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A pilot study on association between phthalate exposure and missed miscarriage

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Abstract. – OBJECTIVE: The incidence of missed miscarriage has been increasing during the past decade in China and the etiology of about half of the cases remains unclear. Exposure to phthalates has been considered as a risk factor. The aim of this paper is to assess the association between exposure to phthalates and missed miscarriage.

PATIENTS AND METHODS: A case-control study was performed including 150 cases of missed miscarriage and 150 matched controls with normal pregnancies. The levels of phthalate exposure were compared between the two groups by measuring 13 phthalate metabolites in urine samples. Blood samples were collected for serum hormone measurement to assess the relationship between serum hormone level and phthalate exposure.

RESULTS: The urinary levels of metabolites of di-(2-ethylhexyl) phthalate (DEHP) and dimethyl phthalate (DMP) were significantly higher in the cases than in the controls. A strong dose-response relationship was observed between urinary metabolite levels and the odds of missed miscarriage. Monomethyl phthalate (MMP), a metabolite of DMP, and mono-2-ethylhexyl phthalate (MEHP), a metabolite of DEHP, each had significant negative correlation with maternal serum hormone levels.

CONCLUSIONS: In the current study, exposure to DEHP and DMP was found to be associated with missed miscarriage. Interruption of hormone synthesis by DMP and DEHP metabolites represents a plausible mechanism of phthalate reproductive toxicity.

Key Words:
Phthalate, Missed miscarriage, DEHP, DMP, Sex hormone.

Introduction

A missed miscarriage, also known as a missed abortion or a silent miscarriage, is an *in utero* death of the embryo or fetus before the 20th week of gestation with retained conception products[1], which happens in about 2% of singleton pregnancies at 10-14 weeks of gestation[2,3]. The incidence of missed miscarriage has been rapidly increasing in China. The incidences of missed miscarriage adjusted for 1000 live births in two hospitals located in different towns had increased by 24- and 13-fold from 2002 to 2012 (Supplemental Figure 1). Well-reported risk factors for missed miscarriage include a deficit in folic acid supplementation, lack of physical exercise and hypoventilation[4]. Environmental risk factors with significant contribution to the increase of missed miscarriage include ionizing radiation[5] and exposure to environmental chemicals, including pesticides[6], polycyclic aromatic hydrocarbons[7], carbon disulfides[8] and endocrine disruptors[9-11]. Phthalate exposure has been recently found to be associated with pregnancy loss[12,13] and preterm birth[13,14]. Several million tons of phthalates are used as plasticizers worldwide every year[15]. Hu-

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man phthalate exposure sources include food, food packaging, PVC flooring, medicinal products, dietary supplements and cosmetic products[9]. Phthalates can be absorbed by the human body through the intestinal and respiratory tracts and by contact with the skin[16]. Although the elimination half-life of phthalate metabolites is less than 24 hours for most metabolites, phthalates and their metabolites can be detected in almost all human samples and in about 98% of children and pregnant women because of continuous exposure[17,18]. Total urinary phthalate metabolite concentration was found to be higher in 2-year and 5-year old children than in pregnant women[17]. Exposure of women to phthalates has been associated with genital malformation, menstrual disorders, endometriosis and breast cancer[19-23]. However, there have been no studies done to investigate the association between phthalate exposure and missed miscarriage. The current study was to test the hypothesis that phthalate exposure is associated with the rapid increase of missed miscarriage in recent years.

Patients and Methods

Population and Study Design

The potential eligible participants were patients having missed miscarriage paired with normal pregnancies recruited from the Fifth People's Hospital of Shanghai. Those who were 22-35 years old and residing in Shanghai from June 1st to December 31st of 2012 were eligible for the current study.

Missed miscarriage was defined as fetal death without expulsion before 20 weeks of gestation and was diagnosed through ultrasonography and measurement of hormone levels. Eligible controls were normal pregnancies within 20 weeks of gestation. The ultrasonography of the controls showed cardiac tube pulsation. Some pregnancy-related variables, including maternal age, parity, gestation and ethnicity, were matched in the two groups. The exclusion criteria were multiple pregnancies, infection, anemia, endocrine disorders, genital malformation, immune dysfunction, organic disease and other complications of pregnancy. A total of 150 cases of missed miscarriage matched with 150 controls were enrolled for the current study. The study was approved by the Institutional Review Board (IRB) of the hospital and informed consent was obtained from all participants before the study was started.

