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RESEARCH ARTICLE

Urinary di(2-ethylhexyl)phthalate metabolite ratios in obese children of South Korea

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Abstract

Phthalate exposure has been reported to be more associated with obesity in children than in adults. The concentration of di(2-ethylhexyl)phthalate (DEHP) was high temporal variability in spot urine, so additional tools of assessing DEHP exposure were required. Therefore, we used relative metabolite ratios (RMRs) as well as concentrations, and RMRs did not need to be corrected to the creatinine concentration. We aimed to evaluate the levels of urinary DEHP metabolites and their RMRs in obese children in South Korea, and to investigate the potential of RMRs for assessing the risks for childhood obesity. We analyzed the four urinary DEHP metabolites (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)) in 240 children aged 5–16 years, using isotope dilution GC-MS/MS. The children were placed into three groups ("normal weight," "overweight," and "obese") according to body mass index (BMI) percentiles. We statistically compared the concentrations and RMRs of DEHP metabolites among these groups. The obese group had lower MEHP levels, and higher secondary metabolite (MEHHP, MEOHP, and MECPP) levels, than the normal weight group. DEHP metabolite levels did not differ significantly between the normal weight and obese groups, whereas RMR_{A2} (as the ratio of the molar concentrations of MEOHP to MEHHP) was found to be negatively associated with BMI percentile ($\beta = -0.236$, $p < 0.01$) and weight percentile ($\beta = -0.282$, $p < 0.001$). Therefore, we suggest that RMRs are an additional tool for assessing the health risks of DEHP.

Keywords Di(2-ethylhexyl)phthalate metabolite · Relative metabolite ratio · Childhood obesity · Urine · GC-MS/MS

Introduction

Di(2-ethylhexyl)phthalate (DEHP) is a major plasticizer used in consumer products, including building materials, food packaging, medical devices, toys, and cosmetics (Hoppe 2002). DEHP is classified as a probable human carcinogen (group B2) by the United States Environmental Protection Agency and the International Agency for Research on Cancer (IARC 2013; U.S. EPA 1988). The most common route of DEHP exposure is oral ingestion with food (Correia-Sá et al. 2018; Erythropel et al. 2014; NTP-CERHR 2006; Wormuth et al. 2006). DEHP is rapidly metabolized to a hydrolyzed monoester (mono-(2-ethylhexyl) phthalate, MEHP) in humans (Frederiksen et al. 2007; Koch et al. 2005, 2006). MEHP is hydroxylated with mono-(2-ethyl-5-hydroxyhexyl) phthalate (MEHHP) and then oxidized to mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP). MEHP is also oxidized to mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP).

Previous studies focusing on the toxicity of phthalates indicated adverse effects on the reproductive system, especially in men (Martino-Andrade and Chahoud 2010; Mendiola et al. 2011, 2012). The urinary concentration of MEHP has been reported to be negatively associated with testosterone, E2, and free androgen index levels in some men (Meeker et al. 2009). Recently, however, many studies on prenatal, childhood, and adult phthalate exposure have reported effects on obesity,

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glucose metabolism, and a relationship with metabolic syndrome (Desai et al. 2015; Giulivo et al. 2016; Hauser and Calafat 2005). Several in vivo and in vitro studies suggest that phthalates may promote obesity through activation of peroxisome proliferator–activated receptors (PPARs) (Feige et al. 2010; Hao et al. 2012). MEHP is a well-known ligand for the PPARs and a mitochondrial toxicant and disruptor of lipid and glucose metabolism (Campioli et al. 2011; Martinez-Arguelles and Papadopoulos 2015).

Because of their behavior patterns (e.g., crawling and mouthing), larger surface area to weight ratio, and enhanced metabolic rate, children are known to be more vulnerable than adults to environmental exposure to phthalates. Several studies have shown that children have significantly higher DEHP levels than adults (Kasper-Sonnenberg et al. 2012; Saravanabhavan et al. 2013). Oxidative phthalate metabolism seems to be slightly favored in neonates and young children compared with adults (Koch et al. 2006). Two recent studies using data from the National Health and Nutrition Examination Survey (NHANES) found that urinary phthalates were associated with higher odds for obesity in children and adolescents (Buser et al. 2014; Trasande et al. 2013).

Phthalates have relatively short elimination half-lives (6–12 h), but are widely exposed to humans; hence, its concentration in excreted urine varies greatly with sampling time. Because of this, in studies evaluating phthalate exposure, collected urine, such as 24-h urine, pooled samples, etc. is preferred over spot urine; the intraclass correlation coefficients (ICCs) for urinary phthalate metabolites were twice as high in the pooled samples (0.24–0.87) than in the first morning urine (0.08–0.69) (Shin et al. 2019). In spot urine from children, the metabolite excretion pattern of DEHP shows high inter- and intrapersonal variability, with ICCs < 0.3 (Johns et al. 2015; Watkins et al. 2014). Therefore, to evaluate the exposure of DEHP with high variability, additional tools were required as well as comparing the concentration of urinary DEHP metabolites. Thus, we suggested relative metabolite ratios (RMRs) that do not require further correction as a tool of DEHP exposure assessment.

