ASSOICATIONS BETWEEN PHTHALATE EXPOSURES AND INTERFERENCE OF TESTICULAR STEROIDOGENESIS PATHWAY IN ADULT MEN

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Introduction

Increased incidence of testicular dysgenesis syndrome, such as hypospadias, cryptorchidism, testicular cancer and male infertility, are thought to be associated with not only heredity but also exposures to environmental endocrine disruptors (EDCs)[1]. Phthalate esters, one of potential EDCs, had been shown to have a weak estrogenic and/or anti-androgenic effect[1]. Di-2-ethylhexyl phthalate (DEHP) and dibutyl phthalate (DBP) were major used for consumer products and usage of those are still allowed in Taiwan. The animal experiments and human epidemiological studies had reported that exposure to phthalate esters resulted not only in the inhibition of testicular testosterone synthesis but also estradiol synthesis[1, 2]. Testosterone biosynthesis starts from cholesterol in the mitochondrial inner membrane where cytochrome P450 cholesterol side chain cleavage enzyme (CYP11A1) first transform it into pregnenolone. Pregnenolone latter diffuses to smooth endoplasmic reticulum, where it is converted to testosterone by the following enzymes: 3β-hydroxysteroid dehydrogenase 1 (3β-HSD), cytochrome P450 17α-hydroxylase/17, 20-lyase (CYP17A1) and 17β-hydroxysteroid dehydrogenase 3 (17β-HSD3)[1, 3]. Aromatase also transforms irreversibly androgens into estrogens[1, 3]. Some of these anti-androgenic or estrogenic effects are possibly caused by the down-regulation of steroidogenic enzymes in Leydig cells[3]. A more recent study also showed that MEHP% was strongly negatively associated with T:E2 ratio in healthy men[4], in contrast with the observations of Meeker et al (2009)[5]. These reflected that phthalates might disorder aromatase or other steroidogenesis enzymes activity in testis. However, the exact mechanism of phthalate exposures on human testicular steroidogenesis has not been well studied. In present study, we report that DEHP and DBP may infered with testicular steroidogenesis in adult men.

Materials and methods

Study population.This cross-sectional study ran from 2010 to 2012. Infertile men (25-45 years old) whose spouses failed to achieve pregnancy after at least 1 year of regular contraceptive intercourse and had no diagnosed reproductive disorder were recruited through infertility clinics in sourth Taiwan. Men with known factors related to male infertility, such as reproductive or urological diseases, using hormonal therapy or steroid medication, occupational exposure to reproductive toxicants were excluded in advance. After signed an informed consent prior to participating, a first spot urine sample and serum sample were collected. Urine and serum samples were frozen at -80℃ until analyses. The questionnaire was filled out by participants.

Urinary phthalate metabolites analyses. Urinary concentrations of mono-iso-butyl phthalate (MiBP), mono-n-butyl phthalate (MnBP), mono-(2-ethylhexyl) phthalate (MEHP), mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono-2-ethyl-5-carboxypentyl phthalate (MECPP) were analyzed by HPLC-MS/MS after well-established solid phase extraction method.

Reproductive hormones analyses. Reproductive hormones, including total testosterone (TT), estradiol (E_2), follicle-stimulating hormone (FSH), luteinizing hormone (LH) were measured by NCKU Hospital Pathology. Sex hormone-binding globulin (SHBG), inhibin B, estrone(E_1), Dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfat (DHEA-S), and androstenedione (AD) were measured by Lezen clinical laboratory.

Statistics. We calculated (in nmol/mL) the sum of DEHP metabolites (ΣDEHP) that MEHP, MEHHP, MEOHP and MECPP were measured, as well as the sum of DBP isomers metabolites (ΣDBPi+n) that MiBP and MnBP were measured. We also calculated MEHP% [MEHP (nmol/mL) / ΣDEHP (nmol/mL)]. Basic descriptive statistics were derived for population characteristics, serum levels of reproductive hormones, and urinary phthalate levels. The adjusted regression coefficient for the changes of urinary phthlate metbolites levels and leydig cell-related hormones were calculated using multiple linear regression with adjustment for age, age squard, BMI, time of blood sampling, and

season. *P*-values for linear trend across quintiles were derived from analyses entering phthalate quintiles variables as ordinal categorical coded using integer values (1–5). The Statistical Product and Service Solutions 17.0 and SAS 9.2 for Windows were used for all statistical analyses.