A standard questionnaire interview was performed for every participant with demographic information including maternal age, education, health insurance, profession, ethnicity, alcohol use, cosmetic use, and obstetric history including parity, gestation, spontaneous abortion and abortion times.

Exposure Assessment

Blood samples were drawn from each participant in the morning, after fasting for 8 hours, for detection of maternal serum progesterone and estradiol on admission to the hospital before any operations were performed. The Bayer ADVIA Centaur assay (Bayer HealthCare LLC, Tarrytown, NY, USA) was used to measure serum progesterone and estradiol, a direct chemiluminescent technology of competitive immunoassay[24]. Urine collections were made from the first morning void samples. The urine samples were centrifuged at 3000 rpm and supernatants were collected and stored at –80°C for later detection of phthalate metabolites within 2 months. None of the cases and controls had intravenous treatment or any treatments, in the hospital, where plastic tubes or syringes were used, before the urine specimen was collected. All the material used in collecting and storing urinary samples were tested to be phthalate-free. A total of 13 phthalate metabolites in the urine were measured using liquid chromatography with tandem triple quadrupole mass spectrometry (LC-MS/MS) as described by Ferguson et al[14]. Briefly, the measurement included enzymatic deconjugation of glucuronidated metabolites, solid-phase extraction, separation through high-performance liquid chromatography and measurement by mass spectrometry. The limit of detection (LOD) for a single metabolite was 0.5 μg/L. A metabolite undetectable in a urine sample was assigned a value of 0.25 μg/L, half of the LOD, for further statistical analysis. Because metabolite concentrations may change with urine dilution, urinary specific gravity (SG) was used to correct the measured concentrations as described[14]. The mean SG of all measured samples was 1.02. The SG-corrected concentrations of phthalate metabolites were LN-transformed for statistical analysis.

Statistical Analysis

The Chi-square test was used to identify significant differences in population and clinical characteristics between cases and controls, including maternal age, education, health insurance, profession, ethnicity, alcohol use, cosmetic

use, parity, gestation, spontaneous abortion and abortion times. The differences in serum hormones and SG-corrected urinary phthalate metabolites between cases and controls were compared using Student's *t*-test to test the association of a single variable with missed miscarriage. Multivariate logistic regression was used to examine the odds of missed miscarriage in association with phthalate metabolite concentrations in full models, adjusting for *a priori* covariates including maternal age, education, health insurance, profession, ethnicity, alcohol exposure and cosmetic use. To examine the dose-dependent effects of exposure, and the potential for nonlinear relationships, SG-corrected phthalate metabolite concentrations were divided into quartiles using non-standardized values from the entire group. Adjusted odds ratios were calculated for each of the top 3 quartiles in comparison with the lowest quartile of exposure in models adjusting for the same sets of covariates used in models with continuous exposure. Tests for trend were conducted by modeling quartiles as a single ordinal variable, again using the same covariates. The correlations between serum hormones and SG-adjusted urinary phthalate metabolites were also analyzed[13]. All analyses were performed using IBM SPSS Statistics software version 19.0 (IBM, Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

Results

Characteristics of Subjects

Of the demographic and clinical characteristics in the case-control study, health insurance and history of spontaneous abortion showed significant differences between cases and controls (Table I). Data analysis showed 61% of the cases and 45% of the controls had health insurance. The odds ratio of spontaneous abortion history for missed miscarriage was 7.02 (95 CI%: 1.56 31.68, $p < 0.05$). Maternal serum levels of progesterone and estradiol were significantly lower in cases when compared to controls (Table II).

Table I. Distribution of population characteristics.

Democratic characteristics	Cases N = 150 (%)	Controls N = 150 (%)	*p*-value
Maternal age			0.807
Average ± SD	28.7 ± 6.0	28.5 ± 6.2	
Education			0.860
Primary school	52 (34.7)	50 (34.7)	
High school/Technical school	54 (36)	60 (40)	
College	44 (39.3)	40 (26.7)	
Health insurance			0.022
Self-pay/Private	59 (39.3)	83 (55.3)	
Medicaid	91 (60.7)	67 (44.7)	
Profession			0.575
Outdoors	3 (2)	7 (4.7)	
Indoors	145 (98)	143 (95.3)	
Nationality			0.756
Han nationality	147 (98)	148 (98.7)	
Other nationalities	3 (2)	2 (1.3)	
Beverage use			0.183
Frequently	15 (10)	25 (16.7)	
Occasionally	135 (90)	125 (83.3)	
Cosmetic use			0.492
Frequently	15 (10)	20 (13.3)	
Occasionally	135 (90)	130 (86.7)	
Spontaneous abortion history			0.014
Yes	13 (8.7)	2 (1.3)	
No	137 (91.3)	148 (98.7)	
Abortion > three time			0.575
Yes	5 (3.3)	7 (4.7)	
No	145 (96.7)	143 (95.3)	
Parity			0.149
Nulliparous	102 (68)	90 (60)	
Multiparous	48 (32)	60 (40)	

Table II. Comparison of serum hormone levels in missed abortion cases and controls.

