Gender-specific effects of prenatal mixed exposure to multiple serum phthalates on neurodevelopment of children aged 2-3 years ： the Guangxi Birth Cohort Study

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Research Article

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Abstract

Phthalates have been shown to have adverse effects on neurodevelopment, which may be gender-specific. However, the association between prenatal mixed exposure to phthalates and children's neurodevelopment remain limited. We measured prenatal phthalate levels and children's neurodevelopment. Logistic regression was fitted to examine the association. Among boys, mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP) has adverse effects on gross motor (OR: 7.39, 95% CI:1.42, 38.5). For gross motor in boys, joint effect was discovered between mono-2-ethylhexyl phthalate (MEHP) and MEHHP. Moreover, synergistic effects were found for MEHP with vanadium and cadmium, and antagonistic effects for MEHP with magnesium, calcium, titanium, iron, copper, selenium, rubidium and strontium. We did not find statistically significant relationships in girls. In the 1st trimester, adverse effects were identified between mono-2-ethyl-5-oxoyhexyl phthalate (MEOHP) and adaptation (P = 0.024), and monomethyl phthalate (MMP) with social area (P = 0.016). In the 2nd trimester, the risk of social development increased with MEHHP. In summary, we found boys may be more vulnerable to the neurotoxicity than girls in gross motor, and the 1st and 2nd trimester might be more sensitive in terms of phthalates on children. To some extent, supplementation of the appropriate metals in the 1st trimester may reduce or inhibit the absorption of phthalates by the foetus, especially in males, so as to prevent neurodevelopmental impairment.

1. Introduction

Phthalates have been widely used in a vast majority of products, such as building materials, cosmetics, plastic toys and food packaging (Philippat, Bennett et al. 2015). Thus, we can be easily exposed to phthalates through many routes, such as ingestion, inhalation and intravenous (Kobrosly, Evans et al. 2014, Jensen, Frederiksen et al. 2016). Phthalates can be detected in a significant amount of human biological materials, such as breast milk, urine and blood (Fromme, Gruber et al. 2011, Frederiksen, Jensen et al. 2014, Jensen, Frederiksen et al. 2016). Evidence from America has shown that prenatal urinary mono-benzyl phthalate (MBzP), mono-iso-butyl phthalate (MIBP) and mono-n-butyl phthalate (MnBP) levels may have an adverse effect on the executive function among 3.5-year-old children (Choi, Villanger et al. 2021).

As phthalates and their metabolites are lipid soluble, and the placental barrier function is limited, phthalate metabolites can penetrate through the placenta and affect fetal growth by interfering with hormone receptors (Calafat, Brock et al. 2006). The placenta is important for foetal growth and development during pregnancy, and its dysfunction will result in foetal growth restriction or even foetal death. Notably, the foetal period is crucial for neurodevelopment, especially in the periods between the second and fourth weeks of gestation (Rice and Barone 2000). Previous studies have indicated that phthalates exposure can have neurotoxicity by interfering with thyroid hormone homeostasis, impairing the dopamine system, disturbing calcium signalling and altering lipid metabolism (Miodovnik, Edwards et al. 2014, Liu, Zhao et al. 2015, Wang, Xu et al. 2016). Huang et al. (2019) evaluated the neurodevelopment of children aged 8–14 years using the Child Behaviour Checklist in Taiwan and found that prenatal urinary mono-2-ethylhexyl phthalate (MEHP) levels were positively associated with children’s internalizing and externalizing problems (Huang, Kuo et al. 2019). Experimental studies on mice also showed that prenatal exposure to phthalates would induce neurobehavioral abnormalities, including anxiety, impairment in social behaviour and cognition (Wang, Xu et

al. 2016, Barakat, Lin et al. 2018, Kougias, Cortes et al. 2018). An antagonism joint action of neurotoxicity on weaning rats after mixing exposure to lead and di-2-ethylhexyl phthalate (DEHP) was observed in the research (Li, Li et al. 2019). Therefore, we used the multiplied term of phthalates and metals to assess the interaction effect on neurodevelopment.

Although the adverse effects of phthalate levels on children's neurodevelopment have been previously reported (Jankowska, Polańska et al. 2019, Daniel, Balalian et al. 2020). However, most studies have only focused on the effects of exposure to a single phthalate, whereas human beings are usually exposed to multiple phthalates simultaneously, and phthalates may have joint effect in human body. To the best of our knowledge, no study has examined the association of prenatal mixed exposure to serum phthalates with the risks to children's neurodevelopment at 2–3 years of age.

Therefore, to address these issues, we selected 750 mother-child pairs, measured 15 kinds of serum phthalate metabolite levels in the pregnant women and evaluated the children's neurodevelopment at age 2–3 years using the Gesell Developmental Schedules (GDS). We then used elastic network and logistic regression to assess the gender- and gestation-specific effects of prenatal phthalates exposure on children's neurodevelopment. We also evaluated the interaction effect of phthalate metabolites and metals on neurodevelopment.

2. Methods

2.1. Study Population

Our data were derived from the Guangxi Birth Cohort Study (GBCS), constructed from July to September in 2015. We recruited pregnant women from 8 Maternity and Child Healthcare Hospitals covering 6 cities of Guangxi (Huang, Huang et al. 2020). More detailed information about the cohort can be found in the literatures (Jiang, Hou et al. 2018, Wei, Cao et al. 2018, Huang, Hou et al. 2019). The flow of study population screening is shown in Fig. S1. A total of 750 mother-child pairs (including 726 singletons and 12 pairs of twins) were included in our main analyses.