Results and discussion

Basic characteristics of participants are shown in Table I. Distributions of urinary phthalate metabolite concentrations are presented in Table II and serum hormone levels in Table III. All phthalate metabolites were detectable in >99% of participants. In quintile analysis (Figure 1), There was a sharp raise in the adjusted mean for change in E_2:TT ratio among $\Sigma DBP_{(i+n)}$ quintile (p for trend = 0.02). A slightly decline was also found in the adjusted mean for change in AD:E_1 ratio among $\Sigma DBP_{(i+n)}$ quintile (p for trend = 0.05). The regression coefficients (95% CI) for change in sex hormones associated with urinary phthalate metabolite concentrations after adjusting for age, age square, BMI, season, and time of day blood sample was collected were collected were shown in Table IV. The postive associations among MnBP, MEOHP, MECPP, ΣDEHP and $\Sigma DBP_{(i+n)}$ and serum E_2 levels were found, and where an IQR increased in them were associated with 6%, 8%, 6%, 6%, and 6% increased in E_2 ($p \leq 0.05$), as well as an IQR increase in MiBP, MnBP, MEOHP, MECPP, ΣDEHP and $\Sigma DBP_{(i+n)}$ were associated with 6%, 7%, 8%, 6%, 6%, and 7% increase in E_2 ($p < 0.05$). Otherwise, the inverse associations among MnBP, MEOHP, $\Sigma DBP_{(i+n)}$ and E_1:E_2 ratio were found, and where an IQR increased in them were associated with 13%, 19%, and 13% drecreased in E_1:E_2 ratio ($p \leq 0.05$). An IQR increased in MEHP% was associated with 9% increased in DHEA:AD ratio (95% CI:1.02~1.17, p=0.01). MiBP, MEHHP, and ΣDEHP were inversely associated with inhibin B, where an IQR increased in them were associated with a 9%, 8%, and 9% decline ($p \leq 0.05$) in inhibin B, as well as an IQR increased in MiBP, MEHHP, MEOHP, MECPP, and ΣDEHP were associated with 11%, 13%, 11%, 11%, and 13% in inhibin B:FSH ratio ($p < 0.05$). It is generally considered that phthalates exert their action by inhibiting Leydig cell synthesis of testosterone. We found increased DHEA/AD ratio with increased MEHP%, which might indicate suppressed 3β-HSD activity was involved in the step of testosterone biosynthesis from DHEA to androstenedione and also androstenediol to testosterone. Androstenedione was invervely assoicated with DBP, DEHP and their matabolites in rat and human[6, 7]. Consistent with the finding, animal and in vitro studies have demonstrated that 3β-HSD activity was inhibited after exposure to DBP, DEHP and MEHP [3, 8]. Furthermore, estrogens are synthesized by 17β-HSD3 and aromatase which are biologically expressed in testis. We found decreased E_1/E_2 ratio with increased MnBP and MEOHP exposures, which might refelect raised 17β-HSD3 activity was involved in interconversion of E_1 and E_2 and also DHEA to androstenediol. We also found increased E_2 and E_2/TT ratio with increased MiBP, MnBP, MEOHP and MECPP exposures, which might represent aromatase was involved in the transformations of androstenedione to E_1 and also testosterone to E_2. This was consistant with a previous report shown that aromatase expression and activity are increased in Leydig cells after phthalate exposure, particularly DEHP[1, 4]. Thus, in turn, could lead to raising estrogen level in testis[1]. Our data might explain why male had ascending serum estradioi level among increasing quintiles of ΣDEHP. We found a postive association between MEHP% with E_2 and TT/E_2 ratio and those results were consistant with the researches of Joensen et al.[4] , but in contrast with the results of Meeker et al.[5]. In conclusion, DEHP and DBP exposures may infere in reproductive hormones by altered the hormone biosynthetic enzymes in synthesis of androgen and estrogen. The sample size in our study was still small, further researches are needed in the future.

