in 155 boys and 148 girls at age 7 years.

Results: Current mercury concentrations in child hair ranged from 0.35 to 6.32 μg/g (geometric mean, 1.71 μg/g for boys and 1.58 μg/g for girls), and were not significantly correlated with the neuromotor parameters. All hand tremor and postural sway parameters, except for tremor intensity at 1–6 Hz with non-dominant hand, were significantly larger in the boys than in the girls. Using multiple regression analysis, some postural sway parameters were related negatively to age in the boys and girls ($p<0.05$), and positively to height ($p<0.05$). Similarly, hand tremor parameters were positively related to age, height and heart rate either in the boys or in the girls ($p<0.05$). Also, there were positive relationships between tremor intensity at 1–6 Hz and transversal and sagittal sways at 1–2 Hz and 2–4 Hz ($p<0.05$).

Conclusions: Heart rate and postural sway, together with age, sex, and height, should be considered in interpreting hand tremor in children. Hand tremor or postural sway may not be so sensitive or specific to methylmercury exposures at levels of less than 7 μg/g in hair.

Key words: hand tremor, postural sway, covariates, methylmercury, child

Introduction

The assessment of neuromotor functions, e.g., postural sway and hand tremor, in children at the developmental stage seems to be troublesome, because various anthropometric factors such as height and body weight interfere with interpretation of the data (1), and children and infants are not little adults and are uniquely vulnerable to environmental factors (2). In addition, few neuromotor tests have been designed to be sensitive enough for the early detection of subtle deficits in involuntary movements (3). For this reason, significant findings have been reported only on the effects of postnatal exposures to hazardous substances such as lead (4, 5), but not on the effects of prenatal exposures to methylmercury (6), which may have been attributable to the difference between neurotoxic substances. As covariates/confounders involved in neuromotor measures for children, age, height, body weight, head circumference, use of alcohol and marijuana during pregnancy, duration of breastfeeding, socioeconomic status, mother's education, and etc. were considered in previous studies (4, 5, 7–9). Most of them have not been associated with neuromotor functions, or the significance of associations with neuromotor functions has not been described. On the other hand, recent research applying the above neuromotor tests to adults reported that postural sway and heart rate affected hand tremor to some degree (10, 11). For the establishment of neuromotor measures in children, therefore, it is crucial to explore such factors affecting postural sway and hand tremor.

In 7-year-old Japanese children, a cross-sectional study on the neurodevelopmental effects of mercury exposure was conducted (12). In the study, the methylmercury exposure level at parturition was estimated to be between 0.11 and 6.86 (median 1.63) μg/g in hair, but any parameters of postural sway

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Reprint requests to: Katsuyuki MURATA
Department of Environmental Health Sciences, Akita University School of Medicine, 1-1-1 Hondo, Akita 010-8543, Japan
TEL: +81(18)884-6085, FAX: +81(18)836-2608
E-mail: winestem@med.akita-u.ac.jp

or hand tremor were not associated with the exposure; at the same time, we could not explain sex difference in the neuromotor parameters. In this case, what sex difference in such parameters resulted from remains unclear. To answer these questions, we tried to scrutinize covariates or confounders affecting the neuromotor function.

Materials and Methods

Subjects

The nature of the procedures used in this study was explained to the parents of the first graders of 28 elementary schools in Akita and Tottori Prefectures, Japan, 14 of which are located near a fishing harbor (12). Children who were born from April 2nd, 1995 to April 1st, 1997 were invited to participate in this study in July–September, 2002 and 2003. In Japan, there were many mines and smelters 30 years ago, and it is probable that soil or water was contaminated by lead, copper or cadmium; therefore, the study population did not include those who came from such areas. Of 327 participating subjects, 24 children were excluded because two children obviously suffered from some neurological disorder (spinal progressive muscular atrophy and epilepsy), one child had a congenital malformation (cleft palate), 20 children had low birth weights (less than 2500 g), and one boy could not perform only the neuromotor test. Thus, 155 boys and 148 girls were enrolled in this study. The study protocol was approved by the ethical review committee at the Akita University School of Medicine.

Methods

Hair samples were collected from the occipital area in all children, by cutting strands of hair close to the scalp. The hair lengths ranged from 1 to 30 (mean 10) cm. Total mercury in aliquots of dried hair samples (15 to 20 mg), rinsed with acetone, was determined by cold vapor atomic absorption spectrophotometry at the National Institute for Minamata Disease (13, 14). A detailed survey of medical records during pregnancy and delivery, including smoking and drinking habits, gestation period, birth weight, and past and present history of illness in the child was conducted, by one medical doctor, at the elementary schools or civic centers where neurodevelopmental examinations were carried out. Also, the medical data were checked by referring to the maternal and child health handbook that mothers had kept for their children.

One trained examiner examined postural sway and hand tremor (11, 12, 15) using the Neurobehavioral Test System (CATSYS 2000, Danish Product Development Ltd., Denmark). Postural sway was measured on a flat floor. The subject was instructed to stand quietly on a platform for 66 s under eyes-open and eyes-closed conditions; Main parameters measured under both conditions were: sway area (mm^2: area traveled by the center of force), transversal sway (mm: mean deviation in the medio-lateral direction, Dx) and sagittal sway (mm; mean deviation in the anterior-posterior direction, Dy). The spectral analysis of Dx and Dy was conducted with the fast Fourier transform (FFT) analysis (16) to identify the specific sway; and, square roots (mm) of the power calculated from the power spectrum within the frequency bands of 0–1 Hz, 1–2 Hz, and 2–4 Hz served as measures of the amount of postural sway for each frequency range. Hand tremor was measured successively for each hand for 16.4 s: the subject was instructed to hold a light stylus as he/she would hold an ordinary pen, with their elbows bent at a right angle and free of body contact or any obstacles. The stylus was held horizontally, parallel to the abdomen at about 10 cm in front of the navel, and the index finger was positioned about 1 cm from the tip of the stylus. The frequency analysis of tremor intensity was conducted with the FFT analysis, and the power spectrum (m/s^2) within the frequency bands of 1–6 Hz, 6–10 Hz, and 10–14 Hz, were calculated. Heart rate was measured using the ECG-Amplifier 1271SP (NEC-Sanei Co., Japan) connected to an analog-to-digital converter (sampling time, 1 ms) and a computer, after the subject lay quietly supine for five min (12).

Statistical analysis

The significance of differences in characteristics between boys and girls was analyzed by the Student (or Welch) t test, Mann-Whitney U test or Fisher exact probability. Also, sex differences in neuromotor parameters were tested by the analysis of covariance to adjust for age, height, body weight, heart rate, gestation period, birth weight, mother's smoking and drinking habits during pregnancy, past history of otitis media, and mercury concentration in child's hair. Mother's smoking and drinking habits during pregnancy and past history of otitis media were scored as "absence"=0 and "presence"=1. Multiple regression analysis was employed to evaluate the relations of age, height, body weight, heart rate, and possible covariates to neuromotor parameters in boys and girls, separately. The partial correlation coefficient, as well as the Pearson product-moment correlation coefficient (r), was calculated to determine the relationships between the sway and tremor parameters after controlling for age, height, and heart rate. All analyses, with two-sided p values, were performed using the Statistical Package for the Biosciences (17).

Results

Characteristics of the 155 boys and 148 girls are shown in Table 1. Age, height or body weight did not differ significantly between both sexes. There were significant relationships

Table 1 Characteristics (mean±SD) of Japanese children and their mothers

	155 boys	148 girls	p values
Age (years)	6.9±0.3	6.9±0.3	0.3068
Height (cm)	119.6±5.2	119.8±4.7	0.6777
Body weight (kg)	23.7±4.3	23.6±3.3	0.8488
Heart rate (beats/min)	81.2±9.6	83.6±10.8	0.0409
Body weight at birth (g)	3326±385	3160±344	0.0410
Gestation period (weeks)	39.1±1.3	39.3±1.2	0.4103
Past history of illness in child			
Otitis media	38.7%	38.5%	1.0000†
Smoking during pregnancy*	9.7%	4.7%	0.1219†
Drinking during pregnancy*	16.1%	9.5%	0.0890†

* Mother's habits during pregnancy.
† Fisher exact probability.

between age and height in the boys ($r=0.389$, $p<0.0001$) and in the girls ($r=0.346$, $p<0.0001$), and significant relationships between height and body weight in the boys ($r=0.677$, $p<0.0001$) and in the girls ($r=0.624$, $p<0.0001$). The mercury in hair ranged from 0.35 to 5.83 (geometric mean 1.71) μg/g in the boys and from 0.55 to 6.32 (geometric mean 1.58) μg/g in the girls, and the hair mercury did not differ between both sexes ($p>0.05$). The log-transformed mercury in hair was not significantly correlated with any neuromotor parameters (i.e., postural sway and hand tremor) either in the boys or in the girls ($p>0.05$ by the Pearson product-moment correlation coefficient).

All neuromotor parameters except for tremor intensity at 1–6 Hz with non-dominant hand were significantly larger in the boys than in the girls (Table 2). In the analysis of covariance, the significant covariates (the correlation and parameter number in parentheses) were age (negative, 15), height (positive, 16), and mother's smoking (positive, 3) and drinking (positive, 8) habits during pregnancy for some postural sway parameters; and, age (positive, 2), height (positive, 2), heart rate (positive, 5), and body weight at birth (positive, 8) for some tremor parameters.

