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Associations between maternal phthalate exposure and cord sex hormones in human infants

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ABSTRACT

It has been speculated that maternal phthalate exposure may affect reproductive development in human newborns. However, the mechanism awaits further investigation. The aim is to evaluate the association between maternal phthalate exposure and cord sex steroid hormones in pregnant women and their newborns from the general population. A total of 155 maternal and infant pair were recruited and analyzed. Levels of urinary phthalate metabolites and sex steroid hormones were determined using liquid chromatography/electrospray tandem mass spectrometry (LC–ESI-MS/MS) and radioimmunoassay (RIA), respectively. No significant correlation was found between each steroid hormones and phthalate metabolites for male newborns, except MMP was marginally significantly correlated with E_2. After adjusting for maternal age, estradiol (E_2) levels in cord serum from male newborns were not correlated with maternal urinary phthalate metabolites. In female newborns, the maternal urinary levels of mono-(2-ethylhexyl) phthalate (MEHP) and mono-(2-ethyl-5-hydroxyhexyl) phthalate (5OH-MEHP) were negatively correlated with the free testosterone (fT) and fT/E_2 levels in cord serum with Pearson correlation coefficients ranging between −0.24 and −0.29 ($p < 0.05$). Additionally, after gestational age was adjusted, the maternal urinary level of DEHP was negatively correlated with the free testosterone (fT) and fT/E_2 levels in cord serum. We suggest that maternal exposure to phthalates may affect sex steroid hormones status in fetal and newborn stage.

1. Introduction

Phthalates are widely used in plastics, building materials, and personal care products, and they are considered to be ubiquitous compounds to which humans are frequently exposed. In recent years, exposure to phthalates has drawn a lot of concern due to their adverse effects on reproductive system. Epidemiological studies have revealed that exposure to several commonly used phthalates such as di-methyl phthalate (DMP), di-ethyl phthalate (DEP), di-*n*-butyl phthalate (DBP), butyl-benzyl phthalate (BBP), and di-(2-ethylhexyl) phthalate (DEHP) can alter sex steroid hormone levels in human subjects (Duty et al., 2005; Main et al., 2006; Pan et al., 2006; Chou et al., 2009). It was recently hypothesized that the effects for prenatal exposure might be more profound because of fasting differentiation and proliferation of gonadal organs (Moore et al., 2001; Lottrup et al., 2006). The health effects of prenatal phthalate exposure and the later effects are of great concern.

Animal studies indicate that prenatal exposure to phthalates could be associated with in various reproductive effects in offspring, especially in male newborns. Prenatal exposure to DBP and/or DEHP is associated with reduced androgen-dependent organ weights (Kai et al., 2005; Howdeshell et al., 2007), decreased fetal serum testosterone (T) levels (Thompson et al., 2004; Borch et al., 2006; Howdeshell et al., 2007; Mahood et al., 2007), increased reproductive malformation (Wilson et al., 2007), alterations of sexual behavior (Dalsenter et al., 2006), increased the number of ovarian atretic tertiary follicles (Grande et al., 2007), reduced Leydig and Sertoli cell function (Mahood et al., 2007; Scott et al., 2007), and reduced anogenital distance (AGD) in exposed offspring (Ema et al., 2000; Barlow et al., 2004; Borch et al., 2006). In addition, prenatal exposure to BBP could be related to reduced body weight, and litter size during lactation, retention of nipples,

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and an increased incidence of male reproductive system malformations in offspring (Nagao et al., 2000; Ema and Miyawaki, 2002; Tyl et al., 2004).

Phthalate exposure has also been reported to be related to distorted levels of reproductive hormones such as free testosterone (fT), estradiol (E_2), follicle stimulating hormone (FSH), and inhibin B (Duty et al., 2005; Pan et al., 2006; Meeker et al., 2009) in adult men. Additionally, a study reported that the DEHP exposure of fertile men is associated with minor alterations of markers of fT (Mendiola et al., 2010). So far, only a few studies have been reported prenatal phthalate exposure in humans (Latini et al., 2003; Swan et al., 2005; Huang et al., 2009). Prenatal exposure to DEHP and/or its metabolite mono-(2-ethylhexyl) phthalate (MEHP) was associated with shorter gestational duration (Latini et al., 2003). Reductions in the AGD in male newborns are correlated with increasing levels of the mono-ethyl phthalate (MEP), mono-butyl phthalate (MBP), and mono-benzyl phthalate (MBzP) corresponding metabolites DEP, DBP, and BBP in human urine samples taken during pregnancy (Swan et al., 2005). Another study recently report that the levels of the phthalate metabolites MBP and MEHP in the amniotic fluid of female newborns were negatively correlated with the anogenital index (Huang et al., 2009).