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Table I Demographic data of adult men recruited from infertile clinic

Item		Infertile men (N = 223)	Median (5th, 95th)
Age (years)		34.6±4.02a	34.1(28.3, 42.0)
Body mass index (kg/m2)		26.1±3.54	25.7(20.1, 32.1)
Waist (cm)		89.9±9.34	90.0(74.4, 105)
Marry duration (years)		3.20±2.30	2.50(1.00, 8.00)
Educationb	High-school	31(13.9)b	
	College	128(57.4)	
	Graduate school	64(28.7)	
Cigarette smoking (yes, n)		66(29.6)	
Alcohol drinking (yes, n)		44(19.7)	
Tea drinking (yes, n)		176(78.9)	
Coffee drinking (yes, n)		92(41.3)	
SIP score c	0	9(4.00)	
	1	64(28.7)	
	2	94(42.2)	
	3	56(25.1)	

a Data are expressed as mean ± SD. b Data are expressed as number (%). cSummary index for plastic material contact (SIP) to ascertain if the subject came into contact with plastic tableware (0/1), water or tea in polyethylene terephthalate bottles (0/1) and food packaging (0/1) (Pan et al. 2006).

Table II The distribution of urinary phthalate monoester and secondary metabolites in infertile men

Phthalate metabolites(ng/ml)	Geometric Mean	Selected Percentiles				
		10 th	25 th	50 th	75 th	95 th
MiBP	7.63	2.29	4.40	7.80	13.3	31.0
MnBP	13.7	4.90	8.42	13.0	23.7	61.0
MEHP	4.32	1.70	2.65	4.11	6.76	16.5
MEHHP	12.6	5.28	7.61	12.1	19.1	51.8
MEOHP	8.45	3.25	5.28	8.20	13.8	31.1
MECPP	16.0	6.22	10.1	15.4	24.1	71.2
MEHP (%)	11.0	5.69	8.12	11.6	15.1	22.1
ΣDEHP (nmol/mL)	0.14	0.06	0.09	0.14	0.21	0.49
ΣDBP(i+n) (nmol/mL)	0.10	0.04	0.06	0.10	0.18	0.43

Table III Summary statistics for serum reproductive hormone levels and hormone ratio in infertile men

Reproductive hormones	Geometric Mean	Selected Percentiles				
		10th	25th	50th	75th	95th
LH (mIU/mL)	4.87	2.66	3.76	5.06	6.38	9.32
FSH (mIU/mL)	4.69	2.63	3.37	4.49	6.42	9.62
Inhibin B (pg/mL)	103	54.4	77.3	102	143	246
SHBG (nmol/L)	25.8	15.1	18.1	25.2	34.7	56.2
DHEA (ng/mL)	5.01	3.18	3.82	4.98	6.34	9.42
DHEA-s (ng/mL)	28.3	16.7	22.0	29.5	38.1	48.9
Androstenedione (AD, ng/mL)	1.50	0.97	1.16	1.48	1.91	2.66
Total Testosterone (TT, ngl/L)	3.94	2.44	3.10	3.89	5.18	7.02
Estradiol (E2, pmol/L)	24.4	16.4	20.1	24.8	31.3	41.2
Estrone (E1, pg/mL)	48.6	34.6	40.8	49.2	57.7	74.8
TT:LH ratio	0.81	0.40	0.59	0.83	1.16	1.91
Inhibin B : FSH ratio	22.0	10.2	14.6	22.9	35.1	70.1
TT:E2 ratio	0.16	0.09	0.12	0.16	0.22	0.31
AD:E1 ratio	0.03	0.01	0.02	0.03	0.04	0.05
E1:E2 ratio	1.99	1.25	1.56	1.95	2.52	3.86
DHEA:AD ratio	3.33	2.29	2.79	3.31	4.06	5.22

Table IV Adjusted [a] regression coefficients [b] (95% CI) for change in sex hormones [c] associated with urinary phthalate metabolite concentrations [c] (N=223)

