Original Article

Association of Co-Exposure to Polycyclic Aromatic Hydrocarbons and Metal(loid)s with the Risk of Neural Tube Defects: A Case-Control Study in Northern China

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Abstract

Objective Exposure to polycyclic aromatic hydrocarbons (PAHs) or metal(loid)s individually has been associated with neural tube defects (NTDs). However, the impacts of PAH and metal(loid) co-exposure and potential interaction effects on NTD risk remain unclear. We conducted a case-control study in China among population with a high prevalence of NTDs to investigate the combined effects of PAH and metal(loid) exposures on the risk of NTD.

Methods Cases included 80 women who gave birth to offspring with NTDs, whereas controls were 50 women who delivered infants with no congenital malformations. We analyzed the levels of placental PAHs using gas chromatography and mass spectrometry, PAH-DNA adducts with ^{32}P-post-labeling method, and metal(loid)s with an inductively coupled plasma mass spectrometer. Unconditional logistic regression was employed to estimate the associations between individual exposures and NTDs. Least absolute shrinkage and selection operator (LASSO) penalized regression models were used to select a subset of exposures, while additive interaction models were used to identify interaction effects.

Results In the single-exposure models, we found that eight PAHs, PAH-DNA adducts, and 28 metal(loid)s were associated with NTDs. Pyrene, selenium, molybdenum, cadmium, uranium, and rubidium were selected through LASSO regression and were statistically associated with NTDs in the multiple-exposure models. Women with high levels of pyrene and molybdenum or pyrene and selenium exhibited significantly increased risk of having offspring with NTDs, indicating that these combinations may have synergistic effects on the risk of NTDs.

Conclusion Our findings suggest that individual PAHs and metal(loid)s, as well as their interactions, may be associated with the risk of NTDs, which warrants further investigation.

Key words: Polycyclic aromatic hydrocarbons; Metal(loid)s; Co-exposure; Neural tube defects; Interaction; Synergistic effects

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INTRODUCTION

Neutral tube defects (NTDs) are a group of congenital malformations caused by the failure of neural tube closure during day 21–28 after fertilization[1,2]. The main subtypes include anencephaly, spina bifida, and encephalocele. NTDs can result in stillbirth and permanent disability, impacting the health and quality of life of affected children as well as sustainable economic and social development[3]. NTD is one of the most common congenital malformations in humans[4]. Folic acid supplementation is an effective method for reducing the occurrence of NTDs. For example, in five counties of Shanxi Province, China, the prevalence of NTDs decreased from 12‰ in 2004 to 3.15‰ in 2014 after the implementation of a folic acid supplementation program by the Chinese government in 2009[5]. Similarly, in the United States, the introduction of mandatory folic acid fortification in 1998 reduced the prevalence of NTDs from 1.07‰ in 1995–1996 to 0.7‰ in 1999–2011[6]. Nevertheless, the estimated average global prevalence of NTDs is about 2‰, resulting in approximately 214,000–322,000 affected pregnancies worldwide each year[7]. It appears that NTD cases cannot be completely eliminated by folic acid supplementation. Overall, investigating the specific causes of NTDs is still of great public health significance for the prevention of NTD and other birth defects.

Accumulating evidence indicates that environmental factors may be associated with the development of NTDs. Shanxi Province, located in northern China, has abundant mineral resources. The prevalence of NTDs in this province was once the highest in the world[5,8]. In the past, coal was the most important production and living fuel in rural households[9]. Numerous substances, including polycyclic aromatic hydrocarbons (PAHs) and metal(loid)s, are generated during the utilization of mineral products[10]. In winter, people light coal stoves in their bedrooms for heating and cooking. In our previous studies, a significant association was found between indoor coal combustion for heating and cooking in Shanxi Province and elevated risk of NTDs[9,11]. Studies have shown that individual exposure to one or more of these substances is associated with the risk of NTDs. A case-control study in the United States demonstrated that maternal occupational exposure to PAHs during the peri-pregnancy period may elevate the risk of spina bifida in offspring, particularly among women who are of normal weight or underweight[12]. Additionally, two other case-control studies conducted in Shanxi revealed correlations of elevated PAH levels in maternal blood and placenta with increased risk of NTDs[2,13]. Some metal(loid)s are reported to be positively associated with NTDs, including uranium (U)[14], barium (Ba)[15], and mercury (Hg)[16], while others have the opposite effect, including calcium (Ca)[15] and cobalt (Co)[8].

However, people are generally exposed to multiple substances simultaneously rather than to a single substance alone[17], and interactions among substances are easily overlooked. Some studies have suggested that PAHs and metal(loid)s may share common pathogenic mechanisms; for example, co-exposure can induce oxidative stress and genetic damage[18,19]. However, no joint effect or biological interaction of PAHs and metal(loid)s on NTDs has been reported, and this possibility must be confirmed based on additive interactions[20,21].

The aim of this study was to investigate the single-exposure, co-exposure, and potential interaction effects of PAHs and metal(loid)s on the risk of NTDs, through a case-control study in Shanxi Province. This study will contribute to comprehensive elucidation of the NTD risks associated with concurrent exposure to multiple substances, thereby decreasing the incidence of birth defects and improving the overall health of the population.