2.2. Measurement of phthalates and metals

Peripheral venous blood samples were collected at morning from 738 pregnant women, including 187 women in the 1st trimester, 432 women in the 2nd trimester, and 119 women in the 3rd trimester. Blood samples were centrifuged, separated and stored at − 80°C until analyses.

We measured the serum levels of 15 kinds of phthalate metabolites using liquid chromatography-triple quadrupole tandem mass spectrometry (LC-MS/MS, Agilent, LC-30AD, USA; SCIEX, SCIEX Triple Quad™ 5500+, USA): monomethyl phthalate (MMP), mono-ethyl phthalate (MEP), mono-iso-propyl phthalate (MIPRP), MEHP, mono-2-ethyl-5-hydroxyhexyl phthalate (MEHHP), mono-2-ethyl-5-oxoyhexyl phthalate (MEOHP), mono-n-butyl phthalate (MBP), MIBP, MBZP, mono-hexyl phthalate (MHXP), mono-iso-nonyl phthalate (MINP), mono-n-pentyl phthalate (MPEP), mono-cyclohexyl phthalate (MCHP), mono (2-ethyl-5-carboxypentyl) phthalate (MECPP), and mono-octyl phthalate (MOP).

Take the phospholipid removal plate and add 6ml of acetonitrile (ACN) to elute 3 times and then add 3ml of ACN. 10ul of 1ppm SS (surrogates standards) and 200μL of serum were added to the 96-well collection plate, and then the mixture was transferred to the phospholipid removal plate. Under vacuum, we connect the thin tube under pressure to the plate to obtain the elution solution; blow the elution solution with nitrogen to about 600ul at 40°C, filter the head, and then continue to blow the nitrogen until it is nearly dry; reconstitute the sample with 50μL MeOH and transfer it to a glass liner injection bottle; add 10ul IS (internal standard) with a concentration of 1ppm to a constant volume and wait for the sample to be loaded. Blank and quality control (QC) samples were analyzed simultaneously with each batch of samples. The recovery rate was ranged from 85–115%, and the coefficient of variation (CV) was below 20%. The intra-batch and between-batch CV of each analyte were both below 15%. The detection rates of 8 phthalate metabolites were above 80.00% and included in our analyses, that is MMP, MEP, MIPRP, MEHP, MEHHP, MEOHP, MIBP and MBP (the detection rates ranged from 97.74 to 100.0%).

Concentrations of 22 serum metals were measured using inductively coupled plasma mass spectrometer (ICP-MS, Thermo Scientific, ICAP Q, Germany), including magnesium (Mg), aluminum (Al), calcium (Ca), titanium (Ti), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), nickel (Ni), cobalt (Co), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), rubidium (Rb), strontium (Sr), molybdenum (Mo), cadmium (Cd), tin (Sn), stibium (Sb), barium (Ba), lead (Pb). Standard reference materials including ClinChek® (human serum controls for trace elements, Level Ⅰand Level Ⅱ, order no. 8882; Recipe Chemicals, Germany) and 1640a (Trace Elements in Natural Water, National Institute of Standards Technology, USA) for QC. The intra-assay and inter-assay CV for all metals were below 10% except As and Zn. Therefore, As and Zn were not included in our analyses. More information about the measurement has been described in the literature(Huang, Huang et al. 2020).

The phthalate metabolite and metal concentrations below the limit of detection (LOD) were assigned a value of LOD/$\sqrt{2}$ (Li, Papandonatos et al. 2019, Day, Collett et al. 2021).

2.3. Neurodevelopmental assessment

To maximize the validity and reliability of the assessment, children's neurodevelopment was evaluated by three well-trained paediatricians in a quiet room using the GDS. The GDS was reviewed and standardized by the Chinese Paediatric Association and mainly includes the adaptation, gross motor, fine motor, language and social area. Results were expressed by the developmental quotient (DQ). We also calculated the overall average DQ by dividing the sum of the development quotients in each field by five. The cut-off value for judging delayed neurodevelopment was 84 (Hudon, Moise et al. 1998): namely, a child with DQ scores < 84 may have a higher probability of delayed neurodevelopment. In this study, all DQ were presented as binary variables based on clinically relevant cut-off values.

2.4. Covariates

According to previous literatures, we have identified some covariates. They may all be related to phthalate levels and DQ but not intermediate variables produced by causal associations (Philippat, Nakiwala et al. 2017, Hyland, Mora et al. 2019, Jankowska, Polańska et al. 2019, Qian, Li et al. 2019). Then, we further selected covariates associated with 2 or more DQ in univariable analysis (*P*-value < 0.2) (Wang, Chen et al.

2016). Finally, nine covariates were included in our analyses: maternal education level (≤ middle school, high school, ≥ college), family annual income (RMB) at the assessment (≤ 49999, 50000 – 10000, ≥ 10000), gestational week of sample collection (1st trimester, 2nd trimester, 3rd trimester; only for gender-specific analyses), childhood tobacco smoke exposure (yes or no), children's gender (boys or girls; only for gestation-specific analyses), birth weight (low birth weight, normal birth weight, fetal macrosomia), children's exact age at assessment, gestational week of childbirth, and feeding status before 6 months (pure breast milk, milk power, mixed).

