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Exposure of methyl mercury *in utero* and the risk of neural tube defects in a Chinese population

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ABSTRACT

To determine if exposure to methyl mercury (MeHg) *in utero* is associated with an elevated risk of neural tube defects (NTDs), we measured its concentration in the placentas of 36 anencephalic and 44 spina bifida cases, as well as in 50 healthy controls. The median MeHg concentration in NTD cases (0.49 ng/g) was higher than that in controls (0.33 ng/g). The crude and adjusted odds ratios (ORs) for a MeHg concentration above the median were 3.54 (95% confidence interval (CI), 1.68–7.49) and 3.64 (95% CI, 1.66–7.99), respectively. Both anencephaly and spina bifida subtypes had higher levels of MeHg than the controls. NTD risk increased for subjects in the second and third highest tertile of MeHg concentrations, with an OR of 2.24 (95% CI, 0.93–5.40) and 2.85 (95% CI, 1.17–6.94), respectively. In summary, higher placental levels of MeHg are associated with an elevated risk of NTDs.

1. Introduction

Neural tube defects (NTDs) are multifactorial disorders arising from a complex interaction of genetic and environmental factors, and are one of the most common and severe congenital malformations of the central nervous system [1–3]. Although it is well known that taking folic acid during the periconceptional period can greatly reduce a woman's risk of having a pregnancy affected by an NTD [4], 10 years after folic acid fortification was introduced, NTD rates remain as high as 1 in 1500 births in the United States [5]. Its prevalence is also high in some areas of China (9.4 per 1000 births), although the government began providing folic acid supplements in 2009 to women who live in rural areas and plan to get pregnant [6]. These facts indicate that there may be other causes for NTDs in addition to maternal folate deficiency.

Prenatal exposure to methyl mercury (MeHg) can induce NTDs or vertebral malformations, or can decrease cell proliferation within the embryonic neural tubes of various animal models such as mice [7,8], rats [9,10], zebrafish [11], and chicks [12]. In an epidemiological study, elevated urinary levels of Hg were found among women in the highest income group who delivered children with NTDs [13]. In our previous case-control study [14], we found that higher placental levels of total Hg were associated with an elevated risk of NTDs, although we did not differentiate between organic and inorganic Hg in the placental tissue. In this study, we examined the relationship between placental levels of MeHg and the risk of NTDs in the same population as we did in our previous study.

2. Materials and methods

2.1. Study design and subjects

The design of this case-control study has been described elsewhere [14,15]. The subjects were recruited from a population-based birth defects surveillance system in a rural area of the Shanxi Province in northern China [16]. The case groups were 36 randomly selected newborns with anencephaly and 44 newborns with spina bifida, and the control group was 50 healthy term newborns. The

Abbreviations: MeHg, methyl mercury; NTDs, neural tube defects; SD, standard deviation; IQR, interquartile range.

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study protocol was approved by the institutional review board of Peking University, and informed consent was obtained from the mothers before the study began in 2002, and each year thereafter.

2.2. Placenta collection and sample preparation for laboratory assessment

The methods of placenta collection were described elsewhere [14,15]. Briefly, before assessment, the placentas were thawed at 4 °C and samples (~5 g) were taken from the middle point between the edge of the placenta and the cord attachment site on the fetus side. The samples were rinsed three times with deionized water, dried with clean tissue paper to remove excess water, and minced with stainless steel scissors. Pieces of placental tissue were weighed, placed in a small glass jar covered with a piece of aluminum foil with holes, and freeze-dried in a CHRIST freeze dryer (ALPHA 2–4 LSC, Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). The jars were put on a rack, which was then placed in a freezer at −70 °C for at least 4 h. The rack was moved to the drying chamber after the vacuum pump was warmed up for 30 min. The freeze drying process lasted for 20–24 h at −86 °C and 0.09 mbar.

2.3. MeHg assessment

2.3.1. Instrument and chemicals

The main instrument used for assessment was the MERX automated methyl mercury analyzer (Brooks Rand Lab, Seattle, Washington USA). Chemicals included 25% KOH methanol (Merck Germany), 25% dichloromethane (Honeywell, USA), hydrochloric acid (Merck), 30% (*w*/*v*) citric acid/10% (*v*/*v*) sodium (Merck) citrate buffer solution, and 1% (*w*/*v*) $NaBEt_4$ solution (1.0 g $NaBEt_4$ dissolved in 100 mL 2% (*w*/*v*) KOH solution, which was shaken, stored in 5 mL teflon bottles, frozen, and kept in the dark).