MATERIALS AND METHODS

Study Design and Participants

As described in our previous studies, the participants in this case-control study were recruited from a population-based birth defect surveillance program[2,22,23]. In that program, a total of 155 cases and 163 controls were recruited from four rural counties in Shanxi Province (Pingding, Xiyang, Taigu, and Zezhou), China, from 2005 to 2007. Cases were women who had given birth to offspring with NTD, while controls were women who had healthy newborns without congenital malformations. According to the original plan, once a case was identified, a control in the same hospital was selected. The controls were matched for infant sex, mother's residence, as well as last menstrual date with cases. However, the initial matching criteria were broken during the actual recruitment process, due to some pregnant women being unable to

provide informed consent. Consequently, for this study, 80 NTD cases and 50 controls were randomly selected from those who agreed to take part in this program voluntarily and signed the informed consent forms before participation. General characteristics of the subjects, including maternal age, education, occupation, reproductive history, lifestyle, and folate supplementation, were collected through a face-to-face interview within one week after delivery or termination of pregnancy. This study was approved by the Biomedical Institutional Review Board of Peking University.

Sample Collection and Laboratory Analysis

Placentae were collected at delivery or termination of pregnancy, placed in plastic bags, and stored at –20 °C until analysis. Approximately 10 g of wet placental tissue was collected from within 2 cm of the fetal side around the umbilical cord junction for the determination of PAH concentrations with an Agilent 7890A-5975C gas chromatograph and mass spectrometer (Agilent, Santa Clara, CA, USA), and lipid weight was determined gravimetrically. Approximately 0.1 g of freeze-dried placental tissue collected within 3 cm of the umbilical insertion point was used to quantify metal(loid) concentrations with an inductively coupled plasma mass spectrometer. Approximately 1 g of wet placenta was used for DNA extraction according to a standard procedure after washing to remove blood. PAH-DNA adduct levels were determined through the nuclease P1-enhanced ^{32}P-post-labeling method, and then quantified and expressed as adducts per 10^8 nucleotides. The details of placental tissue preparation and targeted compound detection were described previously[2,8,22] and are presented in the Supplementary Materials.

List of Target Compounds

The target compounds of this study include 10 PAHs, 38 metal(loid)s, and PAH-DNA adducts. The 10 targeted PAHs included fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene (FLT), pyrene (PYR), benzo[a]anthracene (BaA), chrysene (CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), and benzo[g,h,i]perylene (BPE). The 38 metal(loid)s assessed included Ca, ferrum (Fe), kalium (K), magnesium (Mg), sodium (Na), zinc (Zn), chromium (Cr), manganese (Mn), arsenic (As), selenium (Se), boron (B), aluminum (Al), titanium (Ti), germanium (Ge), strontium (Sr), lithium (Li), Co, nickel (Ni), molybdenum (Mo), argentum (Ag), cadmium (Cd), stannum (Sn), stibium (Sb), Ba, cesium (Cs), U, cuprum (Cu), rubidium (Rb), Hg, plumbum (Pb), lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), samarium (Sm), gadolinium (Gd), dysprosium (Dy), and yttrium (Y). The full names and abbreviations of the target compounds are shown in Supplementary Table S1.

Definition of Covariates

Maternal age is calculated by subtracting the woman's birth date from the survey date, and is divided into three groups: < 25 years old, 25–29 years old, and ≥ 30 years old. Maternal occupation is categorized into farmer and nonfarmer, with the latter including workers, officials or technicians, commercial or service industry employees, and others. Maternal educational level includes primary or lower, junior high school, and high school or above. Parity refers to the times number of this delivery, which only includes deliveries over 20 gestational weeks. Folic acid supplementation and passive smoking refer to whether the woman has taken folic acid supplements and been exposed to secondhand smoke from at least one cigarette in the period spanning three months before to three months after pregnancy, respectively. Fever and/or influenza during early pregnancy refers to whether there has been a fever with a body temperature exceeding 38.5 °C for more than 24 hours or influenza that confirmed by the medical department in the first three months of pregnancy.

Statistical Analysis

Data Cleaning In this study, PAH levels are expressed based on the weight of lipids (ng/g), metal (loid) levels are expressed based on the dry weight of placental tissue (ng/g), and PAH-DNA adduct levels are reported per 10^8 nucleotides. We constructed a total concentration index to represent the total level of 10 PAHs, designated Σ_{10}PAHs. The basic characteristics of the participants have been reported previously with missing values, and shown in Supplementary Table S2[2]. In this analysis, missing values of maternal age (3.1%), education (0.8%), occupation (3.1%), parity (3.8%), previous birth defects history (0.8%), conception season (4.6%), periconceptional folate supplementation (3.8%), fever or flu during early pregnancy (3.8%), and passive smoking (0.8%) were imputed using the multiple imputation method (30 repetitions). The concentrations of target compounds below the limits of detection (LOD) were imputed as 1/2 LOD.

Descriptive Analysis and Inter-group Comparison We compared the basic characteristics between

cases and controls using Pearson's chi-square test and Fisher's exact test. Due to the skewed distribution of these compounds, median and interquartile range (IQR) were used to describe the distribution, and the Mann-Whitney U test was used to compare concentrations between the case and control groups.