As shown in Table 3, postural sway parameters, in general, were related negatively to age and positively to height. Body weight was positively related to the total Dy and the Dy at 0–1 Hz with eyes open in the boys, but negatively to the Dx at 1–2 Hz and Dx at 2–4 Hz with eyes open in the girls. In including mother's smoking and drinking habits during pregnancy in independent variables of the multiple regression analysis, drinking habit, in addition to age and height, was positively related to twelve sway parameters only in the boys ($p<0.05$, data not shown).

The total tremor intensity and component tremor intensities at 6–10 Hz and 10–14 Hz for the dominant hand were positively related to age in the girls, and most of tremor parameters were positively related to height in the boys (Table 4); also, heart rate was positively related to most of tremor parameters in the boys. In adding birth weight and mother's smoking and drinking habits to the independent variables, birth weight, together with age, height and heart rate, was positively related to eight tremor parameters only in the boys ($p<0.05$, data not shown).

With respect to the association between the tremor and sway parameters (Table 5), tremor intensity at 1–6 Hz was significantly correlated with the Dx at 1–2 Hz and 2–4 Hz and Dy at 2–4 Hz with eyes open among the boys and girls. Among the girls, tremor intensity at 1–6 Hz was also significantly correlated with some sway parameters with eyes closed.

Table 2 Differences in postural sway parameters (mean±SD) between Japanese 155 boys and 148 girls after adjusting for ten possible covariates such as age, height, and body weight: results of analysis of covariance

	155 boys	148 girls	p value
Postural sway:			
Eyes open			
Sway area, mm^2	865±550	563±293	<0.0001
Transversal sway (Dx), mm	5.46±1.84	4.45±1.22	<0.0001
Dx at 0–1 Hz, mm	6.82±2.32	5.55±1.49	<0.0001
Dx at 1–2 Hz mm	0.90±0.33	0.75±0.24	<0.0001
Dx at 2–4 Hz, mm	0.39±0.15	0.32±0.10	<0.0001
Sagittal sway (Dy), mm	5.63±1.84	4.80±1.54	<0.0001
Dy at 0–1 Hz, mm	7.04±2.38	5.98±1.88	<0.0001
Dy at 1–2 Hz, mm	1.11±0.47	0.90±0.32	<0.0001
Dy at 2–4 Hz, mm	0.50±0.19	0.41±0.15	<0.0001
Eyes closed			
Sway area (mm^2)	1263±876	808±488	<0.0001
Transversal sway (Dx), mm	6.09±1.90	4.92±1.39	<0.0001
Dx at 0–1 Hz, mm	7.60±2.33	6.15±1.70	<0.0001
Dx at 1–2 Hz, mm	1.26±0.64	0.99±0.34	<0.0001
Dx at 2–4 Hz, mm	0.55±0.26	0.44±0.17	<0.0001
Sagittal sway (Dy), mm	6.23±1.76	5.20±1.43	<0.0001
Dy at 0–1 Hz, mm	7.71±2.20	6.46±1.84	<0.0001
Dy at 1–2 Hz, mm	1.62±0.62	1.32±0.46	<0.0001
Dy at 2–4 Hz, mm	0.71±0.31	0.57±0.22	<0.0001
Tremor intensity, m/s^2:			
Dominant hand			
Total	0.192±0.071	0.169±0.053	0.0034
1–6 Hz	0.144±0.060	0.127±0.044	0.0078
6–10 Hz	0.108±0.036	0.098±0.032	0.0126
10–14 Hz	0.050±0.019	0.044±0.016	0.0054
Non-dominant hand			
Total	0.219±0.079	0.201±0.069	0.0387
1–6 Hz	0.168±0.064	0.158±0.058	0.1705
6–10 Hz	0.119±0.046	0.107±0.038	0.0182
10–14 Hz	0.057±0.026	0.051±0.021	0.0215

Discussion

In this study, the children with hair mercury levels of 0.35–6.32 μg/g showed no significant relations of current mercury exposure to either postural sway or hand tremor parameters. When maternal hair mercury concentration was used as a proxy for mercury exposure at parturition, we failed to find any significant relationships between prenatal methylmercury exposures and neuromotor variables in the same children (12). Similarly, a dose-effect association of methylmercury with postural sway parameters has not been observed in the Faroese birth cohort (6). Methylmercury exposures in both populations resulted mainly from seafood diet (18, 19). On the other hand, postural instability and increased hand tremor have been reported in adults exposed to lead, manganese, arsenic, and organic solvents (11, 20–23). Therefore, it is suggested that postural sway or hand tremor may not be so sensitive or specific to prenatal low-level exposures to methylmercury, at least less than 7 μg/g in hair.

The present study of 7-year-old children showed that postural sway and hand tremor differed between both sexes. In the Faroese birth cohort, difference in postural balance observed between both sexes at age 7 years (24). Also, boys under 10 years swayed more than girls (7, 9). Thus, this difference at the developmental stage may have originated not from anthropometric factors, but from certain biological factors such as sex chromosome or from exposures to a certain neurotoxin other than methylmercury. In addition, Darlington et al. (25) reported that although the menstrual cycle phase had no significant effect on sagittal sway, it did significantly affect transversal sway. In healthy elderly people, however, gender-based balance differences were not shown during quiet standing

Table 3 Relations of age, height, body weight, and heart rate to postural sway parameters in Japanese children: Results of multiple regression analysis

	Standard regression coefficient				Multiple correlation coefficient
	Age	Height	Body weight	Heart rate	
155 boys:					
Eyes open					
Sway area	−0.200*	0.310**	0.024	−0.041	0.318**
Transversal sway (Dx)	−0.176*	0.343**	−0.068	−0.084	0.301**
Dx at 0–1 Hz	−0.191*	0.349**	−0.066	−0.085	0.309**
Dx at 1–2 Hz	−0.094	0.293*	−0.064	−0.091	0.261*
Dx at 2–4 Hz	−0.154	0.288*	−0.065	0.003	0.234
Sagittal sway (Dy)	−0.071	0.074	0.258*	−0.022	0.306**
Dy at 0–1 Hz	−0.089	0.077	0.248*	−0.016	0.298**
Dy at 1–2 Hz	−0.131	0.246*	−0.048	0.048	0.205
Dy at 2–4 Hz	−0.172*	0.319**	−0.095	0.083	0.259*
Eyes closed					
Sway area	−0.194*	0.279*	0.016	−0.088	0.299**
Transversal sway (Dx)	−0.166	0.362**	−0.081	−0.046	0.298**
Dx at 0–1 Hz	−0.169	0.363**	−0.070	−0.046	0.304**
Dx at 1–2 Hz	−0.156	0.212	−0.046	−0.038	0.192
Dx at 2–4 Hz	−0.139	0.280*	−0.122	−0.018	0.208
Sagittal sway (Dy)	−0.063	0.090	0.154	−0.101	0.249*
Dy at 0–1 Hz	−0.073	0.077	0.163	−0.106	0.249*
Dy at 1–2 Hz	−0.169	0.204	−0.042	0.000	0.190
Dy at 2–4 Hz	−0.137	0.228	−0.113	0.046	0.179
148 girls:					
Eyes open					
Sway area	−0.180*	0.234*	−0.069	−0.039	0.219
Transversal sway (Dx)	−0.189*	0.248*	−0.145	−0.082	0.233
Dx at 0–1 Hz	−0.195*	0.248*	−0.145	−0.096	0.241
Dx at 1–2 Hz	−0.159	0.378***	−0.221*	0.033	0.281*
Dx at 2–4 Hz	−0.146	0.322**	−0.219*	−0.005	0.243
Sagittal sway (Dy)	−0.099	0.149	0.097	0.013	0.216
Dy at 0–1 Hz	−0.087	0.157	0.086	0.021	0.211
Dy at 1–2 Hz	−0.174	0.070	0.023	−0.033	0.173
Dy at 2–4 Hz	−0.181*	0.076	−0.036	−0.033	0.170
Eyes closed					
Sway area	−0.263**	0.214	−0.098	−0.057	0.262*
Transversal sway (Dx)	−0.295***	0.271*	−0.102	−0.098	0.314**
Dx at 0–1 Hz	−0.294***	0.263*	−0.088	−0.104	0.314**
Dx at 1–2 Hz	−0.286**	0.283*	−0.172	0.062	0.298**
Dx at 2–4 Hz	−0.218*	0.201	−0.144	0.037	0.224
Sagittal sway (Dy)	−0.221*	0.228*	−0.014	−0.041	0.257*
Dy at 0–1 Hz	−0.219*	0.246*	−0.034	−0.040	0.258*
Dy at 1–2 Hz	−0.285**	0.139	−0.017	0.000	0.267*
Dy at 2–4 Hz	−0.211*	0.184	−0.105	0.050	0.214

* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

(26). In either case, it is important to consider gender-specific neuromotor dysfunctions due to environmental or occupational hazardous substances.