In our study, we evaluated the urinary DEHP metabolite levels in children who may be relatively vulnerable to obesity. These children are classified as normal weight, overweight, and obese children according to the BMI percentile. We also investigated the potential of RMRs as an additional tool for assessing the risk for DEHP exposure.

Methods

Study subjects and sample collection

Study subjects were recruited from children and adolescents aged 5 to 16 years who visited a health clinic in Seoul, Korea, for periodic growth and development check-ups between March 2015 and September 2015. All children and adolescents who visited the health clinic center received a thorough medical history taking and physical examination from two pediatricians. Those with any history or physical characteristics of drug use, genetic disorders, hepatic/renal disorders, and endocrinopathies have been excluded from the study recruitment. Those who had contact with polyvinyl chloride containing medical devices through intravenous procedures in the previous 30 days were also excluded. The experimental protocol was approved by the Institutional Review Board of Inje University Hospital (SGPAIK 2015-01-001-001), and informed written consent was obtained from all volunteers or their parents before enrollment. Height and weight were measured using a stadiometer and an Inbody 720 (Biospace Co. Ltd, Seoul, Korea), while participants wore light clothing and no shoes. BMI was calculated as weight (kg)/height squared (m^2). Sex and age-specific percentiles of height, weight, and BMI were determined on the basis of the Korean National Reference Charts (Moon et al. 2008). Study subjects were grouped into three categories based on sex and age-specific BMI percentile: "normal weight" was defined as BMI < 85th percentile, "overweight" was defined as BMI ≥ 85th and < 95th percentile, and "obese" was defined as BMI ≥ 95th percentile (Moon et al. 2008).

After a 10-hr overnight fast, first morning urine samples were collected in a polyethylene cup. Approximately 5 mL of urine was moved from the urine collection container into polypropylene cryo-vials and stored until analysis at −80 °C.

Sample preparation

Urine samples were analyzed for the following DEHP metabolites: 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). Quantitative analysis was performed according to a previous study (Kim et al. 2014). Briefly, the urine sample (1 mL) was spiked into a 10-μL mixture of isotope-labeled internal standards (each of 1 μg/mL). DEHP forms a glucuronide conjugate, and also remains in free form. So, the spiked sample was enzymatically hydrolyzed with β-glucuronidase from *E. coli*, to detach the glucuronide in the urine. Deconjugated samples were then added to 2 M acetate buffer at pH 4.0–4.5, followed by liquid-liquid extraction with hexane-ether solvents (8:2, v/v). The mixture was centrifuged and the organic solvent was separated by first freezing the aqueous solution, and then evaporating it under nitrogen. The dried residue was derivatized with 50 μL of BSFTA+TMCS (99:1, v/v) at 65 °C for 30 min. Two microliters of the resulting derivative was injected into the gas chromatography tandem mass spectrometer (GC-MS/MS). The gas chromatograph (GC) was obtained using a

7890A Series GC system interfaced with a tandem mass spectrometer (7000) from Agilent Technologies (Palo Alto, CA). The Ultra-2 capillary column (cross-linked 5%-phenyl-methylpolysiloxane, of 25 m × 0.2 mm ID × 0.33-μL film thickness) used for GC was supplied by J&W Scientific (Folsom, CA). The temperature program for the column was as follows: initial temperature, 100 °C, followed by a first ramp at 15 °C/min to 200 °C, and held constant for 5 min, then a second ramp at 10 °C/min to 270 °C, and a third ramp at 30 °C/min to 310 °C, and held for 1 min. The ionization mode used for the mass spectrometer was EI (70 eV), and acquisition occurred in the multiple reaction monitoring (MRM) mode. Injection was performed using a CTC Combi-PAL autosampler, and quantitative analysis was performed using Masshunter software (Agilent Technologies).

Validation

In this study, standard calibration curves were drawn using isotope dilution methods. The concentration of the working standard solution of four DEHP metabolites from 0.02 to 500 ng/mL was prepared and calibrated. The calibration curve was estimated from the linear regression analysis of each analyte based on the ratio of the analyte-IS peak area versus the ratio of the analyte-IS concentration. The limits of detection and quantitation were established at signal-to-noise ratios of 3 and 10, respectively. In children of this study, the precision of the urinary four DEHP metabolites was validated using the pooled urine of laboratory staff. We did not spike the standards in urine because the concentration of four DEHP metabolites in the pooled urine was relatively high, and we analyzed it by including hydrolysis steps for about 20 samples. The precision and accuracy of analytical method were spiked of the standards in the regent water with relatively low levels of phthalate metabolites. Samples containing four DEHP metabolite concentration of 5, 10, and 50 ng/mL in water were evaluated by performing intra-analysis ($n = 5$).