2.5. Statistical analyses

After stratifying by gender and gestation, the data were shown as number (percentage) for categorical variables or as mean (standard deviation) for continuous variables, respectively. Student's t-test or standard analysis of variance (ANOVA) were applied to compare the differences for continuous variables, while the chi-square test was used for categorical variables. The concentrations of phthalates were shown as a median (IQR). The levels of phthalate metabolites were $\log_{10}$-transformed to reduce skewness in our analyses. We used Spearman correlations to explore the correlations among phthalate metabolites. Elastic network regression was fitted to select phthalate metabolites, and multivariable logistic regression was applied to evaluate the association between selected phthalate metabolites and children's DQ. For statistically significant phthalate metabolites, we further examined the dose-response relationships using 3-knot restricted cubic splines (RCS). Knots were placed at the 25th, 50th and 75th percentiles, and the reference value to the median. (Desquilbet and Mariotti 2010).

We evaluated the joint effect of statistically significant phthalate metabolites. We fitted multivariable logistic regression models including the covariates and phthalates respectively, and calculated β_{covi} and β_{PAEi}; then calculated X_{new} as follows:

$$X_{cov} = X_{cov1}*\beta_{cov1} + X_{cov2}*\beta_{cov2} + \ldots\ldots + X_{covi}*\beta_{covi}$$

$$X_{PAE} = X_{PAE1}*\beta_{PAE1} + X_{PAE2}*\beta_{PAE2} + \ldots\ldots + X_{PAEi}*\beta_{PAEi}$$

$$X_{new} = X_{cov} + X_{PAE}$$

Finally, we used X_{new} and the children's DQ to construct a logistic regression to calculate the OR (95% CI). Specifically, logistic regression was used to explored multiplication interaction effect between phthalate metabolites and metals on children's DQ. Specifically, logistic regression was used to explore the multiplication interaction effect between phthalate metabolites and metals on children's DQ.

All statistical analyses were performed using SPSS 26 and R version 4.0.5. The results were considered statistically significant at $P < 0.05$ (two-tailed).

3. Results

3.1. Characteristics of study population and children's DQ

The descriptive statistics of the demographics and DQ of 750 mother-child pairs are shown in Table 1. In gender-stratified analyses, about 70% of mothers of boys and girls were of Han nationality, the mean pre-BMI were normal for mothers of both genders, about 70% of mothers were educated to high school and above, and about half of families (46.13% of mothers of boys and 53.82% of mothers of girls) had an annual income of 50,000 yuan or more, but these characteristics were not statistically significant (all $P > 0.05$). More than 90% of children had a normal birth weight, and there was no statistical difference between boys and girls ($P > 0.05$). There were also no statistical differences in birth length, chest circumference, feeding status before 6 months and follow-up children's ages (all $P > 0.05$). The birth head circumference of girls was slightly bigger than boys, and the difference was statistically significant ($P = 0.030$). Except for the gross motor area, boys had higher rates of delayed neurodevelopmental outcome than girls, but only differences in the fine motor, language and the social area were statistically significant ($P < 0.005$).

In gestation-stratified analyses, the differences of ethnicity among the three groups were statistically significant, ($P < 0.001$). Other maternal characteristics between different trimesters had no statistical difference (all $P > 0.05$). The birth head circumference, chest circumference and follow-up age of the child were statistically different in the gestational age stratification (all $P < 0.001$). In the 3rd trimester, the abnormal rates of the adaptation and gross motor areas were the highest, and the differences were statistically significant ($P < 0.001$ and $P = 0.014$, respectively); while the language retardation had the highest abnormal rate in the 2nd trimester, and the difference was statistically significant ($P = 0.020$).

3.2. Concentrations of phthalate metabolites and metals

The LODs for all phthalate metabolites ranged from 0.05–1.08 ng/mL. The concentration of phthalates [Median (IQR)] are shown in Table 2. The level of MEHP, MEHHP and MIBP in boys were higher than that in girls (the median values were 17.68 nmol/mL vs 16.29 nmol/mL, 0.98 nmol/mL vs 0.81 nmol/mL and 16.02 nmol/mL vs 13.38 nmol/mL), and the differences were statistically significant. The statistically significant differences of MEHHP and MIBP were also observed among 1st trimester, 2nd trimester and 3rd trimester, and the levels were both 1st trimester > 2nd trimester > 3rd trimester ($P = 0.003$ and $P = 0.009$, respectively).

The concentration of metals is shown in Table S1. The LODs of the metals were 0.00-7.74 μg/L, and the detection rates were all 100%, except for Al (87%).

In Spearman's correlation analysis, we observed positive correlations among all phthalate metabolites, with coefficient (r) ranging from 0.24 to 0.90 (all $P < 0.05$) regardless of whether were stratified by gender or by gestational age of sample collection (Fig. S2).

3.3. Screening of phthalate metabolites using elastic network regression models

With the parameters of lambda at the minimum mean-square error in elastic network regression models, phthalates with non-zero coefficients were selected as important predictors for children's neurodevelopment. Among boys, one phthalate metabolite was selected for adaptation (MMP); seven phthalate metabolites for gross motor (MMP, MEP, MIPRP, MEHP, MEOHP, MEHHP and MBP); one phthalate metabolite for fine motor (MMP); one phthalate metabolite for language (MIPRP); four phthalate metabolites for social area (MMP,

MEOHP, MEHHP and MIBP); and eight phthalate metabolites for the average area (MMP MEP, MIPRP, MEHP, MEOHP, MEHHP, MIBP and MBP) (Fig. S3). In girls, MMP was selected for adaptation and gross motor; MEOHP for fine motor; MMP for language and social area; and MEP and MEOHP for the average area (Fig. S4).