Despite the fact that prenatal or neonatal phthalate exposure caused adverse effects for both male and female offspring, limited information is available regarding the effect of phthalate exposure on hormone levels. Our aim was to examine the association between maternal phthalate exposure in the general population and sex steroid hormone levels in cord blood.

2. Material and methods

2.1. Subjects

The usage of plastic material and potential exposure of unknown toxicants from plastics in Taiwanese has been a long and lasting issue in Taiwan, and recent studies have provided certain evidence about the phthalate exposure level in Taiwanese (Huang et al., 2007, 2009, 2010; Chen et al., 2008). The subjects of current study comes from a sub-study of a long-term birth cohort study (Wang et al., 2004, 2005), which also included the exposure assessment of dioxin, PCBs and lead in the same population. Briefly, all pregnant women were invited to visit the local medical center to participate in the study during December 2000 to November 2001. Initially, the spot urine samples of 430 subjects were collected at the third trimester along with their personal data, including reproductive and medical histories and physical parameters. All of the pregnant women were between the ages of 18 and 39, with a single pregnancy, and without clinical complication. Of the 430 subjects, 275 provided urine samples. The remaining 155 subjects with cord blood specimens and complete personal data were included in the present study. The study protocol was reviewed and approved by the Human Ethical Committee of the National Health Research Institutes in Taiwan. This study followed the ethical standards formulated from the Helsinki Declarations of 1964 and revised in 2000 (World Medical Association, 2000). Each participant provided informed consent after receiving a detailed explanation of the study and its potential consequences.

2.2. Phthalate metabolite measurements in maternal urine samples

Maternal urine was collected from subjects during third trimester of pregnancy (28–36 weeks) and spot urine samples were collected using glass beaker and immediately transferred into amber glass bottle at the hospital. Standards of phthalate metabolites including mono-methyl phthalate (MMP), MEP, MBP, MBzP, MEHP, mono-(2-ethyl-5-oxo-hexyl) phthalate (5oxo-MEHP), mono-(2-ethyl-5-hydroxy hexyl) phthalate (5OH-MEHP) and their corresponding $^{13}C_4$-labeled compounds were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Formic acid (FA), acetic acid (AA), buffer salts, and β-Glucuronidase (*Helix pomatia*) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol (HPLC grade) was purchased from Merck (Darmstadt, Germany). Deionized water was acquired from a Millipore system (Milford, MA,USA). β-Glucuronidase enzymatic deconjugation was used to change the glucuronid-conjugated forms of seven phthalate metabolites into their free forms for quantification of the total amounts in urine samples. As previously reported, on-line solid phase extraction (SPE) was used to extract the seven urinary phthalate metabolites (Lin et al., 2004). Briefly, this analytical approach involved the enzymatic deconjugation of phthalate metabolites followed by on-line SPE and quantification using liquid chromatography/electrospray tandem mass spectrometry (LC–ESI-MS/MS). The analytical system consisted of two PE series 200 pumps (PerkinElmer, Norfolk, CT) and an API365 triple quadrupole MS equipped with a TurboIonSpray source (PE Sciex, Throhill, ON, Canada). A PE series 200 autosampler (PerkinElmer, Norfolk, CT) performed the sample introduction, on-line SPE, and chromatographic separation using an electric two-position switching valve (6-ports, Valco Europe, Schenkon, Switzerland), a C18 trap cartridge (2.0 × 55-mm, 3-μm, Merck, Darmstadt, Germany) for SPE, and a Chromolith Flash RP-18e column (4.6 × 50 mm, Merck, Darmstadt, Germany). To deconjugate the samples, aliquots (1 mL) containing 750 μL urine, 50 μL of 2000 ppb $^{13}C_4$-labled phthalate metabolites as internal standards (IS), 200 μL of 100 mM ammonium acetate buffer (pH 6.5), and 10 units of β-glucuronidase were incubated at 37 °C for 90 min. The deconjugation reaction was stopped by the addition of 50 μL of 20% acetic acid/ACN. The mixture was passed through a 0.2 μm PVDF membrane filter (MSF-3, Advantec MFS, Inc., Pleasanton, CA, USA) and stored at 4 °C prior to loading onto the analytical system. The urine mixture was loaded onto the on-line SPE cartridge and washed with 2% formic acid/H_2O at a flow rate of 600 μL min^{-1} for 10 min before the switching valve was triggered to start the LC gradient and initiate chromatography on the Chromolith column. The gradient elution started with the mobile phase from 0.001% formic acid/H_2O at a flow rate of 600 μL min^{-1} to 100% MeOH in 10 min. The LC eluent was diluted 1:20 before entering the mass spectrometer. After gradient analysis was completed, the Chromolith column was washed with MeOH for 5 min and re-equilibrated with 2% formic acid/H_2O for 1 min before the next injection.