In this study, age was negatively associated with postural sway parameters in both boys and girls. Postural sway decreased linearly with age from 2 to 14 years (8); also, sway amplitude in 64 healthy children aged between 3.5 and 17 years decreased with age for boys, but not for girls (7). In another research, Usui et al. (9) reported that upright postural sway decreased markedly between the ages of 3 to 5 years and then slowly after six years of age. By contrast, postural sway in adults is known to increase with advancing age (27, 28). Thus, postural sway appears to decrease with increasing age in children at ages of less than 15 years, although postural stability in adults is worse in elderly people. Nevertheless, it may be difficult to identify the turning-point of aging effects on postural sway, because there seems to be no single anthropometric factor that explains the variations in body-balancing movements during standing (1).

Concerning the hand tremor, age was positively related to two tremor parameters only for the dominant hand of girls (Table 4); while, the age-range interval of our study was within 12 months. On the other hand, the amplitude of finger tremor has been reported to be significantly higher in 22 children at

Table 4 Relations of age, height, body weight, and heart rate to tremor intensity in Japanese children: Results of multiple regression analysis

	Standard regression coefficient				Multiple correlation coefficient
	Age	Height	Body weight	Heart rate	
155 boys:					
Dominant hand					
Total	−0.011	0.249*	−0.091	0.179*	0.240
1–6 Hz	−0.063	0.288*	−0.052	0.165*	0.267*
6–10 Hz	0.079	0.167	−0.146	0.232**	0.262*
10–14 Hz	0.018	0.192	−0.154	0.157	0.201
Non-dominant hand					
Total	−0.151	0.350**	−0.067	0.188*	0.323**
1–6 Hz	−0.124	0.360**	−0.049	0.183*	0.331**
6–10 Hz	−0.154	0.298*	−0.130	0.198*	0.289*
10–14 Hz	−0.168	0.263*	−0.099	0.153	0.255*
148 girls:					
Dominant hand					
Total	0.213*	−0.078	−0.010	−0.003	0.202
1–6 Hz	0.141	−0.029	0.027	−0.055	0.148
6–10 Hz	0.290**	−0.106	−0.052	0.042	0.288*
10–14 Hz	0.267**	−0.134	−0.080	0.041	0.288*
Non-dominant hand					
Total	0.064	0.080	−0.137	0.076	0.156
1–6 Hz	0.065	0.075	−0.129	0.052	0.139
6–10 Hz	0.067	0.037	−0.101	0.142	0.184
10–14 Hz	0.028	0.080	−0.131	0.103	0.156

* $p<0.05$, ** $p<0.01$.

Table 5 Partial correlation coefficients[a] between tremor intensity and postural sway parameters among Japanese children

	Tremor intensity (1–6 Hz)			
	155 boys		148 girls	
	Dominant hand	Non-dominant hand	Dominant hand	Non-dominant hand
Eyes open				
Sway area	0.027	0.008	0.197*	0.129
Transversal sway (Dx)	0.036	0.024	0.091	−0.021
Dx at 0–1 Hz	0.028	0.007	0.102	−0.014
Dx at 1–2 Hz	0.217**	0.166*	0.234**	0.249**
Dx at 2–4 Hz	0.226**	0.174*	0.314***	0.295***
Sagittal sway (Dy)	0.005	−0.070	0.114	0.076
Dy at 0–1 Hz	−0.014	−0.082	0.109	0.083
Dy at 1–2 Hz	0.121	0.107	0.197*	0.234**
Dy at 2–4 Hz	0.220**	0.177*	0.270**	0.278***
Eyes closed				
Sway area	0.026	0.018	0.274***	0.220**
Transversal sway (Dx)	0.039	0.031	0.123	0.067
Dx at 0–1 Hz	0.044	0.029	0.144	0.081
Dx at 1–2 Hz	0.068	0.075	0.154	0.170*
Dx at 2–4 Hz	0.156	0.115	0.224**	0.212*
Sagittal sway (Dy)	0.100	0.086	0.156	0.204*
Dy at 0–1 Hz	0.068	0.053	0.177*	0.219**
Dy at 1–2 Hz	0.107	0.091	0.219**	0.220**
Dy at 2–4 Hz	0.179*	0.153	0.224**	0.238**

[a] Age, height and heart rate were used as confounders in all analyses.
* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

ages 7–9 years than in 22 children at ages 13–15 years (29), which included boys and girls. Also, tremor acceleration in subjects at ages 10–90 years has been reported to increase with advancing age, although the author did not seem to consider sex difference (30). From these reports, hand tremor parameters appear to depend on age, but the relation remains uncertain in children aged between 7 and 15 years.

In the present study, height was positively associated with

postural sway and hand tremor parameters. Usui et al. (9) described that the sway of the center of gravity was larger in tall than in short people, and correction for height was made. On the contrary, some research showed that height and age were negatively associated with sway variables (4, 7). In children at the developmental stage (i.e., aged between 4 and 10 years), the relationship between age and height seems to be considerably strong; for example, the correlation coefficient was 0.96 for 76 children aged between 2 and 14 years (8). For this reason, the relation of height to neuromotor parameters may have been affected by the strong collinearity between age and height in the multivariate analysis. Nevertheless, postural sway would not be independent of height of the center of gravity.

Body weight was related positively to the sagittal sway in the boys of this study, and negatively to the transversal sway in the girls. Also, body weight had a very close relation to height in the boys and girls. Likewise, Riach and Hayes (8) reported that the correlation coefficient between body weight and either age or height was 0.90 or 0.93, respectively. In the latter case, the effect of body weight may be changeable due to the collinearity between height and body weight. Hence, Bhattacharya et al. (4), attempting to clarify the effect of early lead exposure on children's postural balance, used body weight and other possible confounders, together with age, sex, and height, as independent variables of multiple regression analysis. On the basis of these findings, it is speculated that if the study population did not include children with excessive obesity or emaciation, body weight would hardly affect postural sway by controlling for age and height. Rather, too many covariates should not be used to avoid overmatching in an intermediate between exposure and disease (31), and the estimation accompanying such overmatching may be biased toward the null hypothesis (32).

In the present study, heart rate was positively related to tremor intensity at 1–6 Hz and 6–10 Hz in the boys. A similar finding has been observed in female adults (10). Originally, inapparent minor tremors on the human body surface were described in 1930s (33, 34), and Ozaki et al. (35) reported that heart beat, in addition to muscle tonus, might play an important role in minor tremors under various physiological conditions. Likewise, Gallasch and Kenner (36) proved that the R peak on the electrocardiogram triggered microvibrations in the supported arm. In our study, since the subjects were instructed to hold a light stylus, free of body contact or any obstacles, the effect of heart rate on hand tremor may have readily been detectable. In this sense, heart rate should be checked for subjects with palpitation.

Postural sway parameters in the boys and girls of our study were significantly linked with tremor intensity at 1–6 Hz (Table 5). This finding is concordant with two previous reports (3, 11). It is likely that involuntary postural sway at 1–4 Hz intervened directly in finger tremor via the humerus and forearm, because the frequency of tremor was almost similar to that of postural sway.

In conclusion, age, sex, and height are suggested to be the major developmental variables contributing to neuromotor measures for children. Heart rate and postural sway can also affect hand tremor not only in adults but also in children. In addition, body weight at birth and mother's drinking habit during pregnancy were suggested to be associated with the neuromotor function in Japanese boys, but the implication of these two factors awaits further research. The most important thing is that these neuromotor tests in a series of study should be carried out carefully by one trained examiner, although such tests seem to be apparently simple and feasible for examiners. Letz (37) stresses the importance of motivational influences that can affect the reliability and the validity of the tests, and two or more examiners may produce a measurement bias (38). In fact, there are only a few studies that have provided statistically significant findings obtained using the CATSYS system (5, 11, 22, 23, 39, 40, 41).

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ORIGINAL ARTICLE

Katsuyuki Murata · Mineshi Sakamoto · Kunihiko Nakai
Miwako Dakeishi · Toyoto Iwata · Xiao-Jie Liu
Hiroshi Satoh

Subclinical effects of prenatal methylmercury exposure on cardiac autonomic function in Japanese children

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Abstract *Objectives*: The subclinical effects of prenatal exposure to methylmercury from fish consumption on the cardiac autonomic function were assessed in 136 Japanese 7-year-old children recruited for this study. *Methods*: Samples of child's hair and dry umbilical cord preserved were collected, and hair mercury and cord tissue methylmercury concentrations were determined as current and prenatal exposure biomarkers, respectively. Cardiac autonomic indicators of parasympathetic and sympathetic activities were calculated from the electrocardiographic RR intervals measured. *Results*: In the children, the cord tissue methylmercury (0.017–0.367, median 0.089 μg/g) was not significantly correlated with the hair mercury (0.43–6.32, median 1.66 μg/g). The cord tissue methylmercury was related negatively to parasympathetic components of cardiac autonomic indicators ($P < 0.05$) and positively to sympathovagal indices ($P < 0.05$), even after correction for possible confounders such as age and sex, although the hair mercury was not significantly correlated with any cardiac autonomic indicators. *Conclusions*: Despite the potential limitations involved in the retrospective study, these findings suggest that prenatal methylmercury exposure (median of estimated maternal hair mercury at parturition, 2.24 μg/g) may be associated with reduced parasympathetic activity and/or sympathovagal shift.

