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分担研究報告書

The associations between prenatal phthalate exposure and cryptorchidism: The Hokkaido Study in Environment and Children's Health

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研究要旨

Phthalates are chemicals used as plasticizers for polyvinyl chloride, food packaging, cosmetics, personal care products and known endocrine-disrupting effects in rodents. epidemiologic studies of male reproductive disorders such as cryptorchidism and hypospadias using measurements of prenatal or postnatal phthalate levels have been reported, however, in regards to the relationship to prenatal exposure to phthalates is very limited and the results are contradictory. The aim of this study is to investigate potential effects of prenatal phthalate metabolite levels on the occurrence of cryptorchidism in the Hokkaido Study on Environment and Children's Health. This prospective birth cohort study was based on the Hokkaido large-scale cohort, the Hokkaido Study on Environment and Children's Health. We selected 63 cryptorchidism cases and 126 controls based on the birth records and the questionnaires at aged 1, 2, 4, and 7 years old. Controls were 1 to 2 (1 : 2) matched for delivery year ± 1 year and their delivery hospitals. Seven phthalate metabolites were measured from first trimester maternal blood using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS-MS) instrumentation. Cox logistic regression analyses were performed to evaluate associations between prenatal exposure to phthalates and the risk of cryptorchidism. There were no significant associations were observed between cryptorchidism and phthalates metabolite levels, however, a borderline significance was found in the level of MECPP. Prenatal exposure to phthalates did not show adverse effects on cryptorchidism. This was the first study of investigating prenatal phthalates exposure and cryptorchidism in Japanese population. Previous studies also have been reported negative effects of prenatal phthalates exposure on cryptorchidism, continuous investigation is needed as further studies.

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A．研究目的

Phthalates are chemicals used as plasticizers for polyvinyl chloride, food packaging, cosmetics, personal care products and known endocrine-disrupting effects in rodents [1, 2]. Animal studies suggest that prenatal exposure to di-butyl phthalate (DBP) and di-2-ethylhexyl phthalate (DEHP, and di-isononyl phthalate (DiNP) induce anti-androgenic effects on the male fetus. They alter Leydig cell differentiation and function and thus diminish fetal testosterone production and reduced anogenital distance [3-7].

Only three epidemiologic studies of male reproductive disorders such as cryptorchidism and hypospadias using measurements of prenatal or postnatal

phthalate levels have been reported, however, in regards to the relationship to prenatal exposure to phthalates is very limited and the results are contradictory; In Danish study, mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) and mono(4-methyl-7-carboxyheptyl) phthalate (7cx-MMeHP) in maternal amniotic fluid were measured, however, neither di(2-ethylhexyl) phthalate (DEHP) nor di-isononyl phthalate (DiNP) was consistently associated with cryptorchidism and hypospadias [8]. In a prospective Danish–Finnish cohort study, 6 phthalate metabolites from 1–3 months postnatally breast milk sample were measured, however, no association has been reported between phthalate monoester levels and cryptorchidism [9]. In a prospective case – control study of 52 cryptorchidism and 128 control measured monobutyl phthalate (MBP) from breast milk sample 3-5 day after delivery and reported that concentrations were not significantly increased in the cryptorchidism case versus control group although a trend for increased MBP was observed [10].

The aim of this study is to investigate potential effects of prenatal phthalate metabolite levels on the occurrence of cryptorchidism in the Hokkaido Study on Environment and Children's Health.

B．研究方法

Study population

This prospective birth cohort study was based on the Hokkaido large-scale cohort, the Hokkaido Study on Environment and Children's Health [11, 12]. Study details regarding the population, data collection, sampling of biological specimens, and contents of the questionnaire have been described previously [11, 12]. Briefly, native Japanese women living in Hokkaido were recruited in this study at <13 weeks of gestation at 37 hospitals and clinics in Hokkaido between February 2003 and March 2012. From a total of 20,929 pregnant women who were enrolled to The Hokkaido Study of Environment and Children's Health, we selected 19,183 mother-infant pairs who had a baseline questionnaire, birth records, and first trimester maternal blood. From these, we selected 63 cryptorchidism cases and 126 controls based on the birth records and the questionnaires at aged 1, 2, 4, and 7 years old. Controls were 1 to 2 (1 : 2) matched for delivery year ± 1 year and their delivery hospitals.

Assessments of prenatal exposure to phthalates

Seven phthalate metabolites, mono-n-butyl phthalate (MnBP), mono-iso-butyl phthalate (MiBP), mono-benzyl phthalate (MBzP), mono(2-ethylhexyl) phthalate (MEHP), mono(2-carboxymethyl-5-oxohexyl) phthalate (MEHHP), mono(2-ethyl-5-carboxypentyl) phthalate (MECPP), Mono (4-methyl-7-carboxyheptyl) phthalate (cx-MiNP) were measured from first trimester maternal blood. Maternal plasma was analyzed using ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS-MS) instrumentation (Waters, Tokyo, Japan).