The MS/MS data acquisition was initiated by triggering the switching valve. The metabolites and the $^{13}C_4$-compounds were analyzed by monitoring their precursor to product ion transitions using the negative ion mode. The precursor to product ion transitions of MMP, MEP, MBP, MBzP, MEHP, 5OH-MEHP, 5oxo-MEHP, and their corresponding $^{13}C_4$-labeled compounds were the same as those in the previous report (Kato et al., 2005). Other instrumental parameters were optimized to generate the highest signal intensities.

The dynamic concentration range of the MMP, MEP, MBzP, 5OH-MEHP, and 5oxo-MEHP calibration curves was 0.67–1300 (ng mL^{-1}), while the range of calibration curves for MBP and MEHP was 0.67–670 (ng mL^{-1}). We examined the intra-day precision, apparent recovery, and method detection limit (MDL) of the analytical approach for these metabolites (Supp. Table 1A). The intra-day variations of all seven urine phthalate metabolites were below 10%, with intra-day recoveries at 100 ± 20% at three different concentrations, 25%, 50% and 75%, of individual substance. The detection limits of MMP, MEP, MBP, MBzP, MEHP, 5OH-MEHP, and 5oxo-MEHP were 3.4, 2.2, 1.6, 0.99, 0.55, 0.23, and 0.26 ng mL^{-1}, respectively.

The accuracy of the analytical approach was tested against two reference urine samples with different known phthalate

metabolite concentrations. The samples were received from the laboratory intercomparison program (www.g-equas.de) in 2006. These reference urinary samples contained MBP, MBzP, MEHP, 5OH-MEHP, and 5oxo-MEHP, but not MMP and MEP. In both concentrations, the relative errors (RE) of these five urinary metabolites were below 16% (Supp. Table 1B). Our analytical approach can therefore accurately quantify these five urinary phthalate metabolites, but the accuracy for MMP and MEP was not accessed.

Urinary 5OH-MEHP, and 5oxo-MEHP levels were used to represent the whole OH-MEHP and oxo-MEHP levels in urine, respectively. The creatinine-adjusted urinary concentrations of MEHP, OH-MEHP, and oxo-MEHP were summed for assessment of the DEHP exposure.

2.3. Quantification of serum reproductive hormones

Cord blood samples were collected and immediately centrifuged for 20 min at 4 °C. The serum was separated into aliquots and stored at −80 °C before hormone quantitative analysis. Cord blood levels of fT, and E_2 were quantified using radioimmunoassay in a clinical laboratory (Department of Nuclear Medicine, Kaohsiung Medical University, Kaohsiung, Taiwan). We carried out blind duplicates for every 10 samples. The analytical precision values, coefficient of variation (CV), from the duplicate analyses of samples were below 20%. The CV values of fT QC control samples at 3 different concentrations, including 150, 400, 800 ng dL^{-1}, were 7.1%, 5.3%, 7.0%, respectively. The CV values of E_2 QC control samples at three different concentrations, including 80, 250, 350 ng dL^{-1}, were 3.2%, 0.64%, 3.1%, respectively. The detection limits of fT, and E_2 were 20 ng dL^{-1}, and 20 pg mL^{-1}, respectively. For each cord blood sample, the fT was analyzed first and then E_2 was. Due to the limited volumes of cord blood samples, the number of analytical results for fT, and E_2 were 155, and 152, respectively.

2.4. Statistical analysis

To examine sexual differences at varied maternal phthalate metabolite levels, the phthalate metabolite levels were log-transformed and tested using a student *T* test for two independent samples. When the levels of the urinary phthalate metabolites showed geometrical distributions, the data were log10-transformed for further statistical comparison and correlation analyses. If the levels of metabolites or hormones were below their detection limits (DL), they were assigned values equal to half of their DL. We calculated the ratio of fT to E_2 because fT is the precursor of E_2 through the aromatase in metabolism. The Mann–Whitney *U* test was used to examine whether hormone levels, fT/E_2, or demographic data were significantly different between male and female newborns. The Pearson correlation coefficients were used to show the association between the maternal phthalate metabolite level and the sex steroid hormones in cord serum. Potential confounding factors were assessed with a stepwise multivariate regression analysis of the independent variables, including maternal and newborn characteristics. Stepwise multiple regression analysis was completed to control for confounders. A *p*-value of <0.05 was considered to be statistically significant. SPSS version 12 was employed for all statistical tests and correlation analysis.

3. Results

3.1. Subject characteristics

The characteristics of our subjects and their newborn by gender were shown in Table 1. Mean age and BMI of these women were 28.8 ± 3.6 years old and 26.1 ± 3.3. Less than 5% of them had smoking and drinking habit during pregnancy. Only three subjects have ever worked in a chemical factory. The mean age and BMI of the un-followed subjects were 27.9 ± 4.6 years old and 25.4 ± 3.8, respectively. Their smoking and drinking rates were 3.4% and 4.0%, respectively. No significant differences of these variables between participants and un-followed subjects were observed.