Relative metabolite ratios

RMRs were calculated for each sample and differences among groups were compared statistically. The RMRs are the ratios of the products to precursors (Fig. 1). We have previously defined RMR_{A1} as the ratio of the concentrations of MEHHP to MEHP (i.e., RMR_{A1} = MEHHP/MEHP), and RMR_{A2} as the ratio of the concentrations of MEOHP to MEHHP (Song et al. 2013). RMR_{AT} was calculated as the ratio of the concentrations of MEOHP to MEHP. Additionally, RMR_{BT}, which we report here, was defined as the ratio of the concentrations of MECPP to MEHP, taking into account DEHP metabolism into MECPP (via the B-pathway).

Statistical analysis

Statistical analysis included only data for which all DEHP metabolites were detected. The results of urinary DEHP metabolite analysis must be corrected for urinary dilution (Barr et al. 2005; Wittassek et al. 2007). Therefore, the analytical results were corrected by the creatinine concentration of each sample (expressed in nmol/g · creatinine). Metabolite concentrations and their RMRs are reported using the geometric mean (GM) and geometric standard deviation (GSD). All statistical analyses were conducted using PASW Statistics 18 for Windows (SPSS Inc., Chicago, IL). Statistical significance was determined using the nonparametric Kruskal-Wallis and Mann-Whitney U tests to evaluate possible differences among the groups. Variables in which significant differences were found using the Kruskal-Wallis test ($p < 0.05$) were then compared using the Mann-Whitney U test; the groups that we compared were normal weight versus overweight, normal weight versus obese, and overweight versus obese. In the Mann-Whitney U test, the significance level was adjusted using the Bonferroni correction ($p < 0.017$). Ln transformation was applied to molar metabolite concentrations and RMRs to improve the approximation of normal distribution. Multiple linear regression analysis was performed using DEHP metabolite concentrations and RMRs as dependent variables, obesity-related parameters as independent variables.

Results

Characteristics of the study subjects

General characteristics of the study subjects by sex and obesity status are shown in Table 1. A total of 240 children and adolescents (101 boys and 139 girls) were enrolled in this study. The mean age (± SD) of the subjects was 9.1 ± 2.1 years. Out of 101 boys, overweight and obese subjects were 14.8% and 23.8%, while of 139 girls, those were 15.1% and 32.3% respectively. The percentiles of height, weight, and BMI were significantly higher in overweight and obese subjects than normal weight subjects.

Analysis of DEHP metabolites

Figure 2 shows the chromatograms of TMS-derivatized DEHP metabolites measured using GC-MS/MS. As shown in Fig. 2, TMS-derivatized metabolites and their corresponding isotope internal standards were detected between 19.625 (TMS-MEHP-D_4) and 27.367 min (TMS-MECPP). The regression equations, correlation coefficients, and the limits of quantitation values obtained from calibration curves are shown in Table S1. The calibration curves for the four DEHP metabolites showed good linearity ($R^2 > 0.99$), and