Single-exposure Models We used an exposure-wide association study (ExWAS) to estimate the association between single placental exposure and NTDs. The focal compounds were divided into four groups according to their quartiles, and unconditional logistic regression was used to calculate odds ratios (*OR*s) and 95% confidence intervals (*CI*s). The models were adjusted for maternal age, occupation, educational level, parity, folic acid supplementation (yes/no), passive smoking (yes/no), and fever and/or influenza during early pregnancy (yes/no). In these models, exposures were considered as continuous variables and *OR*s were calculated per interquartile range increase in exposure. To control for the false-positive rate caused by multiple comparisons, false discovery rate (FDR; *q*-value) correction was performed[24]. *q*-values were calculated using the following formula: *q*-value = (*P*-value × amount of testing) / ranking of the *P*-values of this test among all tests), and statistical significance was set at *q*-value < 0.05.

Multiple-exposure Models We employed least absolute shrinkage and selection operator (LASSO) penalized regression models to select a subset of significant exposures. We used 10-fold cross-validation to select the value of λ that yielded the simplest model exhibiting a variance range of the lowest mean square error (MSE). For all exposures that were selected through LASSO regression, we investigated their potential combined effects on NTDs (multiple-exposure models) through simultaneous inclusion of these exposures in the unconditional logistic regression model after adjusting for the confounding factors noted above.

Modifying Effects and Additive Interactions Effects We evaluated the modifying effects of metal(loid)s on PAH-NTD associations, as wells as those of PAHs on metal(loid)-NTD associations. Only six exposures with statistical significance in the multiple-exposure models were considered, including PYR, Se, Mo, Cd, U, and Rb. These exposures were divided into low and high groups according to their median levels. We explored PYR-NTD associations in every metal(loid) exposure subgroup and metal(loid)-NTD associations in every PYR exposure subgroup. An interaction term of PYR and metal exposure levels was included in the unconditional regression model to estimate $P\text{-}_{ME}$. Possible joint and additive effects of PYR and the aforementioned five metal(loid)s on NTD risk were explored further. We recorded each pair among PYR, Se, Mo, Cd, U, and Rb as a dummy variable, which stands for low exposure 1/low exposure 2, low exposure 1/high exposure 2, high exposure 1/low exposure 2, and high exposure 1/high exposure 2. The additive interaction was estimated using the relative excessive risk due to the interaction (RERI), proportion attributable to interaction (AP), and synergy index (SI). Statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC, USA, version 9.4) and R software (R Foundation for Statistical Computing, Vienna, Austria, version 4.2.3). A two-sided *P*-value < 0.05 was considered significant, and 95% *CI*s were calculated.

RESULTS

Characteristics of Participants

Table 1 shows the basic characteristics of the 80 cases and 50 controls after multiple imputation. A significantly larger proportion of cases reported experiencing fever and/or influenza during early pregnancy compared to controls (31% *vs.* 8%, *P* = 0.001). No significant differences were observed between the two groups in maternal age, education, occupation, parity, previous birth defects history, conception season of this pregnancy, periconceptional folate supplementation, or passive smoking.

Concentrations of Placental PAHs and Metal(loid)s

Table 2 lists placental concentrations of PAHs (ng/g lipid) and metal(loid)s (ng/g dry weight of placenta) in NTD cases and controls. The LOD and detection rate are presented in Supplementary Table S3. For PAHs, the concentrations of eight PAHs and Σ_{10}PAHs were significantly higher in the NTD case group than the control group, while PAH-DNA adducts showed the opposite tendency (8.12 in cases and 9.92 in controls, *P* < 0.001). For metal(loid)s, the concentrations of 28 out of 38 metal(loid)s differed significantly between cases and controls. Among them, 18 metal(loid)s (Mg, Zn, Cr, Mn, As, Se, Al, Sr, Ni, Mo, Sn, Sb, Ba, U, Cu, Hg, Pb, Y, and Ca) were significantly higher in the case group than the control group. The other nine metal(loid)s (K, Na, Co, Cd, Cs, Rb, La, Ce and Pr) had higher levels in the control group.

Associations of Single-compound Exposomes with NTDs

Unconditional logistic regression was used to explore the associations between individual compounds and the risk of NTDs. *OR*s are presented in Figure 1 after adjusting for seven confounding variables, namely maternal age, occupation, educational level, parity, folic acid supplementation, passive smoking, and fever and/or influenza during early pregnancy. In the single-exposure ExWAS analysis, of 50 exposures, 38 were associated with elevated risk of NTDs at *P*-value < 0.05, and 37 at *q*-value < 0.05, as shown in Figure 1A–B, respectively. Eight PAH congeners (FLU, PHE, ANT, FLT, PYR, CHR, BbF, and BkF) and Σ_{10}PAHs were positively

Table 1. Basic characteristics of pregnant women with neural tube defects (cases) and healthy (controls) infants in the case-control study[a]

Characteristics	Cases (*n* = 80)	Controls (*n* = 50)	*P*[b]
Maternal age (years)			0.333
< 25	32 (40)	17 (34)	
25–29	23 (29)	11 (22)	
≥ 30	25 (31)	22 (44)	
Maternal education			0.229
Primary or lower	15 (19)	5 (10)	
Junior high school	55 (69)	41 (82)	
High school or above	10 (12)	4 (8)	
Maternal occupation			0.162
Farmer	67 (84)	46 (92)	
Nonfarmer	13 (16)	4 (8)	
Parity			0.089
1	49 (61)	23 (46)	
≥ 2	31 (39)	27 (54)	
Previous birth defects history			0.082[c]
Yes	6 (8)	0 (0)	
No	74 (92)	50 (100)	
Conception season			0.431
Spring	19 (24)	14 (28)	
Summer	15 (19)	5 (10)	
Autumn	13 (16)	6 (12)	
Winter	33 (41)	25 (50)	
Periconceptional folate supplementation			0.353
Yes	7 (9)	7 (14)	
No	73 (91)	43 (86)	
Fever or flu during early pregnancy			0.001
Yes	25 (31)	4 (8)	
No	55 (69)	46 (92)	
Maternal passive smoking			0.105
Yes	50 (62)	24 (48)	
No	30 (38)	26 (52)	

Note. [a][*n* (%)], numbers shown in this table were after multiple imputation; [b]Pearson's chi-square test; [c]Fisher's exact test.