In the 1st trimester, MEOHP was selected for adaptation; MMP for the gross motor, fine motor and language area; MMP, MEP, MEHP, MEOHP and MIBP for social area; and MMP for the average area (Fig. S5). In the 2nd trimester, we selected MEHP and MEHHP for adaptation; MMP for gross motor; MEP and MEHHP for fine motor; MEOHP for language; MEP, MEOHP, MEHHP and MIBP for social area; and MMP and MEHHP for the average area (Fig. S6). In the 3rd trimester, elastic network regression models only selected 1 phthalate for each subscale, including MMP for adaptation, and the average area; MEHHP for gross motor; and MEP for fine motor, language and social area (Fig. S7).

3.4. Associations between phthalate metabolites and children's DQ

The results from logistic regression models of phthalate metabolite and DQ are shown in Fig. 1. Among boys, the risk of delayed gross motor development increased per unit increase in MEHHP (OR: 7.39, 95% CI: 1.42, 38.5, $P = 0.018$), and per unit decrease in MEHP (OR: 0.17, 95% CI: 0.06, 0.46, $P < 0.001$). Additionally, with per unit increase in MIBP, the risk of delayed social area and the average area decreased (OR: 0.28, 95% CI: 0.13, 0.64, $P = 0.002$ and OR: 0.38, 95% CI: 0.15, 0.98, $P = 0.045$, respectively). For girls, we did not observe statistically significant relationship.

We used gestation-specific analyses to explore the sensitive period of neurotoxicity of phthalate exposure. In the 1st trimester, a per unit increase in MEOHP concentration was significantly associated with an increased risk of delayed adaptation (OR: 4.22, 95% CI: 1.20, 14.82, $P = 0.024$). The risk of delayed social area development increased per unit increase in MMP and per unit decrease in MIBP (OR: 10.10, 95% CI: 1.54, 66.38, $P = 0.016$; OR: 0.14, 95% CI: 0.03, 0.75, $P = 0.022$, respectively). In the 2nd trimester, the risk of delayed social area increased with per unit increase in MEHHP ($P = 0.029$). We found no statistically significant correlation in the 3rd trimester.

3.5. Dose-response relationship between phthalate metabolites and children's DQ

We also used RCS analyses to observe the dose-response relationships (Fig. 2). For boys, significant linear relationships were found between MEHP, MEHHP and the gross motor area (P for overall = 0.001 and 0.003, respectively), as well as MIBP and the social area and the average (P for overall = 0.001 and < 0.001, respectively). Moreover, in the 2nd trimester, significant linear associations were also observed between MEHHP, MIBP and the social area (both P for overall < 0.001, both P for nonlinearity > 0.05).

3.6. Joint effect of phthalate metabolites on children's DQ

The results of the joint effect of statistically significant phthalate metabolites are shown in Table 3, including MEHP and MEHHP on the gross motor area in boys, MMP and MIBP on the social area in the 1st trimester, and MEHHP and MIBP on the social area in the 2nd trimester. After logistic regression analysis, phthalate metabolites had positive association on the gross motor area in boys (2nd quartile: OR: 2.76, 95% CI: 1.26, 6.07, *P* trend < 0.001). We also observed positive relationships on the social area in the 1st and 2nd trimester (4th quartile: OR: 15.07, 95% CI: 3.27, 69.55, *P* trend < 0.001; 2nd quartile: OR: 2.22, 95% CI: 1.07, 4.61, *P* trend < 0.001, respectively).

3.7. Interaction effect of phthalate metabolites and metals on children's DQ

The results of the interaction effect between phthalates and metals are shown in Tables S2-S4. There were synergistic effects for MEHP with V and Cd on the gross motor area among boys (OR: 1.26, 95% CI: 1.02, 1.55; OR: 1.19, 95% CI: 1.03, 1.37, respectively). On the contrary, antagonistic effects on the gross motor area were found between MEHP and Mg, Ca, Ti, Fe, Cu, Se, Rb and Sr (all $P < 0.05$).

In the 1st trimester, synergistic effects were observed for adaptation between MEOHP and Ca and Ni (OR: 1.14, 95% CI: 1.00, 1.29, $P = 0.043$; OR: 1.20, 95% CI: 1.02, 1.42, $P = 0.029$, respectively), and antagonistic effects were seen for MEOHP with Al and Cu (both $P < 0.05$). Regarding the social area, MMP and Ni had synergistic effect (OR: 1.89, 95% CI: 1.03, 3.47), whereas MIBP and Ba had antagonistic effect ($P = 0.038$). We did not observe statistically significant interaction in the 2nd trimester.

4. Discussion

In this study, we confirmed that prenatal exposure to a multiple phthalates mix had an impact on children's neurodevelopment, and the effects were gender-specific. For the gross motor and social areas, boys were more likely to be affected by a phthalate mix exposure than girls. Positive joint effect on the gross motor area and an interaction effect between phthalates and metals were also found among boys. Additionally, the 1st and 2nd trimester might be the important periods for the impact of the exposure to phthalate metabolites mix, as we observed positive joint effect on the social area in the trimesters. An interaction effect between phthalates and metals was also identified in the 1st trimester.