Metabolite	LH	FSH	DHEA	DHEA-S	TT	E_2	E_1	AD
MiBP	1.02(0.96~1.09)	1.03(0.96~1.10)	1.02(0.96~1.08)	1.01(0.95~1.08)	0.98(0.94~1.02)	1.04(0.98~1.09)	1.02(0.98~1.06)	1.03(0.97~1.08)
MnBP	1.05(0.93~1.13)	1.02(0.95~1.10)	1.02(0.96~1.08)	1.02(0.96~1.08)	0.99(0.95~1.04)	**1.06(1.01~1.12)[d]**	1.02(0.92~1.12)	1.00(0.95~1.05)
MEHP	1.02(0.95~1.10)	1.03(0.96~1.13)	1.01(0.95~1.08)	1.04(0.97~1.11)	0.98(0.94~1.03)	1.03(0.97~1.10)	1.00(0.96~1.04)	0.99(0.93~1.05)
MEHHP	1.04(0.97~1.12)	1.03(0.98~1.14)	0.99(0.93~1.05)	1.01(0.95~1.08)	0.99(0.95~1.04)	1.04(0.98~1.10)	1.02(0.97~1.06)	1.00(0.95~1.06)
MEOHP	1.03(0.96~1.11)	1.05(0.98~1.14)	0.99(0.93~1.06)	1.00(0.94~1.07)	1.00(0.96~1.05)	**1.08(1.02~1.15)[d]**	1.02(0.98~1.06)	1.01(0.96~1.07)
MECPP	1.03(0.96~1.10)	1.03(0.96~1.11)	0.98(0.92~1.04)	1.02(0.96~1.09)	1.00(0.96~1.05)	**1.06(1.01~1.12 [d]**	1.02(0.98~1.06)	0.99(0.93~1.05)
MEHP%	0.97(0.87~1.09)	0.98(0.87~1.11)	1.07(0.96~1.18)	1.06(0.95~1.07)	0.97(0.90~1.04)	0.95(0.87~1.05)	0.96(0.90~1.03)	0.98(0.89~1.07)
ΣDEHP	1.04(0.96~1.12)	1.05(0.97~1.14)	0.99(0.92~1.06)	1.02(0.95~1.09)	0.99(0.94~1.04)	**1.06(1.00~1.13)[e]**	1.02(0.97~1.06)	0.99(0.94~1.06)
$\Sigma DBP_{(i+n)}$	1.05(0.98~1.13)	1.03(0.96~1.11)	1.02(0.96~1.09)	1.02(0.95~1.08)	0.99(0.95~1.03)	**1.06(1.01~1.12)[d]**	1.02(0.98~1.06)	1.01(0.96~1.07)

Metabolite	Inhibin B	SHBG	Inhibin B:FSH ratio	TT:LH ratio	E_2:TT ratio	AD:E_1 ratio	E_1:E_2 ratio	DHEA:AD ratio
MiBP	**0.91(0.83~0.99)[d]**	1.01(0.95~1.08)	**0.89(0.79~0.99)[d]**	0.96(0.89~1.04)	**1.06(1.00~1.12)[d]**	1.01(0.96~1.06)	0.91(0.79~1.07)	0.99(0.95~1.03)
MnBP	0.99(0.91~1.09)	1.01(0.95~1.08)	0.98(0.87~1.10)	0.94(0.87~1.02)	**1.07(1.01~1.13)[d]**	0.98(0.94~1.03)	**0.87(0.75~1.00)[d]**	1.02(0.97~1.07)
MEHP	0.97(0.88~1.06)	1.02(0.95~1.09)	0.93(0.82~1.05)	0.96(0.88~1.06)	1.05(0.99~1.13)	0.98(0.94~1.04)	0.90(0.76~1.06)	1.03(0.98~1.08)
MEHHP	**0.92(0.84~0.99)[d]**	1.03(0.96~1.10)	**0.87(0.77~0.98)[d]**	0.97(0.89~1.06)	1.05(0.98~1.11)	0.98(0.94~1.04)	0.94(0.80~1.11)	0.99(0.95~1.04)
MEOHP	0.94(0.85~1.03)	**1.06(1.01~1.14)[d]**	**0.89(0.79~0.99)[d]**	1.00(0.92~1.10)	**1.08(1.01~1.15)[d]**	0.99(0.95~1.05)	**0.81(0.70~0.95)[d]**	0.98(0.94~1.03)
MECPP	**0.92(0.84~0.99)[d]**	1.03(0.96~1.10)	**0.89(0.79~0.99)[d]**	0.99(0.91~1.08)	**1.06(1.00~1.13)[d]**	0.98(0.93~1.03)	0.89(0.76~1.04)	0.99(0.95~1.03)
MEHP%	1.11(0.96~1.29)	0.96(0.86~1.07)	1.14(0.94~1.38)	0.99(0.87~1.13)	0.99(0.89~1.09)	1.02(0.94~1.09)	1.01(0.78~1.30)	**1.09(1.02~1.17)[d]**
ΣDEHP	**0.91(0.83~1.00)[e]**	1.04(0.98~1.12)	**0.87(0.77~0.99)[d]**	0.96(0.88~1.05)	**1.06(0.99~1.13)[d]**	0.98(0.93~1.03)	0.89(0.75~1.05)	0.99(0.95~1.04)
$\Sigma DBP_{(i+n)}$	0.96(0.88~1.05)	1.02(0.95~1.09)	0.93(0.83~1.05)	0.94(0.87~1.02)	**1.07(1.01~1.14)[d]**	0.99(0.95~1.05)	**0.87(0.75~0.99)[d]**	1.01(0.97~1.06)