Table 2. Concentrations of polycyclic aromatic hydrocarbons (PAHs) and metal(loid)s in placental tissue from NTDs cases and controls

Compounds	Cases[a]	Controls[a]	*P*[b]
PAHs (ng/g lipid)			
FLU	108.33 (80.90–151.19)	79.97 (57.25–96.50)	< 0.001
PHE	308.31 (251.72–479.42)	209.71 (144.69–318.41)	< 0.001
ANT	19.73 (12.08–38.07)	12.31 (7.62–18.99)	0.002
FLT	45.89 (30.20–66.81)	29.56 (22.12–46.74)	< 0.001
PYR	45.09 (27.71–82.15)	23.87 (12.53–29.85)	< 0.001
BaA	3.48 (1.97–5.38)	2.86 (1.52–4.02)	0.097
CHR	11.52 (7.08–22.78)	8.11 (4.38–10.27)	< 0.001
BbF	2.74 (1.51–3.92)	2.12 (1.11–3.11)	0.032
BkF	1.67 (1.08–2.62)	1.18 (0.72–1.65)	0.003
BPE	1.31 (0.52–3.14)	0.87 (0.25–1.71)	0.086
Σ_{10}PAHs	596.72 (466.46–832.89)	392.20 (273.26–538.08)	< 0.001
PAH-DNA adducts (per 10^8 nucleotides)	8.12 (6.49–9.90)	9.92 (6.92–12.12)	0.033
Metal(loid)s (ng/g dry weight)			
Ca (× 10^3)	2.69 (1.75–4.51)	1.92 (1.32–4.04)	0.071
Fe	489.11 (373.39–619.48)	477.76 (385.14–578.21)	0.617
K (× 10^3)	1.46 (1.11–1.82)	1.99 (1.49–2.82)	< 0.001
Mg	431.19 (354.76–526.62)	357.57 (327.17–397.01)	< 0.001
Na (× 10^3)	2.12 (1.55–2.59)	2.57 (1.93–3.47)	0.001
Zn	71.90 (63.63–82.65)	61.31 (55.41–66.91)	< 0.001
Cr	749.67 (397.17–1213.97)	432.45 (289.67–843.32)	0.003
Mn	724.07 (581.57–994.92)	523.47 (428.01–671.44)	< 0.001
As	15.12 (12.14–19.39)	10.42 (8.39–13.97)	< 0.001
Se (× 10^3)	1.30 (1.18–1.47)	1.06 (0.95–1.16)	< 0.001
B	154.73 (111.73–224.56)	145.92 (116.86–199.25)	0.641
Al (× 10^3)	2.41 (1.43–3.99)	1.56 (1.23–2.04)	0.001
Ti	596.20 (545.83–702.10)	566.85 (512.31–620.83)	0.051
Ge	75.55 (59.63–96.81)	72.97 (63.47–81.98)	0.377
Sr (× 10^3)	3.23 (1.39–6.55)	1.41 (0.85–3.06)	0.001
Li	8.21 (5.41–12.63)	6.99 (5.21–11.78)	0.281
Co	17.16 (12.02–22.49)	21.76 (17.15–28.91)	0.001
Ni	106.99 (69.85–245.16)	80.26 (52.98–164.50)	0.030
Mo	38.95 (32.22–43.80)	26.71 (24.10–32.37)	< 0.001
Ag	1.59 (0.82–2.57)	1.67 (1.27–2.24)	0.323
Cd	21.47 (14.29–28.86)	38.92 (29.85–49.87)	< 0.001
Sn	6.36 (4.89–8.91)	4.20 (2.93–6.44)	< 0.001
Sb	1.25 (0.72–2.19)	0.76 (0.60–1.09)	< 0.001
Ba	263.43 (110.87–448.55)	92.57 (60.95–185.14)	< 0.001
Cs	4.29 (3.29–5.35)	5.83 (4.14–8.64)	< 0.001

Continued

Compounds	Cases[a]	Controls[a]	*P*[b]
U	3.14 (0.56–7.91)	0.39 (0.26–1.34)	< 0.001
Cu (× 10^3)	4.32 (3.71–5.37)	3.99 (3.51–4.43)	0.038
Rb (× 10^3)	2.19 (1.61–2.45)	2.92 (2.01–4.34)	< 0.001
Hg	15.77 (9.87–29.64)	7.85 (6.02–16.73)	< 0.001
Pb	136.85 (109.35–196.44)	120.45 (83.35–154.30)	0.019
La	3.23 (2.19–4.95)	4.37 (3.47–6.44)	0.001
Ce	7.23 (4.41–10.51)	9.04 (6.41–12.54)	0.006
Pr	0.59 (0.39–0.83)	0.76 (0.58–1.15)	0.003
Nd	2.28 (1.73–3.21)	2.62 (2.02–3.70)	0.159
Sm	0.33 (0.26–0.48)	0.36 (0.29–0.55)	0.201
Gd	0.35 (0.25–0.46)	0.39 (0.27–0.53)	0.448
Dy	0.21 (0.16–0.30)	0.22 (0.17–0.28)	0.973
Y	0.99 (0.75–1.48)	0.79 (0.69–1.07)	0.014