2.3.2. Sample assessment

Approximately 0.2 g (accurate to 0.001 g) dry placental sample and 5 mL 25% KOH methanol were added to a 50 mL centrifuge tube, which was capped tightly and heated in a water bath at 80 °C for 5 h (shaken once every 30 min) for complete digestion. Then, 3.0 mL HCl and 10 mL CH_2Cl_2 were added slowly after the solution was cooled to room temperature. The mixture was capped and shaken for 30 min and centrifuged for 25 min at a rate of 3000 rpm. The solvent phase was transferred to another 50 mL centrifuge tube and ultrapure water was added to 40 mL. Then, the tube was placed in the water bath at 50 °C for solvent evaporation, after which the residual organic phase was removed by blowing the samples under a stream of nitrogen (200 mL/min) for 3 min at 80 °C. Finally, the samples were diluted with ultrapure water to 40 mL [17,18], and transferred to a pre-cleaned brown bottle (Autosampler vial) that was filled with ultrapure water after 600 μL citric acid/sodium citrate buffer solution and 40 μL $NaBEt_4$ were added. The samples were analyzed on the MERX analyzer.

2.3.3. Quality assurance and quality control

Quality assurance and quality control (QA/QC) were determined using duplicate samples, method blanks, and certified reference materials (TORT-2, NRCC, Canada). The standard curve was in the linear range of 0–200 pg, and the squared correlation coefficient (R^2) was greater than 0.999. The recovery of the blanks ranged from 79% to 96%, and the recovery of TORT-2 ranged from 78% to 108%. The relative standard deviation was less than 15%, and the detection limit was 0.012 ng/g dry weight (DW). During the assessment, case and control status were masked to the technicians.

Table 1
Mean and median concentrations (ng/g DW) of MeHg in placental samples of cases with neural tube defects (NTDs) and its subtypes and healthy controls in a Chinese population.

Groups	n	Mean(SD) (ng/g, DW)	Median (IQR) (ng/g, DW)
Controls	50	0.42 (0.28)	0.33 (0.23–0.55)
NTD cases	80	0.59 (0.40)	0.49 (0.31–0.74)**
Anencephaly	36	0.53 (0.29)	0.50 (0.34–0.58)*
Spina bifida	44	0.59 (0.40)	0.49 (0.31–0.74)*

MeHg: Methyl mercury.
Mann-Whitney U tests in comparison with control group,
NTDs: Neural tube defects.
SD: standard devision.
IQR: Interquartile range.
DW: dry weight.
* P < 0.05.
** P < 0.01.

2.4. Statistical analysis

We compared the demographic characteristic between the case and control groups with a Chi-square test (or Fisher's exact test, if the predicted number of subjects in any category was less than five). The skewed distributions of the MeHg concentrations were described with the median, along with the interquartile range. Nonparametric analysis was used to compare the median levels of MeHg between the case and control groups. An odds ratio (OR) was used to estimate the risk of NTDs in association with exposure to MeHg, and the estimation precision was assessed by its 95% confidence interval (95% CI). We divided all of the subjects, in both the case and control groups, into tertiles to keep the number of subjects consistent at each level of MeHg. This facilitated the assessment of the dose-response relationship between MeHg and NTD risk using the Chi-square test. The dependent variable in the multivariate logistic regression analysis was the presence of NTDs. The independent variables were placental MeHg and other potential confounding factors, such as previous maternal history of birth defects (yes/no) and gestational week. A two-sided p value <0.05 was considered statistically significant. Statistical analyses were performed with SPSS version 18.0.

3. Results

The characteristics of the cases and controls have been described elsewhere [14,15]. Briefly, there were no statistically significant differences between NTD cases and controls with regard to maternal age at the time of delivery or pregnancy termination, maternal education, maternal occupation, self-reported maternal smoking pre- or during pregnancy, self-reported maternal passive smoking pre- or during pregnancy, or parity and self-reported maternal periconceptional supplementation with folic acid. However, a higher proportion of mothers in the case group had a prior history of birth defect-affected pregnancies, a shorter mean gestation period, and self-reported fever or flu during early pregnancy. The median MeHg concentration was 0.49 ng/g DW for total NTDs, 0.50 ng/g DW for anencephaly, and 0.49 ng/g DW for spina bifida, compared to 0.33 ng/g DW for the controls (Table 1).

The risk of NTDs in association with higher levels of MeHg in the placental tissue was analyzed by dichotomizing MeHg concentrations by the median of all of the subjects. Having a concentration above the MeHg median was associated with a 3.54-fold (95% CI, 1.68–7.49) increase in risk for any NTD, a 4.25-fold (95% CI, 1.71–10.59) increase in risk for anencephaly subtype, and a 3.07-fold (95% CI, 1.32–7.15) increase in risk for spina bifida subtype (Table 2).

The association between MeHg levels and NTD risk showed a dose-response relationship. When the lowest level was used