Mean gestational age of newborns were around 39 weeks. Birth weight and head circumference of the male newborns were significantly greater than those of the female newborns ($p < 0.05$). In addition, there was a marginally significant difference in the birth length between male and female newborns ($p < 0.1$).

3.2. Phthalate metabolites in maternal urine samples

Levels and distribution of phthalate metabolites in maternal urine were shown in Table 2. Median levels of urinary MMP, MEP, MBP, MBzP, MEHP, 5oxo-MEHP, 5OH-MEHP and ΣDEHP without creatinine-adjusted were 34.6, 34.6, 65.5, 8.85, 11.7, 17.2, 11.8 and 43.9 ng mL^{-1}, and those with creatinine-adjusted were 54.7, 56.0, 95.9, 15.6, 19.1, 25.6, 19.8 and 68.8 μg g creatinine^{-1}, respectively. For all of these metabolites, the frequencies of samples with phthalate metabolite levels above their MDLs were higher than 98.7%.

3.3. Sex steroid hormones in the cord blood

Levels of cord blood fT, E_2 and the fT/E_2 in male and female newborns are shown in Table 3. All samples had sex steroid hormone levels above the MDL. Median levels of fT in male newborns were

Table 1
Characteristics of the mothers and their newborns according to newborn sex.

Characteristics	Male (*n* = 81)	Female (*n* = 74)	*p* value[a]
Maternal			
Age (mean ± SD, years)	28.8 ± 3.6	29.0 ± 4.5	0.449
BMI (mean ± SD, kg m^{-2})	26.1 ± 3.3	25.6 ± 3.7	0.266
Smoking habit during pregnancy, *n* (%)	0 (0%)	2 (2.7%)	0.138
Drinking habit during pregnancy, *n* (%)	3 (3.7%)	1 (1.4%)	0.358
Ever work history in chemical factories, *n* (%)	1 (1.2%)	2 (2.7%)	0.509
Newborn			
Gestational age (mean ± SD, weeks)	38.8 ± 1.47	38.6 ± 1.64	0.491
Birth weight (mean ± SD, g)	3250 ± 393	3040 ± 414	0.001**
Birth length (mean ± SD, cm)	52 ± 2.4	51 ± 2.7	0.073
Birth head circumference (mean ± SD, cm)	34 ± 1.4	33 ± 1.6	0.039*
Chest girth (mean ± SD, cm)	33 ± 1.8	33 ± 1.5	0.109

[a] Mann–Whitney *U* test or Fisher's exact test.
* $p < 0.05$.
** $p < 0.01$.

Table 2
Unadjusted and creatinine-adjusted concentrations of seven phthalate metabolites in pregnant women's urine samples (n = 155).

Phthalate metabolites[a]	Percentile							Median (range)		
	Min[b]	5th	25th	50th	75th	95th	Max	Taiwan		US[e]
Creatinine-unadjusted (ng mL^{-1})								1st trimester[c]	2nd trimester[d]	3rd trimester
MMP	ND	1.71	18.6	34.6	60.1	127	461	7.1 (0.7–48.4)	4.3 (0.7–237.2)	–
MEP	10.1	12.6	22.3	34.6	61.3	241	397	22.8 (0.5–415)	27.7 (0.7–5466)	–
MBP	9.34	16.4	36.1	65.5	121	275	662	78.0 (8.9–541)	81.8 (13.2–580)	–
MBzP	ND	3.09	5.90	8.85	15.1	40.3	68.0	3.0 (0.7–845)	0.9 (0.9–35.3)	–
MEHP	0.83	5.07	8.85	11.7	16.5	34.6	84.7	24.6 (0.5–1140)	20.6 (0.7–381)	–
5oxo-MEHP	ND	2.76	7.60	17.2	30.2	127	271	–	–	–
5OH-MEHP	ND	2.63	7.33	11.8	22.2	66.1	186	–	–	–
ΣDEHP	11.6	17.3	28.2	43.9	71.1	181	479	–	–	–
Creatinine-adjusted (μg g creatinine^{-1})										
MMP	ND	8.82	26.9	54.7	83.6	184	728	–	10.8 (0.4–363))	–
MEP	9.33	18.9	34.5	56.0	106	346	863	–	68.0 (5.0–13 299)	236 (26.7–5520)
MBP	11.3	29.9	58.4	95.9	169	507	926	–	195 (57.8–1901)	42.6 (21.3–105)
MBzP	ND	4.56	10.1	15.6	25.9	43.9	104	–	3.7 (0.5–69.9))	12.1 (5.6–120)
MEHP	3.73	4.91	10.4	19.1	33.7	100	193	–	60.8 (12.2–1251)	4.6 (1.8–449)
5oxo-MEHP	ND	5.44	14.6	25.6	43.6	158	801	–	–	–
5OH-MEHP	ND	4.17	11.7	19.7	30.7	109	407	–	–	–
ΣDEHP	16.2	25.6	43.2	68.8	105.5	316	927	–	–	–