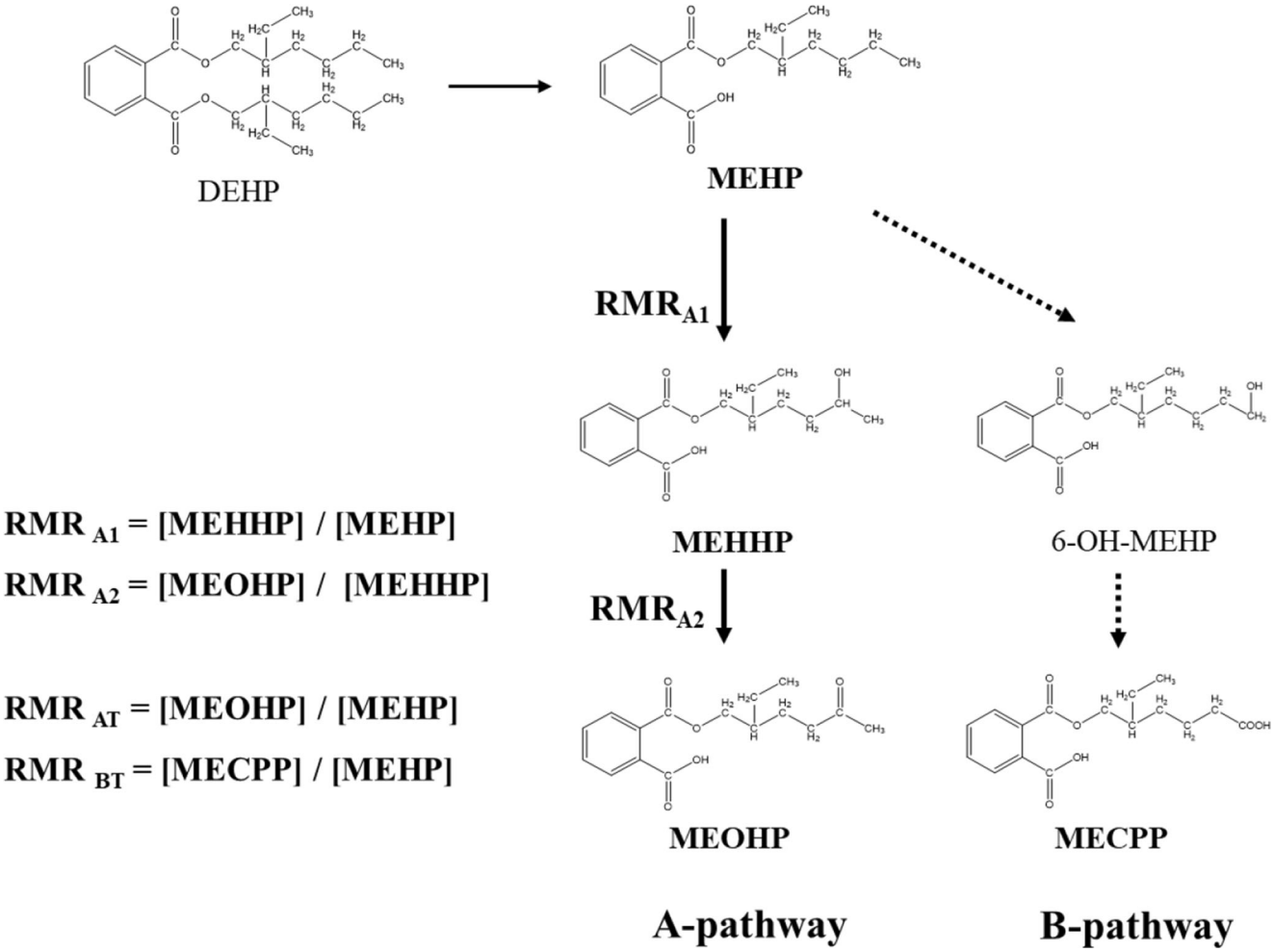

Fig. 1 Description and calculation of relative metabolite ratios (RMRs) for urinary DEHP metabolites: DEHP metabolism based on Koch et al. (2005)

the obtained LOQs ranged from 0.6 to 10 ng/mL. The results of accuracy and precision for this study are shown in Table S2. We repeatedly analyzed pooled urine to evaluate the precision of children's urine. The precision results of the analytes in the pooled urine were relatively high, from 7.54 to 13.36%. The precision and accuracy of the analytical method were obtained in the range of 1.24 to 10.68% and 81.24 to 119.85% respectively at various concentrations (5, 10, and 50 ng/mL).

The concentrations and RMRs of DEHP metabolite

The concentrations of DEHP metabolite with and without creatinine adjustment are shown in Table 2. Of the DEHP metabolites, MECPP was present in the highest concentration, followed by MEHHP, MEOHP, and MEHP. The secondary metabolites were 2–8 times more abundant than the primary metabolites. The concentration of the primary metabolite (MEHP) was lower in the obese group than in the normal weight group by 2.76 nmol/g · creatinine, whereas levels of the secondary metabolites (MEHHP, MEOHP, and MECPP) were higher in the obese group than in the normal weight group, by 17.19, 0.95, and 19.33 nmol/g · creatinine, respectively. The concentration of each metabolite was lowest in the overweight group (Table S3).

The concentrations of DEHP metabolites were 1.3–1.8 times higher (p-value = 0.000 for MEHP, MEHHP, MEOHP, and ∑DEHP) in girls than in boys (Table 3). The concentrations of the metabolites were higher in the 5–9-year age group than in the 10–16-year age group. There were no significant differences ($p > 0.05$) between the normal weight, overweight, and obese groups for DEHP metabolite concentrations by age group and gender.

We found that the metabolic pathway from MEHP to MECPP was the major metabolic pathway for DEHP: in the normal weight group, RMR_{BT} was higher than RMR_{AT} (GM: 5.82 vs 2.17) (Table 4). For boys, all RMRs showed significant differences between normal weight and obese groups, but for girls, only RMR_{A2} showed significant differences between these two groups. In the 10–16-year age group, RMR_{A1} and RMR_{A2} differed significantly between the normal weight and

Table 1 Demographic characteristics of the South Korean children who participated in this study