Sample preparation

A 40 uL of hydrochloric acid (1M) was added to each maternal plasma sample (0.5 mL). Samples were mixed by vortexing and ultrasonic irradiated for 10 minutes. An internal standard, which consisted of 100

ng/mL of MEHP-d4, MiNP-13C4, MEHHP-13C4, MECPP-13C4, OH-MiNP-d4, cx-MiNP-d4 (100 ng/mL of each), 1100 uL of ammonium acetate buffer solution (100 mM, pH 9.0), and 10 uL of β- glucuronidase enzyme were added to each sample to deconjugate glucuronidated phthalate metabolites. The samples were gently mixed and incubated at 37 ℃ for 90 minutes. After incubation, samples were extracted 300 uL into tube and added 900 uL of acetonitrile. The samples were mixed by vortexing to deproteine. After centrifugation (3,500 rpm for 5 min), 500 uL of supernatants were transferred into new tubes and dried under nitrogen gas. After drying, 250 uL of 20 % methanol was added and ultrasonic irradiated for 5 minutes and transferred to a glass sample vial insert.

Instrumental analysis

The reconstituted extract (10 uL) was injected into an ultra-performance LC (ACQUITY UPLC H-Class) coupled to triple quadrupole tandem MS (Xevo TQ-S) (Waters, Tokyo, Japan). The insoluble particulates were filtered by in-line filters (2.1×5 mm, 1.7 um, Vanguard BEH C8, Waters, Tokyo, Japan) preceding the BEH C8 column (2.1×100 mm, 1.7 µm, Waters, Tokyo, Japan). The retention gap technique was used by installing retention gap columns Atlantis T3 (2.1×50 mm, 3 u, Waters, Tokyo, Japan), which improved phthalate metabolites sensitivity by trapping mobile-phase phthalate metabolites (contaminants) in the retention gap column. The column temperature was 40℃. The analytes were quantified using ESI-negative SRM mode with product/precursor ion scans unique for each analyte (Table 1). Analytes were eluted from the column with a linear gradient involving solvent A (2 mM ammonium acetate in water) and solvent B (2 mM ammonium acetate in 95 % acetonitrile) as follows: 2 % B for the initial 1 min, then a gradient of 2 – 98 % B from 1 min to 16 min. The total UPLC cycle time was 20 min including column re-equilibration. An eluent flowrate of 0.3 mL/min was employed for all analyses.

Data analysis

Because our data did not fall into a normal distribution, phthalate metabolites concentrations were converted to a natural log (Ln) scale. For participants with phthalate metabolites concentrations below the MDL, a value equal to half of the MDL was assigned for statistical analyses. We did not include MBzP, MEHHP, and cx-MiNP in the statistical analysis because these compounds were detected less than 50 %.

For analysis of correlations between the children with and without cryptorchidism and characteristics of mothers and infants, we used the Student's t-test and the Chi-square test. To assess risk factors or protective factors for cryptorchidism, binominal logistic regression analyses were used. Crude and adjusted Cox logistic regression analyses were performed to evaluate associations between prenatal exposure to phthalates and the risk of cryptorchidism. In logistic models, we evaluated odds ratios (ORs) for the risk of cryptorchidism with Ln-transformed maternal phthalate metabolite levels. Multivariate analyses were adjusted for confounding variables that influenced cryptorchidism in univariate analyses, possible risk factors reported in previous studies. The fully adjusted model used logistic regression analysis of cryptorchidism adjusted for maternal age at

delivery, gestational week, parity, maternal education, smoking at early pregnancy, alcohol intake at early pregnancy, maternal BMI before pregnancy, and infant birth weight.

All statistical analyses were performed using the Statistical Package for Social Science (SPSS) for Windows, version 22.0 (SPSS, Inc., Chicago, IL, USA) and JMP Pro 12 Statistical Discovery Software for Windows (S.A.S. Institute Inc., Cary, North Carolina). Differences were considered statistically significant at $p < 0.05$.

Ethics

For this study, all participating women provided written informed consent, and the study protocol was approved by the institutional ethical board for epidemiological studies at the Hokkaido University Center for Environmental and Health Sciences.