	Serum hormone levels (mean ± SD		
	Cases	Controls	*p*-value
Estradiol (pg/ml)	309.2 ± 156.0	1213.6 ± 537.0	< 0.001
Progesterone (ng/ml)	12.5 ± 5.9	21.0 ± 5.5	< 0.001

Association Between Exposure to Phthalates and Missed Miscarriage

Nine phthalate metabolites were detected in urine, including monomethyl phthalate (MMP), mono-(3-carboxypropyl) phthalate (MCPP), monoethyl phthalate (MEP), mono-isobutyl phthalate (MiBP), mono-n-butyl phthalate (MBP), mono-(2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), derived from the five parent molecules, dimethylphthalate (DMP), di-n-octylphthalate (DOP), diethylphthalate (DEP), di-n-butylphthalate (DBP), and di-2-ethylhexylphthalate (DEHP) (Table III). Monobenzylphthalate (MBZP), mono-cyclohexylphthalate (MCHP), mono-n-octylphthalate (MOP) and mono-isononylphthalate (MINP) were not detected.

The geometric means of each detected phthalate metabolite were compared between cases and controls (Table III). The levels of each of the four DEHP metabolites, MECPP, MEHHP, MEOHP and MEHP, and the sum of all four DEHP metabolites, were significantly higher in missed miscarriage cases than in normal pregnancies. MMP, a metabolite of DMP, was, also, significantly higher in missed miscarriage cases. Multiple logistic regression analysis showed that only MMP and MEHP were significantly associated with missed miscarriage with odds ratios of 2.28 ($p < 0.01$) and 1.78 ($p < 0.01$), respectively (Table IV).

Results from analysis of nine urinary phthalate metabolite quartiles showed dose-dependent relationships with odds of missed miscarriage. Significant positive trends were observed for MMP, MEHP, MEHHP and MEOHP (Figure 1).

Pearson correlation coefficients between SG-corrected urinary levels of the nine metabolites

Table III. Phthalate metabolite levels in all participants, cases and controls.

Parent compound	Metabolite	Urinary SG-corrected metabolite levels (ug/L) (Geometric Mean ± SD)			
		All	Cases	Controls	*p*-value
DMP	MMP	22.91 ± 6.61	52.48 ± 4.68	10.00 ± 6.31	< 0.001
DOP	MCPP	0.58 ± 2.63	0.62 ± 2.63	0.52 ± 2.57	0.149
DEP	MEP	12.30 ± 3.39	11.74 ± 2.95	12.88 ± 3.89	0.592
DBP	MiBP	31.62 ± 2.75	33.11 ± 2.63	30.20 ± 2.95	0.377
	MBP	46.77 ± 2.88	48.98 ± 2.75	43.65 ± 3.02	0.331
DEHP	MEHP	10.47 ± 2.51	14.45 ± 2.34	7.41 ± 2.34	< 0.001
	MECPP	8.13 ± 2.63	10.23 ± 2.69	6.61 ± 2.40	< 0.001
	MEHHP	6.61 ± 2.63	8.32 ± 2.69	5.25 ± 2.40	< 0.001
	MEOHP	6.17 ± 3.31	8.51 ± 3.39	4.57 ± 3.02	< 0.001
	∑DEHP Metabolites	33.11 ± 2.49	46.77 ± 2.51	26.92 ± 2.29	< 0.001

The compounds analyzed were: monomethyl phthalate (MMP) a metabolite of dimethylphthalate (DMP); mono-(3-carboxypropyl) phthalate (MCPP), a metabolite of di-n-octylphthalate (DOP); monoethylphthalate (MEP), a metabolite of diethylphthalate (DEP); mono-isobutyl phthalate (MiBP) and mono-n-butyl phthalate (MBP), metabolites of di-n-butylphthalate (DBP); mono-(2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP), metabolites of di-2-ethylhexylphthalate (DEHP). Monobenzylphthalate (MBZP), mono-cyclohexylphthalate (MCHP), mono-n-octylphthalate (MOP) and mono-isononylphthalate (MINP) were not detected.