Characteristics	Normal weight	Overweight	Obese	*p*-value
Total				
No. of samples	135	36	69	
Age (years)	9.25 ± 2.02	9.44 ± 2.02	8.64 ± 2.09	
Height (cm)	135.54 ± 15.50	140.63 ± 12.96	138.93 ± 12.93	0.312
Height percentile	59.42 ± 79.78	65.14 ± 30.78	80.79 ± 19.73	0.000
Body mass index (BMI, kg/m^2)	17.51 ± 2.17	21.68 ± 2.38	24.10 ± 2.83	0.000
BMI percentile	48.02 ± 23.89	90.48 ± 3.14	97.82 ± 1.42	0.000
Weight (kg)	33.33 ± 8.70	43.84 ± 12.64	47.62 ± 14.06	0.000
Weight percentile	50.81 ± 24.62	87.23 ± 9.14	97.45 ± 2.12	0.000
Boys				
No. of samples	62	15	24	
Age (years)	10.24 ± 2.09	10.60 ± 1.60	9.79 ± 2.36	
Height (cm)	137.35 ± 20.21	145.42 ± 13.78	145.73 ± 14.01	0.157
Height percentile	37.93 ± 25.51	49.03 ± 33.63	78.04 ± 22.05	0.000
Body mass index (BMI, kg/m^2)	18.62 ± 2.33	23.59 ± 1.49	26.61 ± 2.53	0.000
BMI percentile	51.56 ± 25.67	90.57 ± 3.16	97.87 ± 1.27	0.000
Weight (kg)	36.84 ± 9.84	50.77 ± 12.82	57.50 ± 15.20	0.000
Weight percentile	45.50 ± 25.97	82.69 ± 10.63	97.60 ± 2.19	0.000
Girls				
No. of samples	73	21	45	
Age (years)	8.41 ± 1.53	8.62 ± 1.91	8.02 ± 1.64	
Height (cm)	134.00 ± 9.79	137.21 ± 11.47	135.31 ± 10.38	0.710
Height percentile	77.68 ± 102.75	76.65 ± 23.08	82.26 ± 18.47	0.000
Body mass index (BMI, kg/m^2)	16.57 ± 1.49	20.31 ± 1.90	22.76 ± 1.94	0.000
BMI percentile	45.02 ± 22.01	90.42 ± 3.20	97.79 ± 1.51	0.000
Weight (kg)	30.35 ± 6.28	38.89 ± 10.12	42.36 ± 10.16	0.000
Weight percentile	55.33 ± 22.63	90.48 ± 6.36	97.37 ± 2.10	0.000

Values are mean ± SD; normal weight < 85 BMI percentile; overweight 85–95 BMI percentile; obese ≥ 95 BMI percentile

obese groups. In conclusion, RMR_{A2} differed significantly between the normal weight and the obese group both age groups and gender.

Association of DEHP metabolites with childhood obesity

We used multiple linear regression analysis to explore the associations between DEHP metabolite concentration, RMRs, and obesity-related parameters, after adjustment for age (Table 5). RMR_{A1} and RMR_{A2}, which showed significant differences among obesity groups, were used as independent variables, and multiple regression analyses were performed with obesity-related variables such as BMI percentile, weight percentile, and height percentile as dependent variables. We also performed regression analysis to analyze the associations between these obesity-related variables and the DEHP metabolites of RMR_{A1} and RMR_{A2}. We observed a negative association of RMR_{A2} with BMI percentile ($\beta = -0.236$, $p = 0.005$) and weight percentile ($\beta = -0.282$, $p = 0.001$). The concentration of DEHP secondary metabolites related to RMR_{A2} also showed an association with BMI percentile (MEHHP: $\beta = 0.839$, $p = 0.001$; MEOHP: $\beta = -0.937$, $p = 0.001$) and weight percentile (MEHHP: $\beta = 1.011$, $p = 0.000$; MEOHP: $\beta = -1.044$, $p = 0.000$). However, the concentration of DEHP primary metabolite and RMR_{A1} were not associated with obesity-related indices.

Discussion

We aimed to evaluate the levels of urinary DEHP metabolites and RMRs in obese children in South Korea and to investigate the potential of RMRs as a tool for assessing the health risk of DEHP. The concentrations of DEHP metabolites detected in our study were overall higher than those reported in surveys of children from other countries (CDC 2017; Boas et al. 2010; Koch et al. 2011; Guo et al. 2011; Dirtu et al. 2013)