K. Murata (✉) · M. Dakeishi · T. Iwata
Department of Environmental Health Sciences,
Akita University School of Medicine, 010-8543 Akita, Japan
E-mail: winestem@med.akita-u.ac.jp
Tel.: +81-18-8846082
Fax: +81-18-8362608

M. Sakamoto · X.-J. Liu
Department of Epidemiology,
National Institute for Minamata Disease, Minamata, Japan

K. Nakai · H. Satoh
Department of Environmental Health Sciences,
Tohoku University Graduate School of Medicine, Sendai, Japan

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Introduction

Since 1956, methylmercury pollution has been found near Minamata Bay in Japan, and the pollution, which was discharged from a chemical factory, was so widespread that the number of victims increased to more than 2,000. Also, all children identified as suffering from the most severe form of congenital Minamata disease (i.e., methylmercury poisoning) expressed mental retardation, primitive reflexes, cerebellar ataxia, disturbances in physical growth, dysarthria, and limb deformities (Harada 1995). The diagnosis of Minamata disease was easy in typical and severe cases, but was difficult in "mild" cases ranging from partly damaged to healthy (Igata 1993). On the other hand, recent interests of investigators seem to be the subclinical, but not clinical, effects of low-level exposures to methylmercury on child neurodevelopment. Therefore, it is crucial to discover more sensitive and reliable outcome variables, together with specific exposure biomarkers.

Concerning the neurodevelopmental effects of prenatal methylmercury exposure, the Faroese birth cohort study and Seychelles child development study have provided different conclusions and several uncertainties remain because of issues involved in exposure biomarkers, neurodevelopmental endpoints, and confounders (National Research Council 2000). The implications, judging from these two comparative studies, are that neuropsychological measures may depend on social and cultural factors including race and language, and that a key to resolving whether exposure has harmed the fetus may lie in neurophysiological measures such as brainstem auditory-evoked potentials and electrocardiographic (ECG) RR interval variability (Murata and Dakeishi 2005). In addition, the most serious problem may be the extreme scarcity of data from any place other than the Faroe Islands

and Seychelles (Davidson et al. 1998; Grandjean et al. 1997, 2004; Myers et al. 2003; Sørensen et al. 1999). Since each country has a unique food culture, further research in the third area should be done to draw a conclusion on risk assessment of methylmercury (Clarkson 2002).

Short-term variations in heart rate are seen at all ages and are an important sign of normal homeostatic mechanisms of the cardiovascular system (Finley and Nugen 1995). Especially, beat-to-beat heart rate variability (HRV) with frequency-domain analysis has emerged as a noninvasive method to assess cardiac autonomic activity quantitatively. Also, HRV has been reported to be highly reproducible under standardized conditions (Batten et al. 2000; Massin and von Bernuth 1997; Silvetti et al. 2001). By using the HRV, it has been suggested that subjects with low HRV had an adverse cardiovascular risk profile and an elevated risk of incident coronary heart disease (CHD) and death (Dekker et al. 2000), or that prenatal methylmercury exposure was associated with low HRV (Grandjean et al. 2004). In this way, HRV is considered to be a promising tool to provide a marker of less favorable health (Murata and Araki 1996), and will be much applicable to risk assessment on child neurodevelopment.

We conducted a cross-sectional study on the developmental effects of methylmercury exposure in Japanese 7-year-old children (Murata et al. 2004). According to the food frequency questionnaire (FFQ) done at the time of the examination, fish that their mothers had eaten was sardine, flatfish, mackerel, codfish, skipjack tuna, salmon, eel, swordfish, tuna, etc.; and, a significant relationship between the estimated daily mercury intake from seafood and current hair mercury concentration was observed in the mothers ($r=0.335$), indicating that main methylmercury exposures in Japanese women resulted from fish consumption (Iwasaki et al. 2003). In the study, though we used maternal hair mercury concentration at that time as a proxy for mercury exposure at birth, it might not be the best biomarker of prenatal exposure. Also, it has been suggested that a myth that hair mercury concentration reflects the methylmercury concentration circulating in the blood may collapse if a study population includes subjects with artificial hair-waving (Dakeishi et al. 2005). The mercury level in the umbilical cord tissue has been reported to be associated well with that in cord blood (Dalgård et al. 1994), and most Japanese families treasure a small piece of the umbilical cord of the child as a birth memento (Akagi et al. 1998). For this reason, we tried to collect dry umbilical cord samples of the children from parents who participated in our study. Then, a retrospective study with the methylmercury concentration in the cord tissue was carried out to assess the subclinical effects of prenatal exposure on cardiac autonomic function, as well as the reaction time that was used in both the Faroes and Seychelles studies (Grandjean et al. 1997; Myers et al. 2003).

Materials and methods

Subjects

The nature of the procedures used in this study was explained to parents with a first grader, of 28 elementary schools in Akita and Tottori Prefectures, Japan, 14 of which were located near the fishing harbor. In accordance with the preceding studies on the risk assessment of methylmercury (Grandjean et al. 1997; Murata et al. 1999a), mothers and their children, who were born in the period of April 2nd 1995 to April 1st 1997, were invited for this study during the period of July–September in 2002 and 2003. In Japan, there were many mines and smelters 30 years ago, and it was probable that soil or water has been contaminated by lead, copper or cadmium; therefore, the study population did not include those who came from such areas. Of 327 participating subjects (Murata et al. 2004), 145 provided a piece of dry umbilical cord sample for our proposal. In the light of the previous exclusion criteria (Murata et al. 2004), two children with neurological disorders (spinal progressive muscular atrophy and epilepsy), one with a congenital malformation (cleft palate), and six with low birth weight (less than 2,500 g) were counted out. In total, 136 children were enrolled as subjects of this research. The study protocol was approved by the ethical review committee at the Akita University School of Medicine.

Methods

Hair samples were collected, by cutting strands of hair close to the scalp, from the occipital area in all children. The hair length ranged from 1 to 30 cm (mean 10 cm). Total mercury in aliquots of dried hair samples (15–20 mg), rinsed with acetone, was determined by the cold vapor atomic absorption spectrophotometry method at the National Institute for Minamata Disease (Akagi et al. 2000; Sakamoto et al. 2004). The method involved sample digestion with HNO_3, $HClO_4$, and H_2SO_4 followed by reduction to elemental mercury vapor by $SnCl_2$. The detection limit was 0.01 ng/g. Methylmercury in each umbilical cord sample, after crushing it and removing blood cells from it with tweezers, was determined by gas chromatography with electron capture detector (GC-ECD) according to the method of Akagi et al. (2000), in order to avoid contamination due to inorganic mercury compounds (e.g., mercurochrome of disinfectant). The method involved sample digestion with KOH–ethanol and subsequently, under a slightly acidic condition, the fatty content was removed using *n*-hexane. After extraction with dithizone–toluene, methylmercury was back-extracted with a slightly alkaline sodium sulfide solution. The excess sulfide ions were then removed as hydrogen sulfide by purging with nitrogen gas after slight acidification with HCl solution. Methylmercury was then re-extracted with a small por-

tion of dithizone–toluene. The extract was washed with NaOH solution to remove the excess dithizone, and was then slightly acidified with HCl and analyzed using GC-ECD. The detection limit was 0.01 ng/g. The precision and accuracy of these methods were repeatedly verified by inter-laboratory calibration exercises including the analysis of standard reference material such as IAEA-085, 086 and 142. The methylmercury in the cord tissue and total mercury in hair were used as the prenatal and current exposure biomarkers, respectively.

A detailed survey of medical records during pregnancy and delivery, including smoking/drinking habit, gestation period and birth weight, past/present history of illness in the child, and dietary habits in the mother, was conducted by one medical doctor at the elementary schools or civic centers where neurodevelopmental examinations were done. Also, the medical data were checked by reference to the maternal and child health booklet that she had kept for her child and in which she and medical doctors recorded during pregnancy and at the time of parturition, health examination or medical observation. At that time, height and body weight in the child were measured to compute body mass index (BMI, kg/m^2).

The HRV was measured by a trained examiner, using the ECG-Amplifier 1271SP (NEC-Sanei Co., Japan) connected to an analog-to-digital converter (sampling time, 1 ms) and a computer. After the subject remained quietly in the supine position at least for 5 min, 300 RR intervals were measured, and consecutive 100 RR intervals with the minimal standard deviation were automatically extracted to avoid measurement error (Grandjean et al. 2004; Murata and Araki 1996; Murata et al. 1992). The power spectrum of RR intervals was computed by autoregressive spectral analysis. The spectrum of each of two components, i.e., the high-frequency (HF) component at the center frequency of 0.15–0.4 Hz and low-frequency (LF) component at 0.01–0.15 Hz, was separated by component analysis (Murata et al. 2004). Each component coefficient of variation (i.e., CCV_{HF} and CCV_{LF}, %) was defined as the ratio of the square root of each component power spectral density (PSD_{HF} and PSD_{LF}, ms^2) to the mean value of RR intervals (ms). The CCV_{HF} and PSD_{HF} reflect the vagal activity (Murata and Araki 1996; Pagani et al. 1986; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996), and the CCV_{LF} and PSD_{LF} are thought to represent the sympathetic activity. Also, the LF/HF ratio and %LF are calculated from PSD_{LF}/PSD_{HF} and $100 \times PSD_{LF}/(PSD_{LF} + PSD_{HF})$, respectively. The heart rate-corrected QT interval (QTc) was automatically calculated by the ECG-9202 electrocardiograph (Nihon Kohden Co., Japan), from the RR and QT intervals on ECG according to Bazett's formula (Murata et al. 1999b). Reaction time to a sound stimulus was measured with right and left hands, using the Neurobehavioral Test System (CATSYS 2000, Danish Product Development Ltd, Denmark) (Després et al. 2000; Murata et al. 2004).