Note. [a]Median and interquartile range (IQR); [b]Mann-Whitney *U* test for cases and controls; FLU, Fluorene; PHE, Phenanthrene; ANT, Anthracene; FLT, Fluoranthene; PYR, Pyrene; BaA, Benzo[a]anthracene; CHR, Chrysene; BbF, Benzo[b]fluoranthene; BkF, Benzo[k]fluoranthene; BPE, Benzo[g,h,i]perylene; Σ_{10}PAHs, the total level of 10 polycyclic aromatic hydrocarbons; Ca, Calcium; Fe, Ferrum; K, Kalium; Mg, Magnesium; Na, Sodium; Zn, Zinc; Cr, Chromium; Mn, Manganese; As, Arsenic; Se, Selenium; B, Boron; Al, Aluminum; Ti, Titanium; Ge, Germanium; Sr, Strontium; Li, Lithium; Co, Cobalt; Ni, Nickel; Mo, Molybdenum; Ag, Argentum; Cd, Cadmium; Sn, Stannum; Sb, Stibium; Ba, Barium; Cs, Cesium; U, Uranium; Cu, Cuprum; Rb, Rubidium; Hg, Mercury; Pb, Plumbum; La, Lanthanum; Ce, Cerium; Pr, Praseodymium; Nd, Neodymium; Sm, Samarium; Gd, Gadolinium; Dy, Dysprosium; Y, Yttrium.

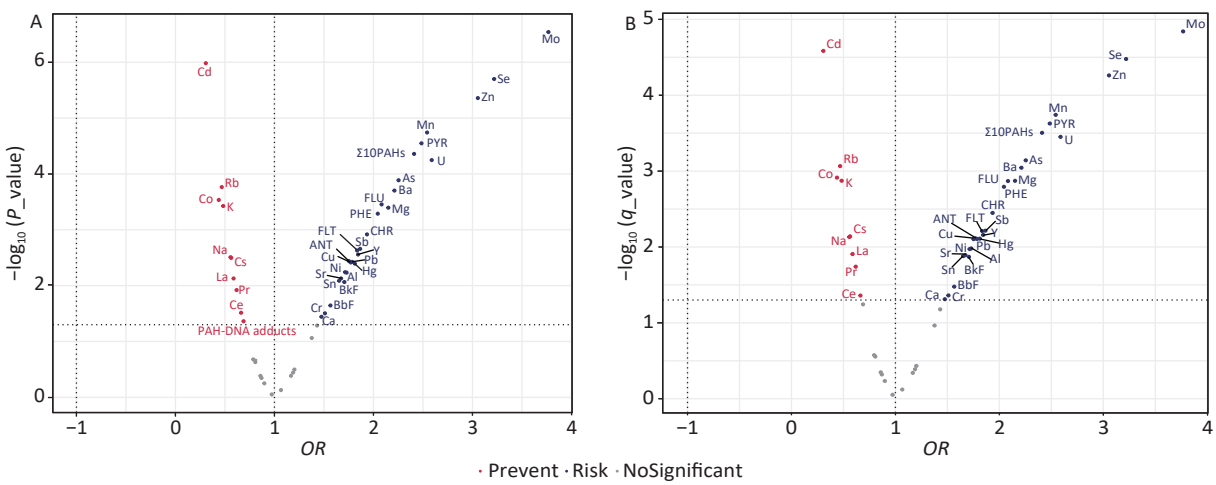

Figure 1. Association between placental single exposure and neural tube defects (NTDs) in a single-exposure exposure-wide association study (ExWAS) model. The model was adjusted for maternal age, occupation, educational level, parity, folic acid supplementation, passive smoking, and fever and/or influenza during early pregnancy. The *P* values are shown in Figure A, while *q*-values (*q*-value = (*P* value × amount of testing)/ranking of the *P* values of this test among all tests) are shown in Figure B. Volcano plot showing significance against odds ratio (*OR*). Black dashed horizontal line at *P* values or *q*-values of 0.05. For continuous variables, *OR*s are calculated per interquartile range increase in exposure.

associated with NTD risk. Placental PAH-DNA adduct concentrations were negatively associated with the risk of NTDs (*OR* = 0.69, 95% *CI*: 0.48–0.99, *P* = 0.043), but the significance of this relationship disappeared after FDR correction (*q* = 0.057). A total of 19 metal(loid)s, such as Mo, Se, Zn, etc., exhibited positive associations with NTD risk, while nine metal(loid)s, such as Cd, Rb, Co, etc., showed negative associations with NTD risk. Two PAH congeners and 10 metal(loid)s were not associated with NTDs before or after correction of *P*-values. *OR*s, 95% *CI*s, *P*-values, and *q*-values are listed in Supplementary Table S4.