Table IV. Multiple logistic regression analysis of metabolites associated with missed abortion.

	p-value	OR	95% CI
MMP	< 0.001	2.28	1.76-2-95
MCPP	0.445	0.86	0.56-1.29
MEP	0.033	0.75	0.57-0.98
MECPP	0.493	0.87	0.58-1.30
MEHHP	0.522	1.17	0.73-1.88
MIBP	0.338	0.85	0.60-1.19
MEOHP	0.253	1.28	0.84-1.95
MBP	0.999	1.00	0.67-1.50
MEHP	< 0.001	1.78	1.35-2.34

and maternal serum levels of estradiol and progesterone were calculated and the results showed that MMP, MEHP, MECPP, MEHHP and MEOHP had significant negative correlations with estradiol while MMP, MEHP, MECPP, MEHHP and MCPP had significant negative correlations with progesterone (Table V). MMP had negative correlation coefficient values greater than 0.3 with both estradiol and progesterone and MEHP had a negative correlation coefficient value greater than 0.3 with estradiol.

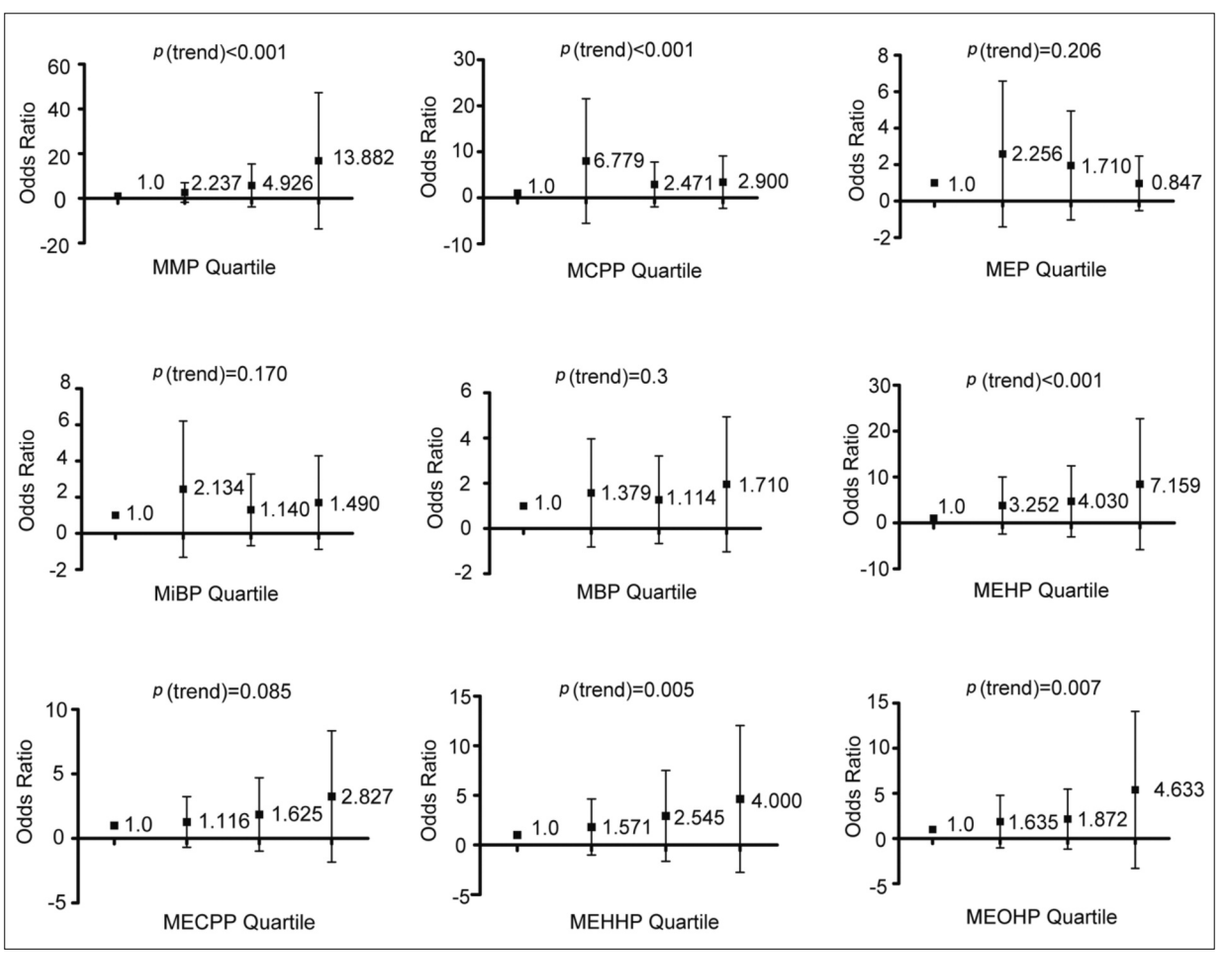


Figure 1. Odds ratio of missed abortion and 95% CI divided by quartiles of adjusted metabolites of phthalate levels. Significant dose-dependent positive trends were observed for MMP, MEHP, MEHHP and MEOHP.