Data analyses

Logarithmic transformation of methylmercury and mercury concentrations was used because of skewed distributions. Likewise, outcome variables excluding %LF, RR and QTc intervals, and reaction time, required logarithmic transformation to obtain a better fit of the regression models. The correlation coefficients between exposure biomarkers and outcome variables were calculated; also, the relations of two exposure levels (i.e., methylmercury in the cord tissue and mercury in hair) to each endpoint were tested by multiple regression analysis to adjust for age, sex, gestation period, and smoking and drinking status during pregnancy (and, plus BMI). Smoking (or drinking) status was scored as "nonsmoker or ex-smoker (or nondrinker)" = 0 and "smoker (or drinker)" = 1. All analyses, with two-sided P values, were performed using the Statistical Package for the Biosciences (Murata and Yano 2002).

Results

Table 1 represents the characteristics at birth and at the time of examination in Japanese children with and without umbilical cord. In the 136 children with umbilical cord, the methylmercury level in the cord tissue ranged from 0.017 to 0.367 μg/g (median 0.089), and the current mercury level in hair was between 0.43 and 6.32 μg/g (median 1.66). The methylmercury in the cord tissue was not significantly correlated with the mercury in hair ($r = -0.107$, $P = 0.217$). Among 136 mothers of the children with umbilical cord, 15 mothers reported drinking a little bit of alcohol during pregnancy, but no mothers ate shark or whale. In the children with umbilical cord, there were seen to be no abnormal ECG findings. The cardiac autonomic indicators or reaction time did not differ significantly between the 75 boys and 61 girls (Table 2).

Among the 136 children with umbilical cord, the methylmercury in the cord tissue was correlated negatively with the CCV_{HF} (Fig. 1) and PSD_{HF} ($r = -0.190$, $P = 0.027$), and positively with the LF/HF ratio (Fig. 1) and %LF ($r = 0.207$, $P = 0.016$), using the Pearson product–moment correlation coefficient; however, the remaining HRV parameters and reaction time were not significantly correlated with either the methylmercury in the cord tissue or mercury in hair ($P > 0.05$). Also, the methylmercury in the cord tissue was significantly related to the CCV_{HF} ($P = 0.022$), PSD_{HF} ($P = 0.023$), LF/HF ratio ($P = 0.017$), and %LF ($P = 0.017$) when controlling for possible confounders (Table 3). These results were similar in adding BMI in independent variables of Table 3.

Table 1 Characteristics at birth and at the time of examination in Japanese children at age 7 years

	136 children with umbilical cord			182 children without umbilical cord		
	Mean (or, Number, %)	SD	5 and 95 percentiles	Mean (or, Number, %)	SD	5 and 95 percentiles
Records during pregnancy and delivery						
Body weight at birth (g)	3,203	359	2,656–3,886	3,133	460	2,420–3,811
Gestation period (weeks)	39.1	1.3	37–41	39.1	1.4	37–41
Natural delivery	120 (88.2 %)			163 (89.6 %)		
Mother's smoking during pregnancy	10 (7.4 %)			14 (7.7 %)		
Mother's drinking during pregnancy	15 (11.0 %)			26 (14.3 %)		
Past history–febrile convulsion	7(5.1 %)			22 (12.1 %)		
Data at the time of examination						
Age (years)	6.9	0.3	6.4–7.4	6.9	0.3	6.4–7.4
Girls	61 (44.9%)			95 (52.2%)		
Height (cm)	120.0	4.7	113–128	119.2	5.4	111–128
Body weight (kg)	24.3	3.9	19.3–32.8	23.1	3.9	18.2–31.2
Body mass index (kg/m^2)	16.8	2.0	14.2–21.6	16.2	2.0	13.7–20.2
Child's hair mercury (μg/g)	1.90	1.00	0.77–4.06	1.88	1.06	0.68–3.98

There were no significant differences in all items except for body weight and body mass index between the two children groups ($P > 0.05$, Student t test or χ^2 test with Yates correction)

Table 2 Cardiac autonomic indicators and reaction time in Japanese boys and girls at age 7 years (see the Materials and methods)

	Geometric mean (5 and 95 percentiles)		P value[a]
	75 boys	61 girls	
ECG RR interval variability			
CCV_{LF} (%)	3.61 (1.65–7.02)	4.14 (2.53–7.40)	0.080
CCV_{HF} (%)	3.35 (1.05–8.18)	3.74 (2.01–7.49)	0.259
PSD_{LF} (ms^2)	673 (138–2670)	842 (262–2935)	0.172
PSD_{HF} (ms^2)	579 (41.8–4384)	686 (155–3312)	0.439
%LF	50.0 (30.6–71.7)	51.2 (39.4–71.6)	0.746
LF/HF ratio	1.16 (0.19–6.42)	1.23 (0.42–6.34)	0.760
ECG parameters			
RR interval (ms)	718 (609–854)	700 (606–835)	0.160
QTc interval (ms)	393 (375–419)	394 (375–419)	0.714
Reaction time			
Right hand (ms)	344 (278–477)	346 (281–458)	0.919
Left hand (ms)	365 (292–503)	370 (297–475)	0.740

[a]Student t test

Discussion

In this study, the feasibility to collect umbilical cord samples depended on the original tradition of keeping a piece of the umbilical cord in a paulownia case. As the result, we were able to analyze the effects of prenatal methylmercury exposures among the 136 children. There were no differences in all items, except body weight and BMI, between the children with and without umbilical cord as shown in Table 1. The methylmercury in the cord tissue was considerably lower in 136 children of this study than in patients with Minamata disease, whose levels were between 0.15 and 4.7 μg/g (median 1.63) (Akagi et al. 1998). The hair mercury equivalent concentration can be calculated from the equation of Akagi et al. (1998): maternal hair mercury at parturition (in microgram per gram) = 25.24× Cord-tissue concentration (in μg/g dry weight). Accordingly, the estimated mercury in maternal hair ranges from 0.43 to 9.26 μg/g (median 2.24) for our subjects, and it was lower than those in the Faroe Islands (0.2–39.1, median 4.5 μg/g) and Seychelles (0.9–25.8, mean 6.5 μg/g) (Grandjean et al. 1992; Myers et al. 2003).

The principal finding of this retrospective study is that methylmercury exposure at parturition was associated with decreased vagal modulation (i.e., low CCV_{HF} and PSD_{HF}) of cardiac autonomic function in Japanese children aged 7 years. This agrees with the outcomes of the Faroes cohort at age 14 years (Grandjean et al. 2004), which included the depressed PSD_{LF}. The PSD_{HF} was significantly lower in 9 patients with fetal Minamata disease than in 13 control subjects (Oka et al. 2002). In workers, vagal hypoactivity appears to result from occupational exposures to lead, solvents, and vibration (Murata and Araki 1996), and shift work (Ishii et al. 2005), but it is unlikely that the Japanese children have been exposed to such hazardous factors in the environment. However, postnatal mercury exposure in this study was not significantly related to the parasympa-

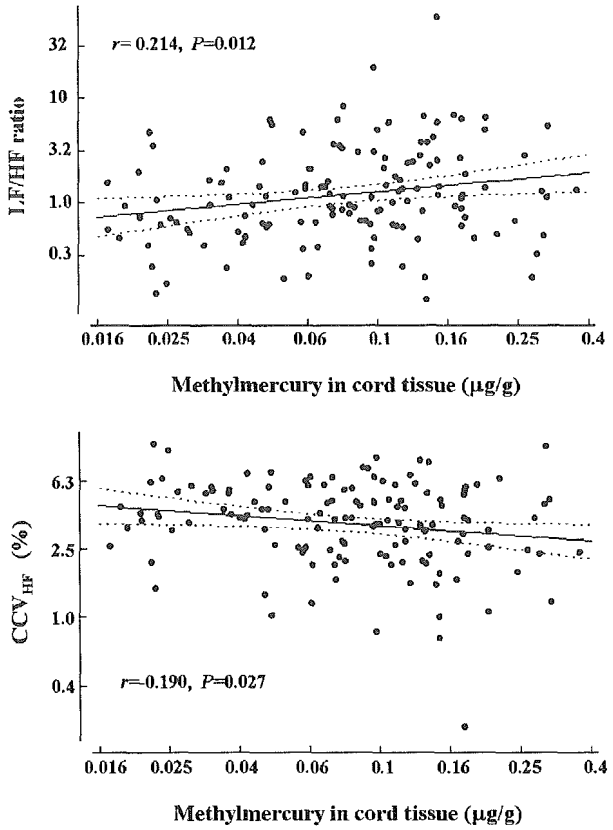

Fig. 1 Pearson product–moment correlations between methylmercury concentrations in cord tissue and cardiac autonomic indicators (*CCV_{HF}* and *LF/HF ratio*) in 136 Japanese children. *Dotted lines* represent a 95% confidence limit of linear regression

thetic tone. Though the statistical significance may be reduced by multiple significance test, it is suggested that increased methylmercury exposures may induce depressed parasympathetic activity in the fetus.