C．研究結果

The characteristics of mother and infant are show in Table 1. There were no statistical significance relationships between cryptorchidism case and control on maternal age at delivery, gestational week, parity, maternal education, smoking at early pregnancy, alcohol intake at early pregnancy, maternal BMI before pregnancy, and infant birth weight, although the trend ($p < 0.1$) for maternal age at delivery and smoking at early pregnancy; mothers who were older and smokers at early pregnancy had higher prevalence of cryptorchidism.

The distributions of phthalate metabolites were shown in Table 2. MnBP, MiBP, MEHP, and MECPP were detected more than 70 % of the samples. Most highly detected metabolite was MnBP, followed as MiBP, MEHP, MECPP.

The comparisons of median concentrations of phthalate metabolites were shown in Table 3. The level of MECPP, a second metabolite of DEHP, was lower in cryptorchidism than controls ($p = 0.034$). Other metabolites were not obtained significant differences.

The Cox regression analysis of the associations between prenatal exposure to phthalates on cryptorchidism were shown in Table 4. There were no significant associations were observed between cryptorchidism and phthalates metabolite levels, however, a borderline significance was found in the level of MECPP (OR, 95% CI: 0.60, 0.35 – 1.03; $p = 0.065$).

D．考察

This was the first study of investigating prenatal phthalates exposure and cryptorchidism in Japanese population. We observed no consistent association between prenatal exposure to phthalates and the risk of cryptorchidism in Japanese populations of large scale birth cohort study. However, a borderline inverse association was found between the level of MECPP and cryptorchidism. Similar findings have been reported from Denmark birth cohort study: higher amniotic fluid levels of MECPP decreased the corresponding OR for cryptorchidism (p for trend = 0.54) [8]. Other two studies have measured postnatal phthalate levels and related it to the occurrence of cryptorchidism or hypospadias.

Main et al. (2006) reported that a study of 68 cryptorchidism cases and 62 controls observed no association between cryptorchidism and metabolites of DMP, DEP, DBP, BBzP, DEHP, and DiNP in breast milk, however, no consistent associations between prenatal phthalates exposure and cryptorchidism or hypospadias [9]. Chevalier et al. (2015) reported that concentrations of MBP from breast milk sample 3-5 day after delivery were not significantly increased in the cryptorchidism case versus control group although a trend for increased MBP was observed in a prospective case – control study of 52 cryptorchidism and 128 control [10]. Moreover, a nested in the EDEN and PELAGIE mother–child cohorts study has been reported inverse associations with maternal urinary phthalate metabolites [13]. Important limitations to these inconsistent interpretations are variations in the case definition, the analytical matrices, and the timing of exposure assessment. Jensen et al. (2015) measured phthalate levels in amniotic fluid from pregnant women who aged more than 35 years old or having high risk of pregnancy [8]. Therefore, study population in this previous study might differ from our population. Main et al. (2006) and Chevalier et al. (2015) measured phthalate levels from breast milk after their delivery [9, 10]. Considering the important time window of fetal masculinization, exposure assessments should be during the first half of pregnancy. The findings from previous studies are still unclear, therefore, continuous investigations are needed.

Animal studies supported that prenatal phthalates exposure alter Leydig cell differentiation and function and thus diminish fetal testosterone production and showed cryptorchidism and reduced anogenital distance [3-7]. The exogenous administration of oestrogens during pregnancy results in increased incidence of cryptorchidism were supported by animal studies (Toppari et al., 1996). In this study, we did not investigate the associations between prenatal exposure to phthalates and hormone levels. We could not confirm the inverse relation between prenatal phthalates exposure and cryptorchidism. In contrast, we observed an unexpected decrease in the risk of cryptorchidism with increased concentrations of maternal phthalate metabolites, for which we have no clear explanation. Therefore, for further study, the associations with hormones such as testosterone, androstendione, and insulin- like factor-3 which is produced by fetal Leydig cells and acts on the gubernaculum of the testis which plays a key role in guiding the testis during its phase of transabdominal descent should be investigated in this study.

The limitations of this study need to be considered. In this study, we used serum for measurements of phthalate met abolites instead of urine. The disadvantage of using blood sample instead of urine, such as conversion from diester to monoester after blood sampling, have been considered [14, 15]. To avoid this conversion, we stored blood samples at -80℃ immediately and added acid to inhibit enzyme activity after defrosting samples. The use of a single

measure to classify exposure levels of phthalate exposure assessments is also one of our limitations. Since phthalates are known as short half-life compounds, exposure levels of phthalates might be varied during pregnancy. Repeated measurements are much appropriate. However, we collected blood samples from first trimester of pregnancy, therefore, our time window of exposure assessment is suitable. The definition of cryptorchidism was based on birth records and the self-reported (mother) questionnaires, therefore some misclassifications might be happened. However, we collected most of cryptorchidism cases from birth records, which are diagnosed by medical doctors. Therefore, we think the misclassifications are held to a minimum. Moreover, cryptorchidism cases appeared in this study might have been not enough to fill the statistical power to analyze the risk of prenatal exposure to phthalates on cryptorchidism, although we selected all cryptorchidism cases from large birth cohort, the Hokkaido Study on Environment and Children's Health. Small sample size could be a possible reason for not observing association between prenatal phthalates exposure and cryptorchidism.