A gender-specific effect on children’s neurodevelopment following prenatal exposure to a phthalate mix was also observed in our results. Kim et al. (2011) observed a strong inverse association between prenatal exposure to MEHHP in the 3rd trimester of pregnancy and the Mental and Psychomotor Developmental Indices (MDI and PDI, respectively) of six-month-old male infants, measured by the BSID-II (Kim, Ha et al. 2011). We also found MEHHP had adverse effect on neurodevelopment, such as the gross motor skills among boys in our study. Kobrosly et al. (2014) illustrated associations between exposure to some phthalates and behavioral problems measured by the Child Behavior Checklist at the age of 6–10 years, and many of the associations appeared to be specific to boys or stronger in boys than girls (Kobrosly, Evans et al. 2014). We also found phthalates had stronger effects among boys than girls. In boys, we observed positive joint effect of phthalates on the gross motor area. The higher sensitivity to phthalate in boys may be because phthalate exposure can interfere with androgens through the androgen signaling pathway, reduce

testosterone levels, prevent brain masculinization, and result in a more feminized version of the brain (Smith, Macdonald et al. 2011, Chen, Hwang et al. 2017, Henrotin, Feigerlova et al. 2020).

In the 1st trimester, we observed MMP and MEOHP were positively associated with children's neurodevelopment, and MIBP was inversely related to children's neurodevelopment, both in the 1st and 2nd trimester. Choi et al (2021) found elevated mid-pregnancy MIBP was associated with more adverse profiles of an executive effect among 3.5-year-old children using a set of instruments, including a parent-and teacher-rated inventory, Behavior Rating Inventory of Executive Function-Preschool [BRIEF-P], and three performance-based assessments (Choi, Villanger et al. 2021). Meanwhile, we observed a negative association between MIBP in the 2nd trimester and neurodevelopment, such as the social area. The associations between phthalates and children's neurodevelopment were attenuated in the 3rd trimester. These results suggest phthalates exposure during different pregnancy has different effects on neurodevelopment. Zhu et al. (2020) found the 1st trimester of pregnancy might be the most vulnerable period regarding neurotoxicity due to phthalates exposure, which is similar to our results (Zhu, Wu et al. 2020).

In humans, neurodevelopment starts as early as the second gestational week, with neurulation (Rice and Barone 2000). Neurogenesis, neuron proliferation, migration, and differentiation of cells of the nervous system, as well as synaptogenesis, happen during foetal and early postnatal life. Disruption of these processes might be deleterious for the nervous system and may lead to neurodevelopmental disorders after birth. Therefore, during this vulnerable period, the rapidly developing brain may be more sensitive to toxic chemicals, such as phthalates.

Based on the results of interaction effect, we found antagonistic effects on the gross motor area in boys between MEHP and V, Cd, Mg, Ca, Ti, Fe, Cu, Se, Rb and Sr. In the 1st trimester, the antagonistic effects of MEOHP with Al and Cu were observed on adaptation; in the 2nd trimester, there was null statistically significant interaction. Li et al. (2019) observed a joint antagonism effect between Pb and DEHP on neurodevelopment in weaning rats (Li, Li et al. 2019), and the mechanism may be due to the two chemical compounds competitively binding to the same receptor, such as N-methyl-D-aspartate receptors (NMDARs) *in vivo* (Cao, Huang et al. 2008, Dai, Yang et al. 2015). The study found prenatal exposure to a mix of phthalates and metals may produce neurotoxicity mainly through perturbations in the metabolism of citric acid (TCA cycle), with possible disruption of mitochondrial oxidative phosphorylation (Sarigiannis, Papaioannou et al. 2021). Based on the results of interaction effect, in the 1st trimester, we can supply appropriate supplementation of metals, such as magnesium, calcium, iron, selenium, etc., to prevent the absorption of phthalates in the foetus, especially in males, so as to prevent neurodevelopmental impairment.

Our study possesses several strengths. First, our sample size was larger than previous studies, so it was sufficient to conduct gender- and trimester-stratified analyses. Second, the study analyzed the interaction effect between metals and phthalates, and provided evidence and methods for reducing the damage of phthalates to foetal neurodevelopment. Third, it is highly relevant to use penalized regression from the exposome notion to select predictive exposures.

Nevertheless, several limitations of this study must be acknowledged. Firstly, serum was used to measure phthalates in our study, instead of urine. Secondly, although GDS is widely used in China to assess children’s

neurodevelopment, it may not be as sensitive as some new scales, for example, the Bailey Infant Development Scale. However, our current scale is concise, and it takes less time to finish. It is suitable for cohort studies with large sample sizes, and the parents' investigation cooperation is high. Thirdly, we did not measure childhood phthalate concentrations, and it is possible that postnatal exposure could also affect neurodevelopment.

5. Conclusions

In summary, we found the effects of prenatal exposure to mixed phthalates on children's neurodevelopment are gender-specific. Boys are more sensitive to the neurotoxicity than girls in the gross motor. The 1st and 2nd trimesters might be the critical periods for the effect of mixed phthalate exposure on children's neurodevelopment. Furthermore, the supplementation of appropriate metals in the 1st trimester may reduce or inhibit the absorption of phthalates in the foetus, especially in males, so as to prevent neurodevelopmental impairment.