Table V. Correlations between adjusted urinary phthalate metabolites and estradiol or progesterone.

	MMP	MEHP	MECPP	MEHHP	MEOHP	MCPP	MEP	MiBP	MBPI
Estradiol	-0.329[a]	-0.311[a]	-0.171	-0.189	-0.246	-0.050	0.061	0.001	-0.002
(*p*-value)	< 0.001	< 0.001	0.003	0.001	< 0.001	0.390	0.293	0.984	0.977
Progesterone	-0.397[a]	-0.212	-0.242	-0.192	-0.095	-0.146	0.036	-0.059	-0.102
(*p*-value)	< 0.001	< 0.001	< 0.001	0.001	0.102	0.011	0.538	0.312	0.078

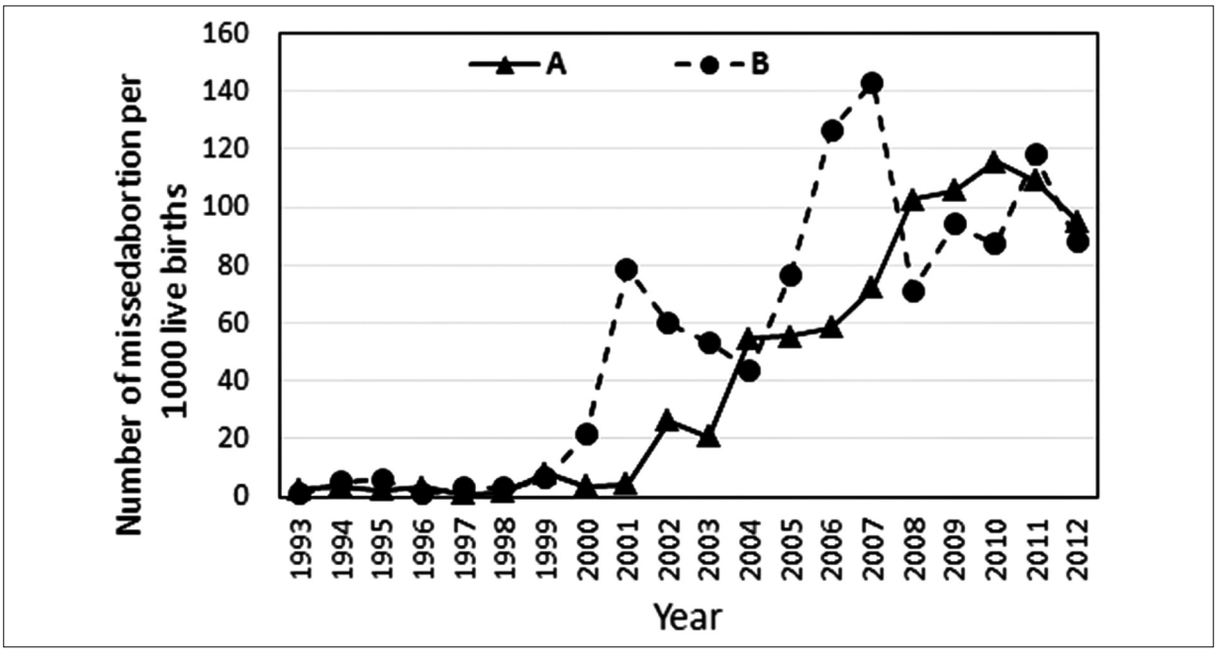
[a]The absolute value of the correlation coefficient was greater than 0.3.