[a] ΣDEHP = MEHP + 5oxo-MEHP + 5OH-MEHP.
[b] ND: not detected. Detection limits (LOD) of seven phthalate metabolites were: MMP, 3.4; MEP, 2.2; MBP, 1.6; MBzP, 0.99; MEHP, 0.55; 5oxo-MEHP, 0.26; 5OH-MEHP, 0.23 ng mL^{-1}, respectively. ND was calculated as 1/2 LOD.
[c] Huang et al., 2009. Taiwanes pregnant women carried female fetus at first trimester (n = 31).
[d] Huang et al., 2007. Taiwanes pregnant women 33.6 ± 3.3 years old (n = 76).
[e] Adibi et al., 2003. New York pregnant women 18–35 years old (n = 25).

Table 3
Levels of cord free testosterone (fT) and estrogen (E_2) in male and female newborns.

Hormones	Percentile								p value[b]
	n[a]	Min	5th	25th	50th	75th	95th	Max	
fT (ng dL^{-1}) Male	81	56	92	140	220	290	490	650	0.029*
Female	74	34	70	120	167	260	370	482	
E_2 (pg mL^{-1}) Male	80	4100	4400	5800	6500	8300	10 000	11 000	0.679
Female	72	2500	3600	5900	6700	9500	7900	9800	
fT/E_2[c] Male	80	0.11	0.13	0.23	0.31	0.39	0.66	1.06	0.019*
Female	72	0.10	0.12	0.20	0.26	0.36	0.48	0.66	

[a] One male and two female newborn do not have enough serum sample to measure estrogen.
[b] Sex differences of hormone levels in cord blood were tested using the Mann–Whitney U test.
[c] fT/E_2 was non-dimensional unit.
* $p < 0.05$.

significantly 1.2-fold higher than those in female newborns (p = 0.029). This difference was similar to a previous report (Dawood and Saxena, 1977). There were also significant differences between sexes for the fT/E_2 in newborns (p = 0.019). There were no gender differences in the concentration of E_2 in cord blood (p = 0.679). Although all 155 samples were tested for fT, three samples were not tested for E_2 due to the limited volumes of the cord blood samples.

3.4. Associations between maternal phthalate exposure and cord sex steroid hormones levels

Correlations between maternal phthalate metabolite concentrations and the cord serum hormone levels of newborns are shown in Table 4. fT concentration in cord blood was negatively correlated with maternal MEP (r = −0.24, $p < 0.05$), MEHP (r = −0.32, $p < 0.01$), 5OH-MEHP (r = −0.28, $p < 0.05$) and ΣDEHP (r = −0.38, $p < 0.001$) for female newborns. In addition, the fT/E_2 ratios were negatively correlated with MEP (r = −0.29, $p < 0.1$), MEHP (r = −0.27, $p < 0.05$), 5OH-MEHP (r = −0.30, $p < 0.05$) and ΣDEHP (r = −0.35, $p < 0.01$). Fig. 1 showed clear negative linear correlations between fT, fT/E_2 and ΣDEHP in female newborns. However, no significant correlation was found between each steroid hormones and phthalate metabolites for male newborns, except MMP was marginally significantly correlated with E_2.

A stepwise multivariate regression model was used to examine the associations between cod sex hormones of newborns, and maternal phthalate metabolites (Table 5). Several potential confounding factors, such as maternal age, BMI, smoking habit, gestational age, pregnant times, and ever using contraceptive drug, which could be associated with phthalate exposure and/or endocrine disruption were included for stepwise multivariate regression analysis.

Gestational age were significantly positively associated with cord blood fT, whereas ΣDEHP showed a negatively correlation with fT (β: −0.23, $p < 0.05$) in female newborns. In addition, we also found that ΣDEHP showed a negatively association with fT/E_2 (β: −0.22, $p < 0.05$). In male newborns, only E_2 level in cord serum was negatively correlated with maternal age (β: −0.01, $p < 0.05$). Additionally, fT/E_2 ratio of male newborns was negatively correlated with pregnant times.

4. Discussion

This is the first report on the association of maternal phthalate exposure and sex steroid hormones in cord serum samples. We

Table 4
Pearson correlation coefficients between levels of maternal phthalate metabolites and cord blood hormone levels.