Association of Multiple Exposures with NTDs

The concentrations of target compounds in this study were correlated, shown in Supplementary Figure S1. Among 10 PAHs, PAH-DNA adducts, and 38 metal(loid)s, eight compounds were selected using LASSO penalized regression models. We used 10-fold cross-validation to select the λ value that yielded the simplest model within a variance range of the lowest MSE (Supplementary Figure S2). We further investigated their potential combined effects on NTDs by simultaneously including these eight compounds in an unconditional logistic regression model after adjusting for the seven confounding factors noted above. PYR (*OR* = 6.44, 95% *CI*: 1.58–26.23, *P* = 0.009), Se (*OR* = 4.76, 95% *CI*: 1.38–16.45, *P* = 0.014), Mo (*OR* = 9.15, 95% *CI*: 1.85–45.27, *P* = 0.007), and U (*OR* = 6.95, 95% *CI*: 1.25–38.56, *P* = 0.026) were positively associated with NTDs. Cd (*OR* = 0.10, 95% *CI*: 0.02–0.43, *P* = 0.002) and Rb (*OR* = 0.23, 95% *CI*: 0.07–0.70, *P* = 0.010) were negatively associated with NTDs. The effects of Zn and Mn were not significant. These results are presented in Table 3.

Additive Interactions of Mo and PYR, or Se and PYR on NTDs

To further explore the co-exposure effects of PAHs and metal(loid)s on NTD risk, we calculated the modifying effects of metal(loid)s on PAH-NTD associations, as well as those of PAHs on metal(loid)-NTD associations (Supplementary Table S5). According to the median values of the six compounds that were significant in the multiple-exposure models, the compounds were divided into two groups: low and high groups. However, the PYR-NTD and metal(loid)-NTD associations did not significantly differ among the groups ($P\text{-}_{ME} > 0.05$).

Table 4 and Figure 2 show possible joint effects of Mo and PYR or Se and PYR on NTDs, as well as their additive interactions on NTD risk. Compared to individuals with low levels of both Mo and PYR, the risk of NTDs increased when either PYR or Mo level was high, or when both PYR and Mo levels were elevated (*OR* = 14.66, 12.99, and 182.02, respectively, all *P* < 0.05). Similarly, compared to those with low Se and low PYR levels, high levels of PYR, Se, or both exhibited significantly increased risk of NTDs (*OR* = 8.75, 9.05, and 108.00, respectively, all *P* < 0.05). According to RERI (Mo and PYR, 155.37, 95% *CI*: 17.24–1413.32; Se and PYR, 91.20, 95% *CI*: 10.14–641.96), the high-exposure groups had a greater risk of NTDs than the low-exposure group. The ratio between the joint effect and sum of the individual effects was greater than 1 (Mo and PYR, 7.06, 95% *CI*: 1.14–43.74; Se and PYR, 6.77, 95% *CI*: 1.21–38.00). Overall, 85% (95% *CI*: 0.03–0.95) of the joint effect was attributed to the interaction of high Mo and PYR in the Mo-PYR co-exposure model, and 84% (95% *CI*: 0.07–0.94) was attributed to the interaction of Se and PYR in the Se-PYR co-exposure model. No significant joint or additive interaction effects were observed in other PAH-metal(loid) groups.

DISCUSSION

In this study, we found that NTDs risk was not

Table 3. Association between exposure levels in placental tissue selected by lasso penalized regression models and the risk of NTDs (Multiple-exposure models)[a]

Compounds	*OR* (95% *CI*)	*P* values
PYR	6.44 (1.58–26.23)	0.009
Zn	0.63 (0.18–2.17)	0.465
Mn	1.62 (0.64–4.08)	0.309
Se	4.76 (1.38–16.45)	0.014
Mo	9.15 (1.85–45.27)	0.007
Cd	0.10 (0.02–0.43)	0.002
U	6.95 (1.25–38.56)	0.026
Rb	0.23 (0.07–0.70)	0.010

Note. [a]For exposures, *OR*s are calculated per IQR increase in exposure; adjusted for maternal age, occupation, educational level, parity, folic acid supplementation, passive smoking, and fever and/or influenza during early pregnancy. NTDs, neural tube defects; PYR, Pyrene; Zn, Zinc; Mn, Manganese; Se, Selenium; Mo, Molybdenum; Cd, Cadmium; U, Uranium; Rb, Rubidium.

only associated with placental single-exposure to eight PAHs, PAH-DNA adducts, and 28 metal(loid)s, but also with the co-exposure to six substances (including PYR, Se, Mo, Cd, U, and Rb). We also observed that women with high levels of Mo and PYR, or Se and PYR, exhibited significantly increased risk of having offspring with NTDs, indicating that these combinations may have synergistic effects on the risk of NTDs.