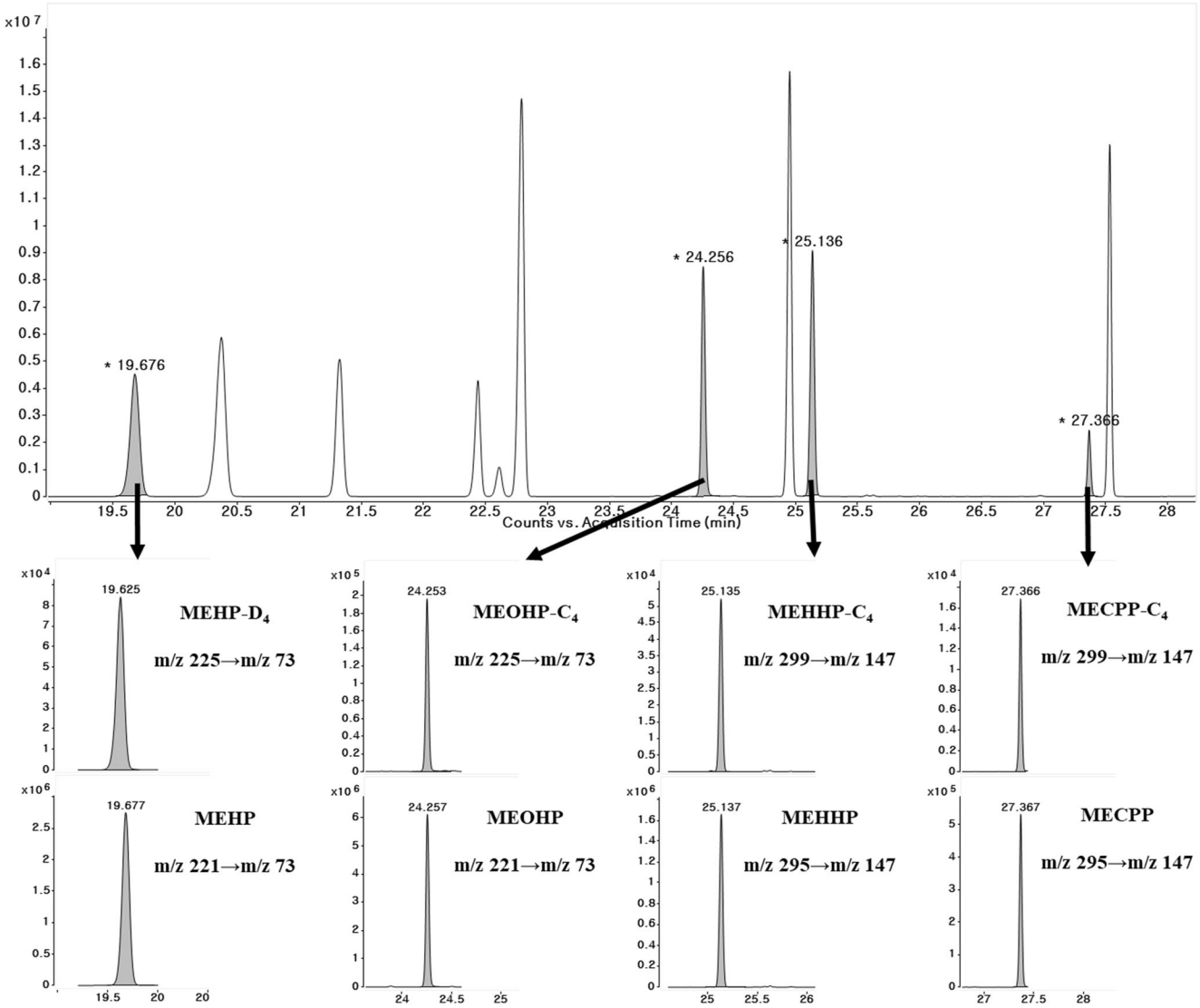

Fig. 2 GC-MS/MS chromatogram of the four DEHP metabolites (1 ng) in the multiple reaction monitoring mode

(Table S4). However, only two children (0.83%) exceeded the HBM I value (500 μg/L) of ∑MEHHP + MEOHP in our study (Apel et al. 2017). Similarly, the levels of MEHP and MECPP that we report are higher than those reported for 3–14-year olds in the German "GerES IV 2003–2006" study (Becker et al. 2009); however, the levels of DEHP secondary metabolites (MEHHP and MEOHP) that we report are lower than those reported in that study.

Table 2 Distributions of DEHP metabolite concentrations with/without creatinine adjustment in 240 children

Compounds	LOQ	%>LOQ	Mean ± SD	GM ± GSD	Min-Max	P50	P95
MEHP (μg/L)	0.6	100	12.93 ± 9.14	10.93 ± 1.73	3.62–67.50	10.04	31.90
MEHP (μg/g creatinine)	-	100	14.54 ± 10.84	11.76 ± 1.89	2.00–64.37	11.00	36.67
MEHHP (μg/L)	1.5	100	44.86 ± 49.83	30.16 ± 2.44	3.02–378.94	30.81	123.91
MEHHP (μg/g creatinine)	-	100	42.51 ± 44.07	32.45 ± 2.05	3.28–532.97	32.36	100.79
MEOHP (μg/L)	3.0	100	34.83 ± 32.71	25.45 ± 2.17	4.71–210.60	24.99	104.48
MEOHP (μg/g creatinine)	-	100	33.96 ± 27.41	27.38 ± 1.90	4.31–273.95	26.85	78.49
MECPP (μg/L)	10.0	100	112.04 ± 133.57	72.57 ± 2.48	10.16–975.94	69.18	402.40
MECPP (μg/g creatinine)	-	100	104.73 ± 102.90	78.08 ± 2.10	9.94–940.14	80.20	277.92