Declarations

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Authors' contributions

Yuan Zheng: Writing - original draft, Writing - review & editing, Formal analysis, Validation. Longman Li: Writing - review & editing, Formal analysis, Validation. Hong Cheng: Writing - review & editing, Formal analysis, Validation.

Shengzhu Huang: Methodology, Investigation. Xiuming Feng: Methodology, Formal analysis, Writing - review. Lulu Huang: Methodology, Formal analysis. Luyun Wei: Methodology, Formal analysis. Dehao Cao: Methodology, Formal analysis. Sida Wang: Methodology, Formal analysis. Long Tian: Data curation, Validation. Weijun Tang: Data curation, Validation. Caitong He: Data curation, Validation. Chunhua Shen: Data curation, Validation. Bangzhu Luo: Data curation, Validation. Maoling Zhu: Data curation, Validation. Tao Liang: Data curation, Validation. Baohong Pang: Data curation, Validation. Mujun Li: Data curation, Validation. Chaoqun Liu: Data curation, Formal analysis, Validation. Xing Chen: Data curation, Formal analysis, Validation. Fei Wang: Data curation, Formal analysis, Validation. Zengnan Mo: Conceptualization, Investigation, Funding acquisition, Validation. Xiaobo Yang: Conceptualization, Validation, Writing - review & editing, Funding acquisition.

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Data availability

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Competing interest

The authors declare that they have no competing interests.

Ethical approval

The Medical Ethics Committee of First Affiliated Hospital of Guangxi Medical University (ID: 2015(028)) have approved all study procedures.

Consent to participate

All participants have signed the informed consents for this study.

Consent for publication

The manuscript is approved by all the authors for publication.

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Tables

Table 1

Characteristics of the study population (n = 750).

Characteristics [a]	Gender			Gestation			
	Boys (n = 468)	Girls (n = 282)	*P* [b]	1st trimester (n = 192)	2nd trimester (n = 438)	3rd trimester (n = 120)	*P* [b]
Maternal/household							
Ethnicity			0.573				< 0.001
Han	200 (70.9)	348 (74.4)		136 (70.8)	314 (71.7)	98 (81.7)	
Zhuang	72 (25.5)	104 (22.2)		46 (24.0)	112 (25.6)	18 (15.0)	
Other	10 (3.55)	16 (3.42)		10 (5.2)	12 (2.7)	4 (3.3)	
Age (years)	28.5 (4.47)	28.7 (4.59)	0.659	29.1 (4.21)	28.5 (4.56)	28.4 (4.99)	0.303
Pre-pregnancy BMI (kg/m2)	20.7 (3.32)	20.5 (2.96)	0.325	20.5 (2.82)	20.5 (3.23)	20.6 (3.10)	0.993
Education status			0.738				0.079
middle school and below	86 (30.5)	137 (29.3)		46 (24.0)	143 (32.6)	34 (28.3)	
high school	83 (29.4)	130 (27.8)		54 (28.1)	117 (26.7)	42 (35.0)	
college and above	113 (40.1)	201 (42.9)		92 (47.9)	178 (40.6)	44 (36.7)	
Family annual income (RMB)			0.061				0.053
< 49999	152 (53.9)	216 (46.2)		86 (44.8)	218 (49.8)	64 (53.3)	
50000-100000	113 (40.1)	207 (44.2)		83 (43.2)	184 (42.0)	53 (44.2)	
>100000	17 (6.03)	45 (9.62)		23 (12.0)	36 (8.22)	3 (2.50)	
Children							
Childhood tobacco smoke exposure			0.397				0.361

Yes	149 (54.0)	225 (50.4)		88 (47.3)	224 (53.2)	62 (53.9)
No	127 (46.0)	221 (49.6)		98 (52.7)	197 (46.8)	53 (46.1)

Table 1 (continued)

Characteristics [a]	Gender			Gestation			
	Boys (n = 468)	Girls (n = 282)	*P* [b]	1st trimester (n = 192)	2nd trimester (n = 438)	3rd trimester (n = 120)	*P* [b]
Preterm			0.539				0.374
Yes	12 (4.26)	26 (5.56)		10 (5.21)	19 (4.34)	9 (7.50)	
No	270 (95.7)	442 (94.4)		182 (94.8)	419 (95.7)	111 (92.5)	
Birth weight [c]			0.390				0.744
Low birth weight	14 (4.96)	24 (5.13)		11 (5.73)	20 (4.57)	7 (5.83)	
Normal birth weight	264 (93.6)	430 (91.9)		176 (91.7)	406 (92.7)	112 (93.3)	
Fetal macrosomia	4 (1.42)	14 (2.99)		5 (2.60)	12 (2.74)	1 (0.83)	
Birth length (cm)	50.3 (1.62)	50.3 (1.82)	0.724	50.3 (1.54)	50.3 (1.82)	50.3 (1.81)	0.924
Birth chest circumference (cm)	32.6 (1.43)	32.7 (1.40)	0.312	33.0 (0.83)	32.6 (1.47)	32.2 (1.75)	< 0.001
Birth head circumference (cm)	32.7 (1.30)	32.9 (1.28)	0.030	33.0 (0.75)	32.9 (1.28)	32.4 (1.82)	< 0.001
Follow-up children's ages	31.0 (1.78)	30.9 (1.67)	0.736	29.1 (1.12)	31.1 (1.13)	33.2 (1.07)	< 0.001
Feeding status before 6 months			0.064				0.471
Pure breast milk	171 (60.6)	243 (51.9)		102 (53.1)	244 (55.7)	68 (56.7)	
Milk powder	27 (9.57)	51 (10.9)		15 (7.81)	50 (11.4)	13 (10.8)	
Mixed	84 (29.8)	174 (37.2)		75 (39.1)	144 (32.9)	39 (32.5)	
Adaptation			0.058				< 0.001