PAHs and PAH-DNA adducts have been reported to be associated with NTDs based on single-exposure models. PAHs are a class of persistent organic pollutants with lipophilicity, and thus tend to accumulate in adipose-rich internal organs after exposure[25]. Previous studies have found that PAHs and PAH-DNA adducts can persist in the human or rat body for a long period of time[26-29], providing a basis for studying the chronic toxic effects of PAHs. Similar to our current findings on the placenta, previous research has shown that maternal serum PAHs are also associated with an increased risk of NTDs[2,13]. Both serum and placenta contain high

Table 4. Possible joint and the additive interaction effects of Mo and PYR, Se and PYR on neural tube defects (NTDs) risk

Compounds	Cases [*n* (%)]	*OR* (95% *CI*)[a]	Additive interaction indicator		
			RERI	AP	SI
Mo and PYR			155.37 (17.24–1413.32)	0.85 (0.03–0.95)	7.06 (1.14–43.74)
Both low	6 (15.8)	1.00 (Ref)			
Mo low and PYR high	19 (70.4)	14.66 (3.67–58.51)			
Mo high and PYR low	19 (70.4)	12.99 (3.36–50.24)			
Both high	36 (94.7)	182.02 (22.87–1448.91)			
Se and PYR			91.20 (10.14–641.96)	0.84 (0.07–0.94)	6.77 (1.21–38.00)
Both low	8 (19.5)	1.00 (Ref)			
Se low and PYR high	16 (66.7)	8.75 (2.17–25.25)			
Se high and PYR low	17 (70.8)	9.05 (2.24–36.60)			
Both high	39 (95.1)	108.00 (17.57–663.90)			

Note. [a]Unconditional logistic regression model, adjusted for maternal age, occupation, educational level, parity, folic acid supplementation, passive smoking, and fever and/or influenza during early pregnancy. RERI, the relative excessive risk due to the interaction; AP, proportion attributable to interaction; SI, synergy index (ratio between combined effect and individual effects); Mo, Molybdenum; PYR, Pyrene; Se, Selenium.

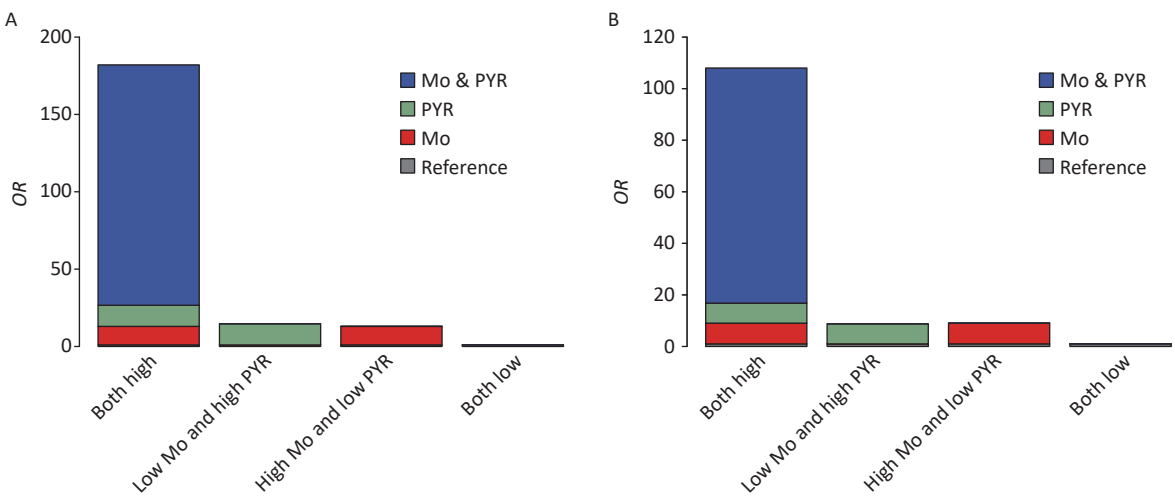

Figure 2. Additive interaction effect between Mo and PYR (A), Se and PYR (B) on the risk of NTDs. Mo, Molybdenum; PYR, Pyrene; Se, Selenium.

concentrations of PHE, FLU, and PYR. However, while ANT, ACE, and BAA pose higher risks in serum, PYR, FLU, and PHE present greater effect on NTDs risk in the placenta[13]. This may reflect tissue differences in the distribution and toxicity of PAHs. For PAH-DNA adducts, our study found that in the placenta it was negatively associated with NTDs[22], although the correlation was no longer significant after FDR correction (q-value > 0.05). This contrasts with previous research which showed a positive correlation between PAH-DNA adducts in the umbilical cord and the risk of NTDs[30]. These results suggested that the metabolic capacity of the mothers for PAHs may have been weaker in the case group than the control group, with fetuses being more susceptible to DNA damage than mothers[31].

Some placental metal(loid)s, such as Ag, U, Ba, Hg, Al, Mn, Mo, Se, and Zn, were positively associated with NTDs, whereas Cs and Co showed the opposite trend. These results were reported in our previous studies with a larger sample size (408 cases and 593 controls)[8,14-16,32-34]. Most of the results in this study are the same as those reported previously, except that Ag was not found to be associated with NTDs. This consistency demonstrates that the results of this small sample size study of 80 cases and 50 controls are generally reliable. In addition, although NTDs occur early during development and the placenta is acquired after childbirth, the uninterrupted progression from trophoblast to placenta provides a valid surrogate for assessing environmental exposures that may have occurred earlier in gestation[2].