The cardiac autonomic function in children of the Faroes study, together with our study, was assessed by one researcher in the same manner (Grandjean et al. 2004), but the two outcomes did not agree in regard to postnatal mercury exposures. In our study, only one standardized regression coefficient between the CCV_{LF} and child's hair mercury was positively significant ($P = 0.041$). By contrast, the CCV_{LF} and PSD_{LF} at age 7 years in the Faroes study were negatively associated with mercury levels in the child's hair (Grandjean et al. 2004); while, any cardiac autonomic endpoints at age 14 years were not affected by postnatal mercury exposures. One possible explanation for this discordance is as follows: prenatal mercury exposures were considerably higher in the Faroes study than in our study, but postnatal mercury exposures were conversely low in the former study (i.e., geometric mean of hair mercury, 0.60 μg/g for 7-year-old children and 0.96 μg/g for 14-year-old children). Also, a significant correlation between the cord blood mercury and 7-year-old child's hair mercury was observed in the Faroes study. For this reason, Grandjean et al. (2004) described that the Faroes study might not allow a clear separation of the effect of exposures at different developmental stage. Apart from autonomic nervous effects of methylmercury, the CCV_{LF} and PSD_{LF} seem to be reduced by chronic high-level exposures to lead and alcohol, but these indicators tend to be unaffected by low-level exposures (Murata and Araki 1991, 1996; Murata et al. 1995). Thus, prenatal methylmercury exposure levels in the Faroes cohort are speculated to have been high enough to affect the sympathetic activity. Taken together, these findings suggest that postnatal exposures to methylmercury at levels of less than 4 μg/g in hair may not influence cardiac autonomic function potently, and such an effect may be reversible, if it exists.

In our research, the LF/HF ratio and %LF, representing a sympathovagal balance (Pagani et al. 1986;

Table 3 Relations of methylmercury in cord tissue (MHg), total mercury in child's hair (HHg) and possible confounders to cardiac autonomic indicators and reaction time in 136 Japanese children at age 7 years: results of multiple regression analysis (see the Materials and methods)

Endpoints	Standardized regression coefficient							R^2
	Log(MHg)	Log(HHg)	Age	Sex	Gestation period	Smoking	Drinking	
ECG RR interval variability								
Log(CCV_{LF})	−0.018	0.179*	−0.157	0.160	−0.048	−0.027	0.036	0.077
Log(CCV_{HF})	−0.196*	0.060	−0.086	0.117	−0.157	−0.014	0.149	0.097
Log(PSD_{LF})	−0.036	0.154	−0.146	0.129	−0.071	−0.031	0.077	0.064
Log(PSD_{HF})	−0.195*	0.041	−0.076	0.089	−0.164	−0.017	0.169*	0.099
%LF	0.205*	0.081	−0.044	0.011	0.138	−0.008	−0.137	0.098
Log(LF/HF ratio)	0.204*	0.091	−0.042	0.011	0.134	−0.008	−0.135	0.098
ECG parameters								
RR interval	−0.078	−0.087	0.029	−0.113	−0.113	−0.025	0.184*	0.081
QTc interval	−0.0002	0.071	−0.012	0.051	0.025	0.120	−0.023	0.020
Reaction time								
Right hand	−0.080	0.060	−0.150	0.004	−0.026	−0.078	0.072	0.041
Left Hand	0.040	−0.017	−0.223*	0.000	−0.015	−0.148	0.038	0.074

*$P < 0.05$

Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996), were positively connected with prenatal methylmercury exposure. This finding suggests that a sympathodominant state may have been induced by vagal hypoactivity due to increased mercury exposures, which were within low levels compared to those in the Faroes study (Grandjean et al. 2004). By the way, an increased mortality from ischemic heart disease was found in male Slovenian miners (standardized mortality ratio, 1.66), who had been exposed to inorganic mercury (Boffetta et al. 2001). Also, a high intake of mercury from freshwater fish was involved in an excess risk of myocardial infarction, as well as death from CHD, in eastern Finnish men with hair mercury levels of less than 15.7 μg/g (Salonen et al. 1995) and the risk factor-adjusted odds ratio for myocardial infarction in the highest as compared with the lowest quintile of toenail mercury was 2.16 (95% confidence interval, 1.09–4.29) in an European study (Guallar et al. 2002). On the contrary, American data did not support an association between total mercury exposure and the risk of CHD (Yoshizawa et al. 2002) and the mortality from heart diseases was not increased among 1,483 patients with Minamata disease (Tamashiro et al. 1985). Rather, fish consumption appears to be inversely associated with fatal CHD (He et al. 2004). Apart from mercury exposures, a sympathodominant state and depressed vagal tone have been reported to be associated with the risk for developing CHD (Liao et al. 1997; Lombardi et al. 1987). In spite of several problems related to the type, route, level, and phase of exposures, therefore, an assumption that the increased risk for CHD in adults exposed to mercury may originate from sympathovagal shift of the HRV remains pending. A prospective study of adults with data of HRV parameters, as well as the mercury level, will be necessary to examine this.

Concerning the ECG parameters, cardiac abnormalities such as irregular pulse, ventricular ectopic beats and prolongation of the QT interval, following ethylmercury exposure have been reported in patients who were hospitalized during the Iraqi poisoning epidemic in 1956 (Jalili and Abbasi 1961). In our study, however, no significant link was observed between mercury exposure biomarkers and either the ECG RR interval or QTc. Since the former was not subclinical but clinical cases, this inconsistency would have been attributable to the difference of exposure levels.

Reaction time in the children of our study did not have a close relation to the cord tissue methylmercury or current hair mercury. Likewise, the Seychelles study, with 9-year-old children, failed to find any significant relationship between the mercury in maternal hair at birth and continuous performance task including hit reaction time, attentiveness, and risk-taking (Myers et al. 2003). In contrast, the Faroes study, using a continuous performance test consisting of total number of missed responses and the average reaction time, observed a significant association with the mercury in cord blood in 7- and 14-year-old children (Grandjean et al. 1997, 2002); also, this association was statistically significant considering the effect of PCB (Grandjean et al. 2001). For this reason, we could not derive a conclusion from the available facts with regard to the neuromotor effect of methylmercury. In fact, the type of stimulus presented in the reaction time test differed among three studies.

One limitation of this retrospective study is that coexposure levels of common contaminants (PCB, chlorinated pesticides, lead, cadmium, etc.), selenium, and omega-3 fatty acids (Grandjean et al. 1992, 2001; Guallar et al. 2002; National Research Council 2000; Sakamoto et al. 2004; Yoshizawa et al. 2002), other than methylmercury could not be determined. The major studies addressing the risk assessment of methylmercury (Davidson et al. 1998; Grandjean et al. 1997, 2004; Kjellström et al. 1989; Myers et al. 2003), except for one Faroes study (Grandjean et al. 2001) did not evaluate the interactions of these coexposures. Such substances in Japan will be assessed in the Tohoku Study of Child Development that is now ongoing (Nakai et al. 2004). Recall bias also could not be avoided more or less, although we confirmed medical records by reference to the maternal and child health booklet. Regarding a possibility that children whose mothers ate too much fish during pregnancy were not included in this study, the children residing in both nonfishing areas and fishing areas in Japan were enrolled as the study population (Murata et al. 2004). Two percent of Japanese reproductive aged females have been reported to exceed the hair mercury level of 5 μg/g (Yasutake et al. 2003), and the estimated hair mercury levels at parturition in our study seem to have shown a similar distribution. In addition, possible confounders such as age, sex, and gestation period were considered in the data analysis.

Another possible limitation is about the HRV. The Task Force on HRV Standards points out that spectral analysis of 24-h recordings may not provide detailed information about autonomic modulation of the RR interval due to the lack of stationarity, and recommends that controlled 5-min recordings processed in the frequency domain be used to study pathophysiologic mechanisms (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology 1996). We measured 300 RR intervals in the supine position (i.e., approximately for 4 min in children or for 5 min in adults), and HRV parameters were calculated from consecutive RR intervals with minimal deviation; i.e., the process of RR-interval data extraction for spectral analysis does not depend on examiners. Liao et al. (1997) computed LF- and HF-PSDs of HRV from 2-min beat-to-beat heart rate data in the atherosclerosis risk in communities (ARIC) study. On the other hand, it is also indicated that the power of heart-rate modulation in the frequency range considered to reflect sympathetic activity (PSD_{LF}) tends to be lower than that of the actual activity and may be misinterpreted as a deficiency of sympathetic

outflow (Hilz and Dütsch 2005). In applying spectral analysis with fast Fourier transformation, the ARIC study used records of beat-to-beat data of 2.5 min to capture a few cycles of low-frequency heart rate fluctuation (Liao et al. 1997). We have employed the autoregressive spectral model, and the model does not seem to have caused such problems (Grandjean et al. 2004; Ishii et al. 2005; Murata et al. 1992, 1995, 2005)). Thus, it is suggested that our findings were not heavily influenced by measurement error. In any case, it may make no sense to compare the value of PSD_{LF} or CCV_{LF} between two studies conducted by different researchers.