Table 3 The concentrations of DEHP metabolite by gender and age group

Metabolites	Obesity status (GM ± GSD)			*p*-value
	Normal weight	Overweight	Obese	
By gender				
∑DEHP				
Boys	394.15 ± 1.98	311.13 ± 1.81	442.52 ± 1.70	0.456
Girls	658.09 ± 1.87	499.43 ± 1.52	623.99 ± 2.09	0.144
MEHP				
Boys	37.64 ± 2.00	26.91 ± 1.62	31.37 ± 1.78	0.169
Girls	51.00 ± 1.82	45.36 ± 1.62	48.35 ± 1.86	0.603
MEHHP				
Boys	79.98 ± 2.13	71.69 ± 2.00	104.61 ± 1.70	0.305
Girls	142.28 ± 1.91	111.58 ± 1.53	139.82 ± 2.11	0.212
MEOHP				
Boys	77.38 ± 1.91	59.80 ± 1.81	83.27 ± 1.65	0.396
Girls	116.09 ± 1.84	89.03 ± 1.52	105.73 ± 2.04	0.137
MECPP				
Boys	188.67 ± 2.10	148.73 ± 1.86	217.01 ± 1.81	0.367
Girls	337.15 ± 1.96	244.67 ± 1.61	316.52 ± 2.23	0.117
By age group				
∑DEHP				
5–9 years	625.27 ± 1.92	466.42 ± 1.52	599.19 ± 2.07	0.133
10–16 years	383.92 ± 1.97	360.49 ± 1.88	426.22 ± 1.57	0.873
MEHP				
5–9 years	48.81 ± 1.95	43.42 ± 1.56	44.72 ± 1.87	0.435
10–16 years	37.89 ± 1.85	30.67 ± 1.80	32.71 ± 1.88	0.567
MEHHP				
5–9 years	135.65 ±1.95	107.10 ± 1.49	134.98 ± 2.10	0.212
10–16 years	76.41 ± 2.14	80.41 ± 2.04	101.68 ± 1.55	0.337
MEOHP				
5–9 years	114.18 ±1.84	83.65 ± 1.46	104.82 ± 2.00	0.076
10–16 years	72.86 ±1.90	68.01 ± 1.90	76.06 ± 1.54	0.960
MECPP				
5–9 years	313.34 ± 2.04	226.70 ± 1.63	302.08 ± 2.23	0.130
10–16 years	187.81 ± 2.09	174.40 ± 1.94	209.74 ± 1.63	0.864

unit, nmol/g · creatinine; ∑DEHP = MEHP + MEHHP + MEOHP + MECPP; *NOR*, normal weight group; *OW*, overweight group; *OB*, obese group

We observed that the concentrations of DEHP secondary metabolites (MEHHP, MEOHP, and MECPP) were higher than those of the primary metabolite (MEHP) in our study. In a study of DEHP exposure (Guo et al. 2011; Kato et al. 2004), urinary levels of MEHHP and MEOHP were approximately 10-fold higher than those of MEHP. During DEHP metabolism, MEHP is excreted mostly as free metabolites; the secondary metabolites are excreted in the urine, mostly as glucuronide conjugates (Samandar et al. 2009; Silva et al. 2003). Due to its low water solubility, MEHP is excreted into urine much more slowly than the secondary metabolites (Koch et al. 2004): after 24 h, the two secondary metabolites of DEHP (MEHHP and MEOHP) accounted for 38.5% of the oral DEHP dose, whereas MEHP accounted for only 7.3% of the dose. We found that the concentrations of MEHP and secondary metabolites of DEHP showed opposite trends in the obese group compared to the normal weight group. For children of 6–19 years, our findings are consistent with those obtained using the NHANES 2007–2010 data (Buser et al. 2014): levels of the DEHP secondary metabolites (MEHHP, MEOHP, and MECPP) were higher in the obese group than normal weight group, and no association was found between DEHP metabolite levels and obesity. And phthalate concentrations did not differ significantly between the normal weight group and the overweight or obese groups in New York City children of 6–8 years (Teitelbaum et al. 2012).

Our results, in which the DEHP metabolite levels in children decreased significantly with increasing age, are consistent with those of several studies (Becker et al. 2009; Teitelbaum et al. 2012; Wang et al. 2013; Zhang et al. 2014; Smerieri et al. 2015). For example, Smerieri et al. (2015) reported that DEHP metabolite concentrations were higher in prepubescent than in pubescent children. These differences in phthalate exposure may have been caused by differences in susceptibility to perturbation in sex hormone levels.