[a]All models were adjusted for age, age square, BMI, season, and time of day blood sample was collected. Testosterone and estradiol were additionally adjusted for SHBG. [b]Coefficient represents the change in hormone level for a unit change in phthalate metabolite concentration after back-transformation of the phthalate metabolite concentrations. For a unit change in phthalate metabolite concentration, a coefficient equal to 1.0 indicates no change in hormone level, a coefficient < 1.0 indicates a decrease in hormone level, and a coefficient > 1.0 indicates an increase in hormone level. [d]P-value<0.05. [e]P-value<0.1. [c]Ln-transformations of phthalate metabolites and men sex hormones were used.

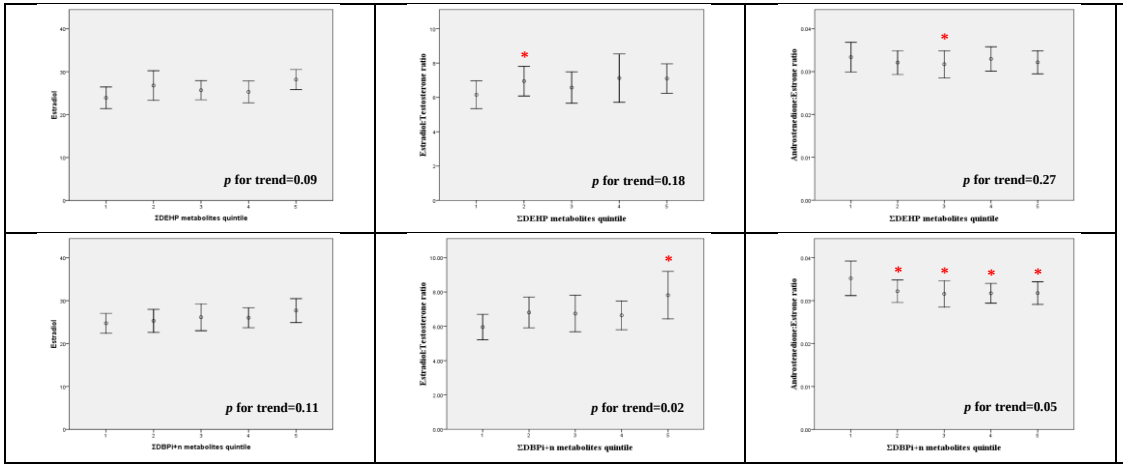

Figure 1. Levels of selected steroid hormone enzyme index by quartiles of ∑DEHP quintile (above) and ∑DBP_{i+n} quintile (down); quartile 1 is lowest and 5 is highest. ∑DEHP metabolites quintile (range): 1 (0.01–0.0.07), 2 (0.08–0.12), 3 (0.13–0.16), 4 (0.16–0.24), 5 (0.25–5.59). ∑DBP_{i+n} metabolites quintile (range): 1 (0.007–0.04), 2 (0.05–0.08), 3 (0.08–0.11), 4 (0.12–0.19), 5 (0.20–1.15). Scales on the y-axis correspond to estimated mean values; whiskers indicate 95% CIs. *p < 0.05 compared with first (lowest) quartile of ∑DEHP or ∑DBP_{i+n}.