Considering the strong correlations found between these compounds, we used the LASSO regression model to select important exposures that impact NTDs[35]. Then, the multiple-exposure model was used to estimate the effects of these exposures. PYR, Se, Mo, and U were positively associated with NTDs, while Cd and Rb were negatively associated with NTDs, consistent with the single-exposure model. Few studies have reported the toxicity of PYR, but its metabolite 1-hydroxypyrene (1-OH-PYR) is considered an internal exposure marker of PYR or PAHs and is widely used in the evaluation of health effects[36]. Researchers have reported that exposure to PAHs may interfere with or alter normal neuronal plasticity and diversity adversely affecting early neurodevelopment[37,38]. For example, Li et al. found that 1-OH-PYR in urine was associated with increased probability of autism spectrum disorder[39]. In our study, a neurotoxic effect of PYR was observed, which might be due to the high fat content of placental tissue, where PYR tends to accumulate rather than being rapidly metabolized. Se and Mo are essential trace elements that participate in redox reactions and are essential for maintaining the normal growth and development of the body[40]. Nevertheless, studies have indicated that elevated levels of Se and Mo are associated with neurological disorders. Case-control studies have demonstrated that maternal and paternal exposures to Se are associated with increased risk of having a child with spina bifida[41], and that the developing central nervous system is particularly vulnerable to the effects of childhood exposure to Mo[42]. As the heaviest element in nature, U is associated with reduced body weight and abnormal bone development in rat offspring[43], and its possible reproductive and developmental toxicity should be taken seriously. Overall, this study further demonstrates that the effects of such exposures on NTDs are relatively stable through LASSO regression and multiple-exposure models.

We further explored the joint and interaction effects of co-exposure to PYR and five metal elements (Se, Mo, U, Cd, and Rb), which has not been reported previously. Nevertheless, previous studies have provided useful evidence about interaction effects. Studies of coal coke oven workers in China found that co-exposure to PAHs and metals (such as Sb, Al, and Ti) might increase early health damage by altering the expression of microRNA[19], while combined exposure to 1-hydroxynaphthalene, Sb, and Mo might increase DNA damage and the mosaic loss of chromosome Y[44]. Data from the National Health and Nutrition Examination Survey in the United States show that co-exposure to heavy metals and PAHs is positively associated with osteoarthritis[45]. Increasing evidence suggests that PAHs and metal(loid)s may have common pathogenic mechanisms, such as genetic damage, oxidative stress, and cardiac autonomic dysfunction[19]. These shared mechanisms could be a significant contributing factor to the combined reproductive toxicity exhibited by the two substance types, and joint exposure and interaction effects of PAHs and metal(loid)s are worth exploring further. In addition, we demonstrated synergistic effects between PYR and Mo, as well as between PYR and Se, and no modification of these effects was found in the stratified analysis. Although evidence on the interactions between PAHs and metal(loid)s is limited, animal experiments and population studies of single exposures provide some evidence of teratogenicity. A cross-sectional study showed that

employees in waterpipe venues had higher concentrations of Mo and 1-OH-PYR in their urine[46]. We speculated that exposure to coal burning and passive smoking might be the main sources for women in our study. Experiments on chicken embryos indicate that Mo disulfide can increase the rate of embryo death, while also causing growth retardation and organ malformation[47]. Population studies have shown that both Mo and Se may have reproductive and growth toxicity; for example, Mo in newborns was associated with stunting, and maternal Se was associated with congenital heart defects in offspring[48,49]. Further research is warranted to investigate the underlying mechanism through which co-exposure to PAHs and metal(loid)s increases the risk of NTDs.

Our study has several strengths. First, the area chosen for this study provides an excellent setting for investigating the associations between environmental exposures and NTDs due to its high prevalence of NTDs as well as air pollution from coal combustion and passive smoking. Second, we simultaneously measured multiple internal exposure biomarkers in the placenta and comprehensively assessed their exposure levels; the use of single-exposure models, LASSO regression, and multiple-exposure models allowed us to select the substances with the most robust effects. Third, to our knowledge, this is the first study to investigate the joint and interaction effects of co-exposure to PAHs and metal(loid)s, indicating that exposure to PYR and Mo or PYR and Se might have synergistic effects on NTD risk. However, this study also has limitations. First, the case-control study design may have led to recall bias, although the confounding factors were important information that was unlikely to have been remembered incorrectly. Also, we collected questionnaires as early as possible, within 1 week after delivery or termination of pregnancy, thereby reducing recall bias as much as possible. Second, our study included only 80 NTDs cases and 50 controls, resulting in a large CI in the interaction effect analysis. This small sample size was due to NTDs being a rare disease with a limited target population, and a small sample allows for the measurement of a greater variety of substances. Despite the small sample size, our study addresses a gap within this field, and the preliminary findings of our exploration provide valuable insights for future research. Prospective studies with larger sample sizes are needed to verify the conclusions of this study.

CONCLUSION

The results of this study suggest that single-exposure and co-exposure to PAHs and metal(loid)s are associated with the risk of NTDs. PYR and Mo, as well as PYR and Se, might have synergistic effects on the risk of NTDs. Animal experiments and prospective population studies with larger sample sizes are needed to verify and explore the mechanisms underlying these findings.

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Competing Interests Authors declare no competing interests.

Ethics This study is exempt from ethical review.

Authors' Contributions Conceived the study: Zhiwen Li and Xiaoqian Jia; Collected questionnaire information and conducted the experimental analysis: Yuan Li, Yali Zhang, Jufen Liu, Lei Jin, Le Zhang, Linlin Wang, and Aiguo Ren. Performed the data analysis, and wrote the first draft of the manuscript: Xiaoqian Jia and Yuan Li. Reviewed and edited the manuscript: Yali Zhang, Jufen Liu, Lei Jin, Le Zhang, Linlin Wang, Aiguo Ren, and Zhiwen Li. Managed the program: Zhiwen Li. All the authors contributed to and approved the final version of the manuscript.

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