E．結論

In conclusion, prenatal exposure to phthalates did not show adverse effects on cryptorchidism. This was the first study of investigating prenatal phthalates exposure and cryptorchidism in Japanese population. Previous studies also have been reported negative effects of prenatal phthalates exposure on cryptorchidism, continuous investigation is needed as further studies.

F．研究発表

1.論文発表

該当なし

2.学会発表

該当なし

G．知的財産権の出願・登録状況（予定を含む。）

該当なし

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Table 1. Characteristics of mothers and infants

		total		case		control		p value
		n	%	n	%	n	%	
Maternal education	=<12 yr	90	44.1	30	44.1	60	44.1	1.000
	>12yr	114	55.9	38	55.9	76	55.9	
Household income	< 4	120	67.0	36	62.1	84	69.4	0.330
(million yen/year)	>= 5	59	33.0	22	37.9	37	30.6	
Alcohol intake during early pregnancy		27	13.0	9	13.0	18	13.0	1.000
Smoking during early pregnancy		22	10.6	10	14.5	12	8.7	0.202
Prity	< 1	95	45.9	32	46.4	63	45.7	0.921
	>= 2	112	54.1	37	53.6	75	54.3	
Maternal age at deliver	Mean±SD	30.8	4.9	31.6	4.3	30.5	5.1	0.129
Gestational week	Mean±SD	38.6	1.6	38.4	1.8	38.6	1.6	0.277
BMI before pregnancy	Mean±SD	21.2	3.6	21.8	4.3	21.2	3.2	0.282
Birth weight (g)	Mean±SD	3075	443.4	3006.7	452.5	3108.7	436.5	0.121
X2 test or t-test								

Table 2. Distributions of phthalate metabolites

	MDL	<MDL (%)	Min	25%	Median	75%	Max
MnBP	0.57	100	2.7	19.75	41	67.25	150
MiBP	0.44	99	0.22	3.4	5.3	8.13	19
MBzP	0.19	2.9	<LOD	<LOD	<LOD	<LOD	4.7
MEHP	0.23	98.5	<LOD	0.7	1.2	6.75	72
MEHHP	0.23	5.3	<LOD	<LOD	<LOD	<LOD	1.9
MECPP	0.11	86.4	<LOD	0.16	0.26	0.37	1.4
cx-MiNP	0.12	0.97	<LOD	<LOD	<LOD	<LOD	0.13
BPA			0.012	0.14	1.1	4.9	30

MDL: method of detection limits

Table 3. Comparisons of phthalate metabolites

		median	(25th - 75th)		p value
MnBP	Case	39.00	(20 -	56.0)	0.363
	Control	41.00	(18.5 -	7.00)	
MiBP	Case	5.30	(3.0 -	8.2)	0.581
	Control	5.30	(3.65 -	8.15)	
MEHP	Case	1.30	(0.68 -	9.1)	0.566
	Control	1.20	(0.7 -	6.4)	
MECPP	Case	0.21	(0.13 -	0.33)	0.011
	Control	0.28	(0.19 -	0.42)	
BPA	Case	0.99	(0.14 -	4.75)	0.637
	Control	1.20	(0.14 -	4.9)	

Mann-Whitney U-test

Table 4. The Cox regression analysis of the associations between prenatal exposure to phthalates on cryptorchidism

		OR	95% CI		p value
MnBP	Crude	0.64	0.37	1.10	0.106
MnBP	Adjusted	0.64	0.34	1.20	0.164
MiBP	Crude	0.64	0.37	1.10	0.104
MiBP	Adjusted	0.63	0.34	1.15	0.133
MEHP	Crude	1.13	0.77	1.67	0.540
MEHP	Adjusted	1.18	0.78	1.79	0.435
MECPP	Crude	0.64	0.40	1.03	0.068
MECPP	Adjusted	0.60	0.35	1.03	0.065
BPA	Crude	0.85	0.60	1.19	0.338
BPA	Adjusted	0.87	0.60	1.26	0.459

All phthalate metabolites were Ln-transformed.
Adjusted for birth weight, maternal age at delivery, parity, gestational week, maternal education, smoking during early pregnancy, alcohol intake during early pregnancy, and maternal BMI before pregnancy