Discussion

The current study is the first demonstration of an association between urinary phthalate levels and risk of missed miscarriage. Exposures to DEHP and DMP, but not other phthalates, were associated with increased risk of missed miscarriage. Urinary levels of the DMP metabolite, MMP, and the DEHP metabolites, MEHP, MEHHP and MEOHP, showed dose-dependent relationships with odds of missed miscarriage. The highest quartile of urinary MMP had an odds ratio of 13.9, while the highest quartile of urinary MEHP had an odds ratio of 7.2. Our results support and complement the findings of studies performed by Ferguson et al[14] and Toft et al[12] with endpoints of preterm birth and pregnancy loss associated with DEHP exposure, respectively. It is not surprising to observe a spectrum of adverse reproductive effects in human studies based on the well-reported reproductive toxicities induced by DEHP in both *in vivo* and *in vitro* experiments with animals[25,26]. DEHP exposure resulted in a decrease of the serum levels of sex hormones and disruption of the hypothalamus-pituitary-ovarian axis in rats[27]. MEHP, the toxic DEHP metabolite, interferes with steroid production such as estradiol production and aromatase synthesis through the peroxisome proliferation-activated receptor (PPAR) pathway but had no effect on progesterone production[28,29]. Low levels of estrogen and progesterone have been associated with human fetal loss and the prognostic levels of progesterone and estradiol for normal pregnancy were above 12.3 ng/ml and 350 pg/ml, respectively[30]. Levels of serum progesterone and estradiol were both found to be markedly reduced in the missed miscarriage cases in comparison to matched controls. Although high urinary levels of DEHP metabolites and the DMP metabolite were significantly correlated with low levels of sex hormones, based on the current study, it is impossible at this time to conclude that phthalate exposure caused the reduction of sex hormones because the hormonal drop could be the result of fetal death.

Elevated levels of MBP, a DBP metabolite, were found to be related to odds of preterm birth, and a dose-dependent relationship was observed in Ferguson et al's study[14]. However, MBP had no relationship with pregnancy loss in Toft et al's study[12] or missed miscarriage in the current study. Studies in rodents[31,32] have shown that DBP exposure caused a reduction in the number of litters, litter size, live births and increased risk of mid-pregnancy abortions. However, the concentrations used in these rodent studies were 100 times higher than the average daily exposure in the general population[33]. We speculate that adverse reproductive endpoints in pregnant women are associated with DBP exposure levels. Exposure levels that are not high enough to cause pregnancy loss or missed miscarriage may still affect other birth outcomes, such as preterm birth.

An unexpected finding in the current study was that DMP exposure was highly correlated with missed miscarriage. The relationship was even stronger than that for DEHP. The average level of MMP, the DMP metabolite, in missed miscarriage cases was about 5 times that of controls, while the average level of summed DEHP in missed miscarriage cases was about 2 times that of controls. The odds ratio of the 4th quartile vs the 1st quartile was nearly 14 for MMP while it was 7 for MEHP (Figure 1). The level of MMP also had the strongest inverse relationship with levels of estrogen and progesterone among all the phthalate metabolites in the current study. Unlike DEHP whose reproductive toxicities have been widely studied, there is only one animal study showing that DMP exposure had no effect on pregnancy at doses up to 2 mg/kg when administered during early gestation to Sprague-Dawley rats through intraperitoneal injection[34]. Unfortunately, MMP was not measured in the



Supplemental Figure 1. All cases of missed abortion from two hospitals located in different cities were collected from 1993 to 2012. The incidence of missed abortion was calculated as the number of cases per 1000 living births in each year. The incidences started to rapidly increase from 2001 and 2002 respectively in each hospital. ***A,*** The Fifth People's Hospital of Shanghai. ***B,*** Fujian Maternity and Children Health Hospital.

two previous human studies[12,14]. However, findings in the current study strongly suggest that more toxicological studies on DMP need to be done. If still proven to be non-toxic, DMP could be a good biomarker for the prediction of missed miscarriage associated with phthalate exposure.

Spontaneous abortion history was a risk factor for missed miscarriage with an odds ratio of 7.02 (1.56-31.68) and contributed to about 8% of total missed miscarriage cases in the current study. It can either be an independent risk factor or related to previous pregnancy loss caused by phthalate exposure as discussed before[12]. Health insurance was shown to be associated with missed miscarriage; this may reflect the fact that women who have health insurance are more likely to visit a physician when they feel that there is something abnormal with their pregnancy.

Conclusions

This pilot study is the first to demonstrate a significant association between DEHP and DMP exposures and missed miscarriage. However, the size of this case-control study is small. Cohort studies with more cases are needed to confirm current findings. More investigations are needed with DMP to understand its toxicities on the reproductive system and process. Urinary screening for levels of DMP or DEHP metabolites in early gestation can be used to estimate the risk of phthalate exposure-associated missed miscarriage.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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