Phthalate metabolites (μg g creatinine^{-1})[a]	Male newborns			Female newborns		
	fT (ng dL^{-1}) (n = 81)	E_2 (pg mL^{-1}) (n = 80)	fT/E_2 (n = 80)	fT (ng dL^{-1}) (n = 74)	E_2 (pg mL^{-1}) (n = 72)	fT/E_2 (n = 72)
MMP	0.11	0.21$^{\#}$	0.00	−0.01	0.03	−0.01
MEP	−0.10	0.02	−0.13	−0.24*	0.01	−0.29*
MBP	−0.11	0.05	−0.15	−0.07	−0.07	−0.06
MBzP	0.05	0.14	−0.03	−0.18	−0.20$^{\#}$	−0.10
MEHP	−0.07	−0.07	−0.04	−0.32**	−0.19	−0.27*
5oxo-MEHP	0.11	0.02	0.11	−0.19	−0.07	−0.20
5OH-MEHP	0.16	0.07	0.15	−0.28*	−0.09	−0.30*
ΣDEHP[b]	0.06	−0.00	0.07	−0.38***	−0.17	−0.35**

[a] Phthalate metabolites were all log-transformed.
[b] ΣDEHP = MEHP + 5oxo-MEHP + 5OH-MEHP.
$^{\#}$ p value for Pearson correlation: $p < 0.1$.
* p value for Pearson correlation: $p < 0.05$.
** p value for Pearson correlation: $p < 0.01$.
*** p value for Pearson correlation: $p < 0.001$.

found that both the fT concentration and fT/E_2 ratio in the cord blood of female newborns were negatively correlated with two DEHP metabolites, MEHP and 5OH-MEHP, in maternal urine. Alteration of the reproductive hormone concentrations in newborns might be associated with maternal, perinatal, and pregnancy factors that are related to hormone-related diseases (Troisi et al., 2003). According to our data, prenatal phthalate exposure may alter sex steroid hormone levels and proportions, which may be related to the factors for subsequent cancer risk. fT and E_2 cord blood concentrations were consistent with those in an earlier report, which identified the fT and E_2 levels in a general population (Savarese et al., 2007). The ratio of fT to E_2 is considered as an index of aromatase activity as fT is the metabolic precursor of E_2 through aromatase. Furthermore, the fT/E_2 ratio has been related to coronary artery disease (Kajinami et al., 2004; He et al., 2007), gynecomastia (Beck, 1981), and hepatocellular carcinoma (Tanaka et al., 2000). In the present study, male newborns had different fT/E_2 ratios in cord blood than female newborns. In female newborns, the fT/E_2 level was associated with maternal DEP and DEHP exposure, indicating that prenatal phthalate exposure may alter the proportion of sex steroid hormones.

One study reported the levels of urinary phthalate metabolites at second trimester from pregnant women in southern Taiwan (Huang et al., 2007). Among the phthalate monoesters, median levels of MMP, and MBzP were 4-to-5 folds higher than those in Huang's report, whereas those of MBP and MEHP were 2-to-4 folds lower (Table 2). Similar phthalate metabolite profile at first trimester from pregnant women in southern Taiwan was observed in Huang's another report (Huang et al., 2009). These different exposure profiles could be possibly caused by the difference exposure sources of phthalates, like dietary habits, from subjects with difference area. In our study, the levels of the urinary phthalate metabolites MBP, MBzP, MEHP, 5OH-MEHP, and 5oxo-MEHP were validated using authentic samples from a continuous laboratory intercomparison program, which allowed us to provide validated reference values for urinary phthalate metabolite levels in pregnant women in Taiwan.

Compared to the median levels of urinary MEP, MBP, MBzP, and MEHP in pregnant New York women (Adibi et al., 2003), median level of MEP in this study was 4-fold lower, and that of MEHP was 4-fold higher (Table 2). The median levels of MBP and MBzP were similar between the two reports. Another previous report identical identified phthalate metabolite concentrations (ng mL^{-1}) in pregnant women (Swan et al., 2005). For comparing their urinary metabolite levels with our data, we assumed that the urinary creatinine levels of the previous report and our study were similar. We used the 0.8 g L creatinine^{-1} concentration identified by our study to adjust the urinary metabolite levels in the previous report. With the exceptions of MMP, MBP, and MEHP, the metabolite medians were similar. Among DEHP-derived metabolites, the levels of the secondary metabolites 5oxo-MEHP and 5OH-MEHP were higher than the primary metabolite MEHP. This finding was consistent with an earlier report (Swan et al., 2005).