In conclusion, our data suggest that prenatal methylmercury exposure may be associated with decreased vagal tone or sympathovagal shift in Japanese children, despite the potential limitations involved in the retrospective study. This evidence possesses two significances. The data were provided from another area other than the Faroe Islands and Seychelles. Another is that the usefulness of HRV for subclinical risk assessment on child neurodevelopment was stressed.

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REPRODUCTIVE TOXICOLOGY

Norio Sugawara · Kunihiko Nakai
Tomoyuki Nakamura · Takashi Ohba · Keita Suzuki
Satomi Kameo · Chieko Satoh · Hiroshi Satoh

Developmental and neurobehavioral effects of perinatal exposure to polychlorinated biphenyls in mice

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Abstract Because behavioral deficits associated with gestational exposure to polychlorinated biphenyls (PCBs) have been a concern, we studied the developmental and neurobehavioral effects of perinatal exposure to Aroclor 1254 (A1254), a commercial mixture of PCBs, in mice. The PCB mixture (A1254; 0, 6, 18, and 54 mg/kg body weight) was administered to pregnant mice (C57BL/6Cr) every 3 days by gavage from gestational day (GD) 6 to postnatal day (PND) 20. Compared with the control, treatment with A1254 did not alter the maternal body weight during the gestation and lactation periods. The body weight of the offspring did not differ among treatments. To assess the effects on offspring following such exposure, physical and neurobehavioral development (i.e., pinna detachment, hair growth, eye opening, incisor eruption, grasp reflex, righting reflex, walking, negative geotaxis, and cliff avoidance) was observed before weaning. At PND 7, poor adult-like responses in negative geotaxis were observed in all exposed groups. When the offspring were at 8-week old, the PCB-treated (18 mg/kg body weight) mice showed a decreased walking speed in the open-field test, and a prolonged time to reach the platform in the water maze test. Spontaneous locomotion activity was not affected by PCB exposure at 9 weeks . These results showed that perinatal exposure to PCBs produces several behavioral alterations in mice. Although dose-dependent changes were not observed, the neurobehavioral effects such as a decreased walking speed in the open-field test and a prolonged time to reach the platform in the water maze test remained in adulthood after the seeming recovery from the transient delay in development before weaning.

N. Sugawara (✉) · K. Nakai · T. Nakamura · T. Ohba
K. Suzuki · S. Kameo · C. Satoh · H. Satoh
Department of Environmental Health Sciences, Tohoku University Graduate School of Medicine, 2-1 Seiryomachi, Aoba-ku, Sendai, 980-8575, Japan
E-mail: nsuga@ehs.med.tohoku.ac.jp
Fax: +81-22-717-8106

Keywords PCBs · Neurobehavioral effect · Development · Aroclor1254 · Perinatal exposure

Introduction

Polychlorinated biphenyls (PCBs) are widespread environmental pollutants that are known to be neurotoxic in humans and other animals (Seegal 1996; Tilson and Kodavanti 1998). The chemical inertness, thermal stability, and physical properties of a mixture of PCB congeners led to their industrial use as dielectric fluids, flame retardants, plasticizers, and heat-transfer fluids and dispersants for pesticides (WHO 1993; Carpenter 1998). Although the production of PCBs has stopped in most countries, they persist in the environment due to their chemical and thermal stability (Tanabe 1988), and their lipophilic nature has facilitated the bioaccumulation of these compounds and their metabolites in the food chain (Safe 1994).

The concern for their influence on humans has emerged from two tragic cases of poisoning from rice oil contaminated with PCBs and dibenzofurans. Children perinatally exposed to these chemicals showed a delayed development and various physical, emotional, and intellectual problems (Rogan et al. 1988; Hamada 1996; Yoshimura 1996). Studies on environmental exposure to PCBs demonstrated the association of the level of exposure with delays in neurological and cognitive development in humans (Nakai and Satoh 2002). In neonates, significant relationships were found between prenatal PCB exposure and performance impairments using the neonatal behavioral assessment scale (Stewart et al. 2000b). In 7-month-old children, negative associations between the concentrations of PCBs in breast milk and mental development were reported (Winneke et al. 1998). These developmental and neurobehavioral defects are primarily due to the exposure of the fetus through placental and lactational transfer.

Behavioral deviation, thyroid hormone perturbation (Crofton et al. 2000; Zoeller et al. 2000), and impair-

ments in long-term potentiation in the dentate gyrus (Gilbert et al. 2000) are observed in animals that are perinatally exposed to a commercial PCB mixture, Aroclor 1254 (A1254). A1254 is considered to have developmental neurotoxicity, because it contains a high proportion of highly chlorinated biphenyls. A1254 congeners comprise a significant proportion of ingested food and accumulated body burdens of PCBs (Dewailly et al. 1999; Patandin et al. 1999; Stewart et al. 2000a). In addition, reference doses derived from animal studies using A1254 and other commercial mixtures are used in the risk assessment of PCBs to humans (Cogliano 1998).

In this study, we studied the developmental and neurobehavioral effects of perinatal exposure to A1254 in mice. In addition, we aimed to establish if pre-weaning developmental delay induced by perinatal exposure to A1254 persists into adulthood. We performed the follow-up observation in mice up to 9-week old. For the assessment of physical and neurobehavioral development, we observed some developmental landmarks and reflexes before weaning. Afterwards, we carried out an open-field test, a water maze test and a spontaneous locomotion to evaluate the neurobehavioral effects of A1254 on mice on 8- and 9-week olds.

Materials and methods

Animals and exposure

Pregnant C57BL/6Cr mice (Nippon SLC Co. Ltd, Hamamatsu, Japan) were purchased on gestation day (GD) 2. The day when the vaginal plug was observed was defined as GD0. Upon arrival at the laboratory, the mice were housed individually in plastic cages (22×15×13 cm). They were kept in a temperature-controlled room (23±2°C) with a 12-h light–dark cycle (light phase, 0800–2,000). Pellet food (Crea Japan Inc., Tokyo, Japan) and tap water were available ad libitum. The mice were divided into four treatment groups (n=5–7). They were administered 0, 6, 18, and 54 mg/kg body weight of Aroclor1254 (A1254; AccuStandard Inc., New Haven, USA, Lot 124–191) in 10 ml/kg corn oil (Nacalai tesque Inc., Kyoto, Japan) every 3 days by gavage from GD6 to postnatal day (PND) 20. We chose the PCB dose range by taking into consideration previous results that showed clear neurobehavioral alterations following a perinatal exposure to A1254 (Lowest Observed Adverse Effect Level; LOAEL=11 ppm in diet; Storm et al. 1981). The day when the birth of offspring was observed in the morning was defined as PND0. On PND1, the litter size was adjusted to five–six and the all mice of both sexes were assigned to the assessment of development before weaning. The offspring were weaned at PND21. After weaning, we used only male mice to exclude the influence of the estrous cycle. Two or three males from each litter of each treatment group were used for three tests after 8-weeks. This study was carried out in accordance with the Guide of Animal Experimentation of Tohoku University Graduate School of Medicine.

Assessment of development before weaning

Before weaning, physical and neurobehavioral development was observed on PNDs 4, 7, 10, 12, 14, and 16 for the mice of both sexes. Pinna detachment, hair growth, day of eye opening, and incisor eruption were also recorded. Pups were tested according to the method of Fox (1965). The tests were conducted during the light phase between 1400 and 1900, each subject being tested at approximately the same time of the day. The following reflexes and responses were examined: grasp reflex (a pup grips a wire with its forepaws); righting reflex (a pup turns over on its four feet on the ground when placed on its back); walking (a pup moves using its four feet with the abdomen not touching the ground); negative geotaxis (when a pup is placed on a board inclined at 45° with the head pointing downward, it has to turn around 180°); and cliff avoidance (a pup turns and crawls away from the edge of a cliff when placed over it with the forepaws and face over the edge).

Behavioral test

An open-field test and the Morris water maze test were carried out when the male offspring were 8-week old as described by Kim et al. (2000). All the animals were handled by the same experienced person so that they would become used to being handled.

The open-field test was performed in a 50×50 cm square open-field apparatus. The floor was white and surrounded by a 20-cm-high white wall. The field was illuminated at 800 lx with fluorescent light 1.5 m above the apparatus. Each mouse was transferred from the home cage directly to the center of the open field, and a small box made of opaque plexiglas was then placed over the mouse. After the box was removed, the movement of the animal was recorded for 2 min using a CCD camera. The movement was then analyzed using an image analyzer (AXIS 60 video-tracking system, Neuroscience Inc., Tokyo, Japan); the latency in the start of walking, distance traveled by the mouse, and mean walking speed were calculated over a period of 2 min. The numbers of incidences of defecation and urination in the open field were counted by the observer. Before each trial, the floor was cleaned with 70% ethanol followed by wiping with wet cotton. All trials were carried out between 1000 and 1200.