(-)	268 (57.3)	182 (64.5)		135 (70.3)	257 (58.7)	58 (48.3)
(+)	200 (42.7)	100 (35.5)		57 (29.7)	181 (41.3)	62 (51.7)
Gross motor			0.466			0.014
(-)	356 (76.1)	207 (73.4)		159 (82.8)	319 (72.8)	85 (70.8)
(+)	112 (23.9)	75 (26.6)		33 (17.2)	119 (27.2)	35 (29.2)

Table 1 (continued)

Characteristics [a]	Gender			Gestation			
	Boys (n = 468)	Girls (n = 282)	*P* [b]	1st trimester (n = 192)	2nd trimester (n = 438)	3rd trimester (n = 120)	*P* [b]
Fine motor			0.040				0.188
(-)	363 (77.6)	237 (84.0)		154 (80.2)	343 (78.3)	103 (85.8)	
(+)	105 (22.4)	45 (16.0)		38 (19.8)	95 (21.7)	17 (14.2)	
Language			0.020				0.144
(-)	220 (47.0)	158 (56.0)		107 (55.7)	208 (47.5)	63 (52.5)	
(+)	248 (53.0)	124 (44.0)		85 (44.3)	230 (52.5)	57 (47.5)	
Social area			0.020				0.020
(-)	342 (73.1)	228 (80.9)		160 (83.3)	320 (73.1)	90 (75.0)	
(+)	126 (26.9)	54 (19.1)		32 (16.7)	118 (26.9)	30 (25.0)	
Average area [d]			0.135				0.266
(-)	325 (69.4)	211 (74.8)		146 (76.0)	306 (69.9)	84 (70.0)	
(+)	143 (30.6)	71 (25.2)		46 (24.0)	132 (30.1)	36 (30.0)	

Abbreviations: BMI, body mass index;

[a] Values were number (%) for categorical variables and Mean (SD) for continuous variables; (-) indicates normal neurodevelopment, namely the DQ scores ≥ 84; (+) indicates neurodevelopmental delay, namely the DQ scores < 84.

[b] *P*-values were calculated by Student's t-test, standard analysis of variance (ANOVA) or chi-square test for different variables.

[c] Low birth weight: < 2500g; Normal birth weight: 2500-4000g; Fetal macrosomia: > 4000g.

[d] Average area = (Adaptation + Gross motor + Fine motor + Language + Social area) / 5.

Table 2

The serum phthalate concentrations of pregnant women [Median (IQR)].

Phthalates (10-3 nmol/mL)	Detection rate (%)	Gender		P^a	Gestation			P^b
		Boys (n = 468)	Girls (n = 282)		1st trimester (n = 192)	2nd trimester (n = 438)	3rd trimester (n = 120)	
MMP	99.6	4.15 (2.50, 9.37)	3.97 (2.38, 8.22)	0.333	4.39 (2.61, 10.19)	4.06 (2.43, 9.34)	3.39 (2.26, 6.70)	0.050
MEP	99.2	2.07 (1.28, 3.53)	1.86 (1.27, 3.02)	0.111	2.03 (1.26, 3.78)	1.97 (1.32, 3.18)	1.94 (1.14, 3.14)	0.412
MIPRP	97.74	1.89 (0.45, 3.91)	1.64 (0.39, 3.60)	0.113	2.39 (0.50, 4.11)	1.62 (0.39, 3.63)	1.80 (0.45, 3.67)	0.067
MEHP	100	17.68 (10.93, 35.46)	16.29 (9.35, 30.53)	0.037	18.39 (11.47, 84.00)	16.90 (9.89, 31.21)	15.81 (10.17, 28.64)	0.058
MEOHP	97.47	0.14 (0.09, 0.20)	0.13 (0.09, 0.20)	0.156	0.14 (0.10, 0.22)	0.13 (0.09, 0.20)	0.14 (0.08, 0.18)	0.145
MEHHP	99.87	0.98 (0.61, 1.93)	0.81 (0.50, 1.56)	0.002	1.13 (0.62, 2.50)	0.90 (0.56, 1.63)	0.79 (0.57, 1.57)	0.003
MIBP	99.87	16.02 (9.08, 37.47)	13.38 (7.16, 30.40)	0.020	17.52 (9.72, 55.43)	14.32 (8.35, 29.28)	13.63 (7.30, 35.21)	0.009
MBP	99.87	28.42 (16.71, 50.58)	26.68 (14.12, 46.93)	0.180	29.33 (18.34, 64.27)	27.86 (15.10, 48.54)	25.80 (16.50, 40.70)	0.140

Abbreviations: MMP, monomethyl phthalate; MEP, mono-ethyl phthalate; MIPRP, mono-iso-propyl phthalate; MEHP, mono-2-ethylhexyl phthalate; MEOHP, mono(2-ethyl-5-oxohexyl) phthalate; MEHHP, mono(2-ethyl-5-hydroxyhexyl) phthalate; MIBP, mono-isobutyl phthalate; MBP, mono-n-butyl phthalate.

[a] *P*-values were calculated by Mann-Whitney U Test.