Various human and animal studies have demonstrated that phthalate exposure has anti-androgenic effects on reproductive systems, especially in males (Fisher, 2004; Swan et al., 2005; Latini et al., 2006). In a recent report, male workers exposed to DBP and DEHP had a decreased serum fT level (Pan et al., 2006). It has been suggested that phthalates affect the reproductive system by influencing Leydig cell function in the testis (Lottrup et al., 2006). In human and animal models concerning male offspring, many studies have focused on the effects of prenatal phthalate exposure on the reproductive system, especially DBP, BBP and DEHP (Ema et al., 2000; Ema and Miyawaki, 2002; Thompson et al., 2004; Barlow et al., 2004; Swan et al., 2005; Dalsenter et al., 2006; Foster, 2006; Howdeshell et al., 2007; Mahood et al., 2007; Scott et al., 2007; Wilson et al., 2007). However, we did not identify an association between fT concentrations in the cord blood of male newborns and prenatal phthalate exposure. Additionally, another report showed that maternal fT was correlated with the fT concentrations in the cord blood of male, but not female, newborns (r = 0.34) (Troisi et al., 2003). Therefore, the effects of prenatal phthalate exposure on the fT levels in the cord blood of male newborns may be veiled by the effect of maternal fT. Meanwhile, several studies have demonstrated that prenatal phthalate exposure also affects the reproductive system of female offspring. Female offspring exposed to BBP in utero and during lactation had an increased AGD, but the levels of sexual hormones were not associated with phthalate exposure (Nagao et al., 2000). Prenatal exposure to BBP has been related to delayed puberty in female offspring, although BBP exposure has more reproductive effects for male offspring (Tyl et al., 2004). In addition, an increase in the number of ovarian atretic tertiary follicles has been observed in female offspring exposed to DEHP in utero and during lactation (Grande et al., 2007).

The phthalate exposure assessment was dependent upon the phthalate metabolite levels in spot urine samples from pregnant women in their third trimester. We assumed that the maternal phthalate exposure during pregnancy was consistent, as a number of earlier reports have supported the assumption that daily human phthalate exposure was consistent (Hoppin et al., 2002; Hauser et al., 2004). However, if this was not true, it is possible that misclassification of exposure levels based on spot urine samples could minimize the prenatal exposure effects on reproductive hormones.