Several days later after the open-field test, the offspring were subjected to the water maze test. The water maze was made of a 100-cm-diameter circular plastic pool, filled with water at a temperature of approximately 20°C. The water was made opaque by adding milk to prevent the mice from seeing a submerged platform and to increase the animal-background contrast for digital analysis. A round, 10-cm-diameter platform was placed

at a fixed location (e.g., at the center of one quadrant of the pool), submerged 0.5 cm below the surface of the water. Different visual cues were placed around the pool. Each mouse was released into the water at a fixed position, from the inside of the pool wall. A CCD camera, mounted at the center above the pool, recorded the movements of each mouse. The movements were then analyzed using an image analyzer; the time taken and the distance traveled by the mouse to reach the submerged platform, and the mean swimming speed were calculated. The mice were subjected to three trials on each of 5 days. When the mouse could not find the platform within 2 min, the test was terminated and the mouse was placed on the platform and left there for 20 sec. In this case, the time necessary to reach the platform was considered to be 2 min.

Spontaneous locomotion activity

The spontaneous locomotion activity of offspring was determined over a period of 24 h. Each mouse was put in a separate plastic cage similar to the home cage and left there for at least 12 h before the recording commenced. Each cage was placed in a small isolated chamber, which was kept at 23 ± 2°C and illuminated at 150–200 lx with a small fluorescent light with a 12 h-light-dark cycle (light phase, 0800—2000). Each cage was placed 15 cm below an infrared sensor (Model NS-AS01, Neuroscience Inc.) connected to a host computer, and spontaneous locomotion activity data were collected at 1-min intervals using an analysis system (AB system, Neuroscience Inc.).

Statistical analysis

Data are presented as mean ± SEM. One-way ANOVA was performed to determine statistically significant differences in open-field test, water maze test, and spontaneous locomotion activity data. Post hoc comparisons were performed using the Dunnet test. Body weight was evaluated by repeated-measures ANOVAs. With regard to data on physical and neurobehavioral development, adult-like responses and the complete appearance of a somatic feature were evaluated by the chi-square test for each time point. A value of $p < 0.05$ was considered significant.

Results

Maternal weight and pregnancy outcome

Compared with the control, treatment with A1254 did not alter maternal body weight during gestation and lactation. Moreover, the number of pups and implant sites was not affected by the treatment. There was no significant difference in the number of surviving offspring on PND1.

Physical and neurobehavioral development before weaning

The body weight of male offspring did not differ among treatments. The data on physical and neurobehavioral development (Fig. 1) showed some alterations in the ontogenic profile of perinatally exposed mice. For negative geotaxis, the ratio of adult-like responses of the three A1254-treated groups was lower than that of the control on PND7. For the righting reflex, the ratio was higher for that A1254-treated group with 6 mg/kg bod weight as compared the other A1245-treated groups on PND4. All the other responses (e.g., pinna detachment and hair growth) were not affected by treatments.

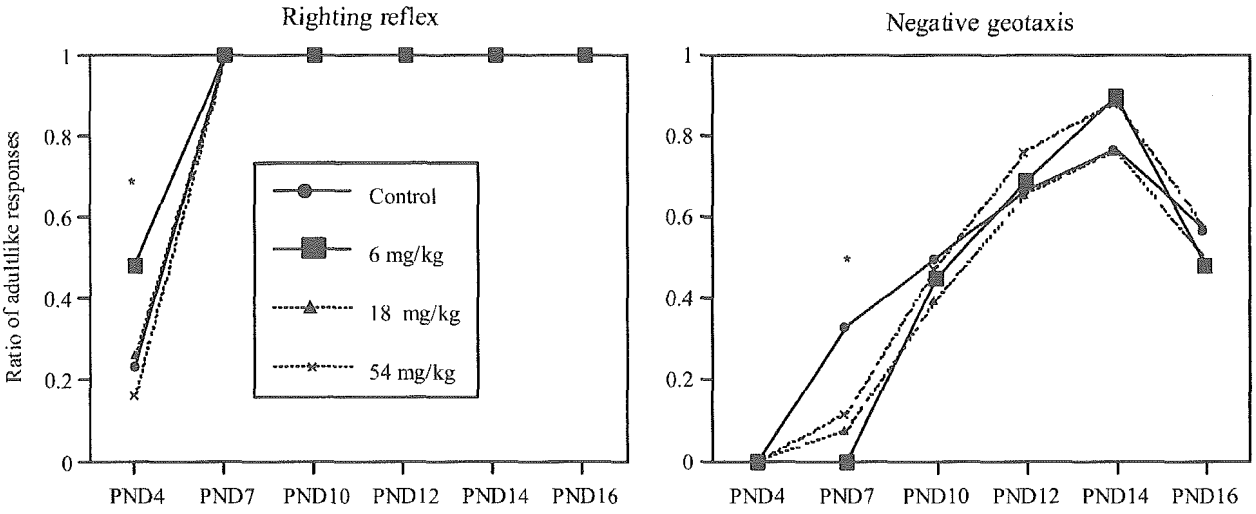

Fig. 1 Righting reflex and negative geotaxis of pups perinatally exposed to A1254. *Asterisk* indicates a significant difference among the treatment groups

Open-field test

The results of the open-field test are shown in Fig. 2. Exposure to 18 mg/kg body weight of A1254 significantly decreased the walking speed, whereas that 54 mg/kg body weight exposure tended to decrease it. No other effects on all the other parameters were observed.

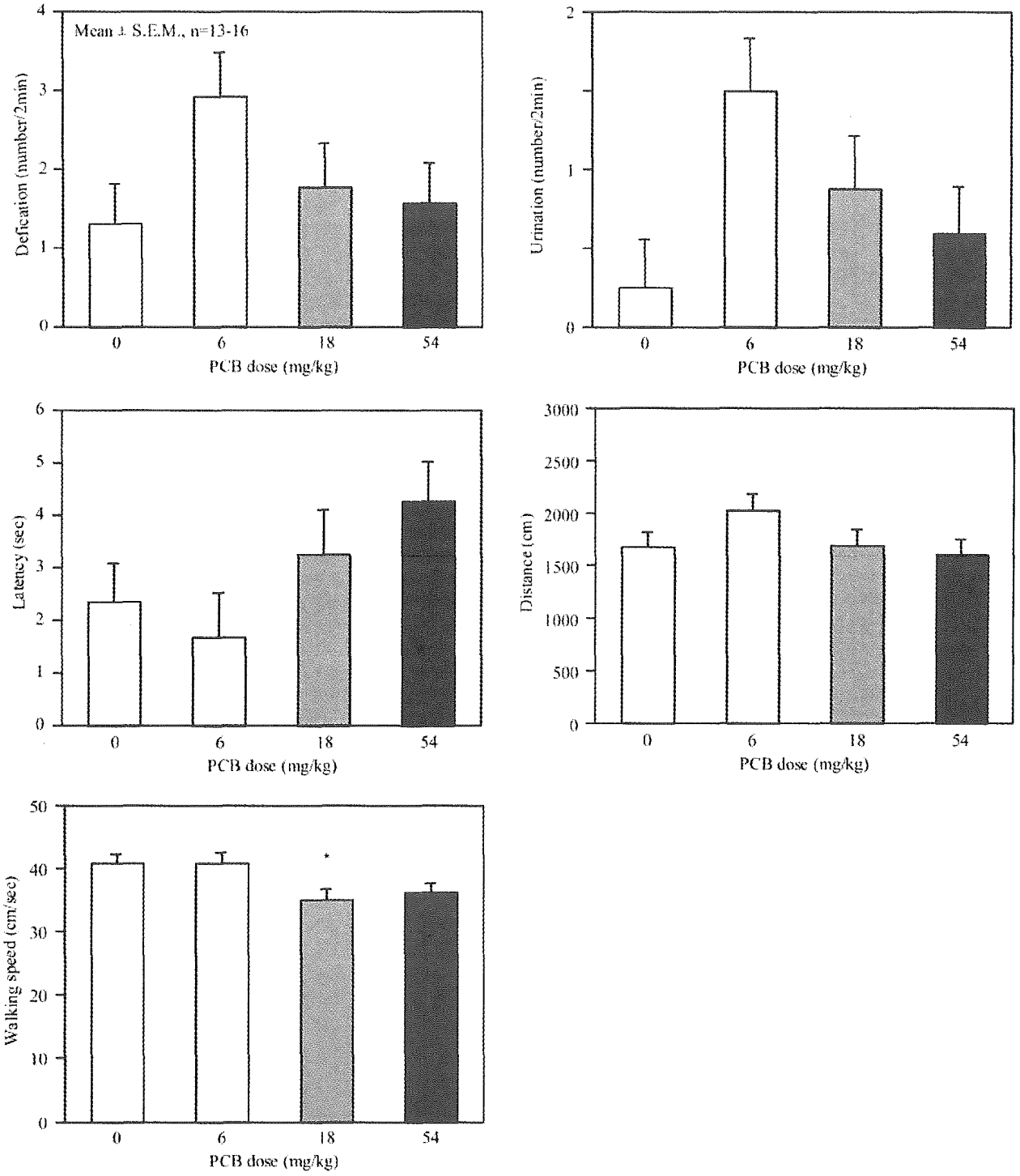

Fig. 2 Defecation, urination, latency, distance, and walking speed in open-field test of offspring perinatally exposed to different doses of A1254. *Asterisk* indicates a significant difference from control