[b] *P*-values were calculated by Kruskal-Wallis H Test.

Table 3

Combined effect of phthalate metabolites on children's DQ.

Variables [a]	OR (95% CI)	*P* [b]
Boys		
Gross motor		
Q1	Reference	
Q2	2.76 (1.26, 6.07)	0.012
Q3	3.86 (1.79, 8.31)	0.001
Q4	7.18 (3.41, 15.15)	< 0.001
P trend		< 0.001
1st trimester		
Social area		
Q1	Reference	
Q2	2.67 (0.49, 14.52)	0.254
Q3	3.29 (0.63, 17.18)	0.159
Q4	15.07 (3.27, 69.55)	0.001
P trend		< 0.001
2nd trimester		
Social area		
Q1	Reference	
Q2	2.22 (1.07, 4.61)	0.032
Q3	2.43 (1.18, 5.01)	0.016
Q4	7.06 (3.54, 14.08)	< 0.001
P trend		< 0.001

[a] The combined effect of MEHP and MEHHP on gross motor in boys, MMP and MIBP on social area in the 1st trimester, as well as MEHHP and MIBP on social area in the 2nd trimester, respectively.

[b] *P*-values were derived from logistic regression adjusted for maternal education level (≤ middle school, high school, ≥ college), family annual income (RMB) at assessment (≤ 49999, 50000 - 10000, ≥ 1,0000), gestational week of sample collection (1st trimester, 2nd trimester, 3rd trimester; only for gender-specific analyses), childhood tobacco smoke exposure (yes or no), children's gender (boys or girls; only for gestation-specific analyses), birth weight (low birth weight, normal birth weight, fetal macrosomia), children's exact age at assessment, gestational week of childbirth, and feeding status before 6 months (pure breast milk, milk power, mixed).

Figures

Figure 1

Associations of phthalate metabolite levels with the risks of children's adaptation (A), gross motor (B), fine motor (C), language (D), social area (E) and average area (F)

The data were presented as odds ratio (OR) and 95% confidence interval (CI) using logistic regression models. Models were adjusted for maternal education level (≤ middle school, high school, ≥ college), family annual income (RMB) at assessment (≤ 49999, 50000 - 10000, ≥ 1,0000), gestational week of sample collection (1st trimester, 2nd trimester, 3rd trimester; only for gender-specific analyses), childhood tobacco smoke exposure (yes or no), children's gender (boys or girls; only for gestation-specific analyses), birth weight (low birth weight, normal birth weight, fetal macrosomia), children's exact age at assessment, gestational week of childbirth, and feeding status before 6 months (pure breast milk, milk power, mixed).

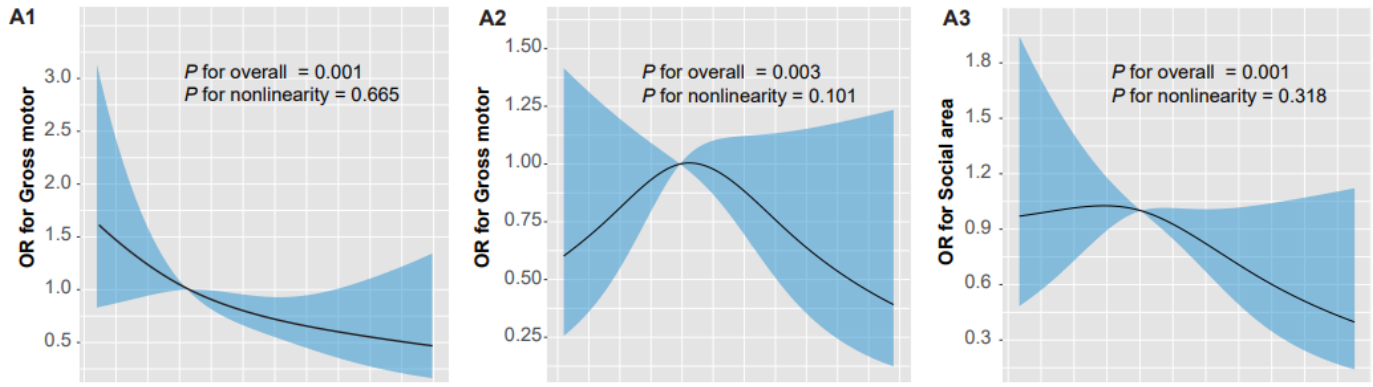

Figure 2

The restricted cubic spline for the association between phthalate metabolite and DQ in boys (A1-A4), the 1st trimester (B1-B3) and the 2nd trimester (C1-C2)

The lines represent adjusted odds ratios (OR) based on restricted cubic splines for phthalate metabolite in the logistic regression model. Knots were placed at the 25th, 50th, and 75th percentiles of phthalate metabolite distribution, and the reference value was set at the 50th percentile. All analyses were adjusted for maternal education level (≤ middle school, high school, ≥ college), family annual income (RMB) at assessment (≤

49999, 50000 - 10000, ≥ 1,0000), gestational week of sample collection (1st trimester, 2nd trimester, 3rd trimester; only for gender-specific analyses), childhood tobacco smoke exposure (yes or no), children's gender (boys or girls; only for gestation-specific analyses), birth weight (low birth weight, normal birth weight, fetal macrosomia), children's exact age at assessment, gestational week of childbirth, and feeding status before 6 months (pure breast milk, milk power, mixed).

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