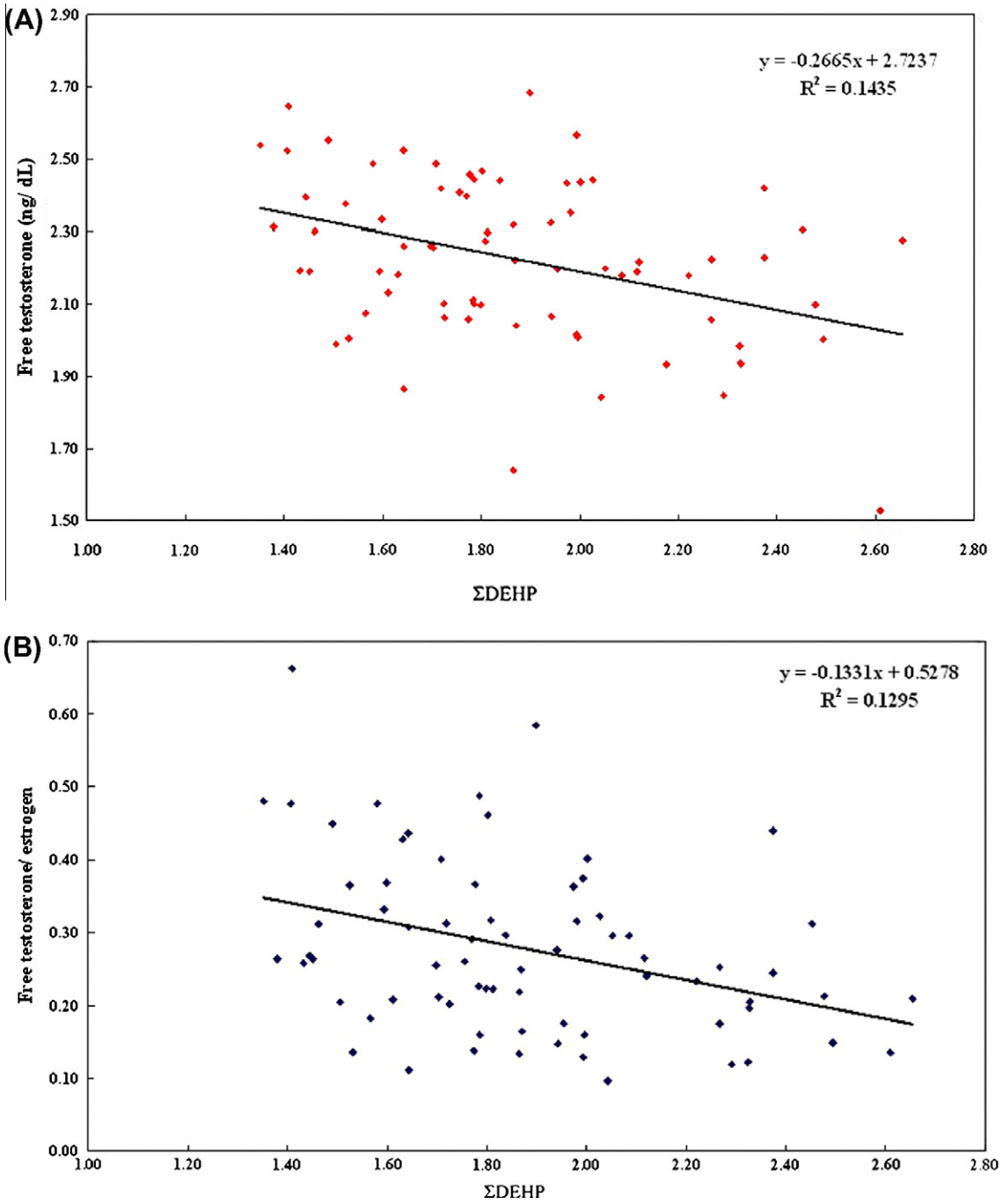

Fig. 1. Scatter plot of linear regression: (A) free testosterone (fT) and ΣDEHP; (B) fT/estrogen (E_2) and ΣDEHP.

More significant effects of phthalate exposure on hormone levels should be observed when there is less exposure misclassification. Therefore, we believe that using spot urine samples for the assessment of phthalate exposure would not cause a false significant correlation.

In the present study, we found that prenatal phthalate exposure might be related to a reduction in the cord blood fT concentration of female offspring. Interestingly, pregnant women exposed to endocrine disruptors, including polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and polychlorinated biphenyls (PCBs), had reduced fT concentrations in the cord blood of female newborns (Cao et al., 2008) but not in male newborns. Such observations indicate that endocrine disruptors affect the reproductive systems of female offspring through different mechanisms than male offspring. As the enzyme aromatase is responsible for the conversion of T to E_2, the endocrine disruption effect of phthalates may be a result of aromatase activity regulation, resulting in the distortion of T and E_2 concentrations (Lovekamp-Swan and Davis, 2003). For illustrating the mechanism of ovarian action of DEHP, they proposed a model which DEHP or/and its active metabolites cause decreased serum E_2 levels through the suppression of aromatase. However, the model is not consistent with our observations, which may be resulted from our observation based on the human and/or prenatal phthalate exposure. A previous report showed that brain aromatase activity is inhibited at low DEHP doses and increased at high DEHP doses in rat male offspring after in utero or

Table 5
Stepwise multivariate linear regression between sex steroid hormones and phthalate exposure in cord blood of newborns.

Newborn gender	Male		Female	
Hormone/Phthalate metabolites[a]	E_2 (pg mL^{-1})	fT/E_2	fT (ng dL^{-1})	fT/E_2
	Estimate	Estimate	Estimate	Estimate
Constant	4.11**	−1.42**	0.99	−1.17**
Age	−0.01**	0.03	−0.03	0.05
BMI	−0.04	−0.01	0.19#	0.16
Gestational age	0.06	0.89	0.04*	1.86
Smoking	–	–	0.08	0.01#
Times of pregnant	0.13	−0.04*	0.00	−0.08
Ever using contraceptive drug	−0.07	−0.17	−0.21	−0.23#
MMP	0.15	0.03	0.06	0.06
MEP	−0.02	−0.17	0.02	−0.02
MBP	−0.02	−0.22	−0.01	−0.01
MBzP	0.11	−0.01	0.00	0.10
ΣDEHP[b]	−0.05	0.01	−0.23*	−0.22**
R^2	0.12	0.07	0.26	0.16
p value	0.003	0.031	0.000	0.001

[a] Creatinine-adjusted.
[b] ΣDEHP = MEHP + 5oxo-MEHP + 5OH-MEHP.
# $p < 0.1$.
* $p < 0.05$.
** $p < 0.01$.

lactational treatment. In female offspring, the aromatase activity was increased regardless of the doses, which enhanced the conversion of T to E_2 (Andrade et al., 2006). The enhancement of the T to E_2 conversion in female offspring is similar to our observation of a decreasing fT/E_2 ratio with an increasing phthalate exposure level. Whether the endocrine disruption effect of phthalates offers a similar mechanism remains to be studied.

5. Conclusion

This is the first report associating prenatal phthalate exposure with reproductive hormone concentrations in human cord blood samples. The levels of DEHP metabolites in maternal urine were negatively correlated with the fT and fT/E_2 levels in the cord blood of the female newborns. A negative association was also observed between the level of DEHP and the fT/E_2 ratio in the cord blood of female newborns. Our results suggest that prenatal phthalate exposure may affect the hormone levels of newborns at the time of delivery. The long term adverse effects of prenatal phthalate exposure on the reproductive systems of female newborns should be of great concern.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chemosphere.2010.12.079.

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