Compared with the normal weight group, RMR_{A1} was higher in the obese group in both age groups and gender. RMR_{A1} and RMR_{A2} are significantly different between normal weight and obese groups. In the NHANES 2001–2012 study (Yaghjyan et al. 2016), BMI was associated with the MEHHP/MEHP ratio (corresponding to RMR_{A1} in our study), but not with the MEOHP/MEHHP ratio (corresponding to RMR_{A2} in our study). However, we found significant negative associations between RMR_{A2} and BMI percentile. The NHANES 2001–2012 study was restricted to adults with BMI <30, so there may be differences from our results. Some studies have reported that there may be a negative association between DEHP metabolites and childhood obesity. For 8–10-year-old girls in China, Zhang et al. (2014) reported significant negative associations ($p < 0.05$) between obesity and urinary levels of MEHP, MEHHP, and the sum of DEHP metabolites. Wang et al. (2013) also reported that levels of only secondary metabolites (MEHHP and MEOHP) were significantly associated with BMI for 8–11-year-olds. We suggest that secondary metabolites of DEHP, rather than MEHP, may affect childhood obesity.

Our finding shows that the value of RMR_{A2} was close to 1 in the normal weight group, while it was substantially lower than 1 in the obese group. This finding could be explained that MEHHP is almost continuously metabolized into MEOHP. Barr et al. (2003) reported as evidence of relatively low RSD among individuals for the MEOHP/MEHHP ratio.

Table 4 The relative metabolite ratios (RMRs) of DEHP metabolite by gender and age group

RMRs	Obesity status (GM ± GSD)			*p*-value	*p*-value[a]		
	Normal weight	Overweight	Obese		NOR vs OW	OW vs OB	NOR vs OB
By gender							
RMR_{AT}							
Boys	2.06 ± 1.70	2.22 ± 1.47	2.65 ± 1.39	**0.049**	0.529	0.225	**0.014**
Girls	2.28 ± 1.54	1.96 ± 1.55	2.19 ± 1.65	0.379			
RMR_{BT}							
Boys	5.01 ± 1.78	5.53 ± 1.59	6.92 ± 1.48	**0.028**	0.403	0.069	**0.012**
Girls	6.61 ± 1.75	5.39 ± 1.67	6.55 ± 1.92	0.268			
RMR_{A1}							
Boys	2.12 ± 1.96	2.66 ± 1.67	3.34 ± 1.48	**0.004**	0.190	0.166	**0.001**
Girls	2.79 ± 1.61	2.46 ± 1.58	2.89 ± 1.79	0.421			
RMR_{A2}							
Boys	0.97 ± 1.28	0.83 ± 1.19	0.80 ± 1.17	**0.000**	0.055	0.214	**0.000**
Girls	0.82 ± 1.14	0.80 ± 1.14	0.76 ± 1.17	**0.025**	0.614	0.132	**0.007**
By age group							
RMR_{AT}							
5–9 years	2.34 ± 1.59	1.93 ± 1.44	2.34 ± 1.61	0.191			
10–16 years	1.92 ± 1.62	2.22 ± 1.59	2.33 ± 1.50	0.338			
RMR_{BT}							
5–9 years	6.42 ± 1.79	5.22 ± 1.36	6.75 ± 1.85	113			
10–16 years	4.96 ± 1.73	5.69 ± 1.87	6.41 ± 1.53	0.167			
RMR_{A1}							
5–9 years	2.78 ± 1.68	2.47 ± 1.45	3.02 ± 1.74	0.210			
10–16 years	2.02 ± 1.91	2.62 ± 1.77	3.11 ± 1.55	**0.022**	0.140	0.443	**0.008**
RMR_{A2}							
5–9 years	0.84 ± 1.17	0.78 ± 1.13	0.78 ± 1.17	**0.010**	0.095	0.721	**0.004**
10–16 years	0.95 ± 1.29	0.85 ± 1.17	0.75 ± 1.16	**0.001**	0.151	0.025	**0.000**

[a] The significance level was adjusted by the Bonferroni method (*p*-value = 0.017); *NOR*, normal weight group; *OW,* overweight group; *OB*, obese group
Bold, significant differences at *p*-value < 0.05

According to the DEHP concentration results of our study, levels of MEHHP were significantly higher in the obese group than in the normal weight group, whereas levels of MEOHP were similar in these groups. It was expected that hydroxylation from MEHP to MEHHP is promoted, or metabolic efficiency of MEHHP to MEOHP is decreased, but we could not explain why. However, we suggested that the secondary metabolites of DEHP are important factors

Table 5 Regression analysis of associations between DEHP metabolite concentrations, RMRs and, obesity-related characteristics

	BMI percentile β (95% CI)	Weight percentile β (95% CI)	Height percentile β (95% CI)
Metabolites[a]			
MEHP	−0.021 (−0.211, 0.169)	−0.012 (−0.198, 0.175)	0.007 (−0.187, 0.201)
MEHHP	**0.839*** (0.349, 1.328)**	**1.011*** (0.530, 1.492)**	**0.502* (0.001, 1.002)**
MEOHP	**−0.937*** (−1.474, −0.401)**	**−1.044*** (−1.571, −0.517)**	−0.476 (−1.024, 0.072)
RMRs			
RMR_{A1}	−0.057 (−0.218, 0.104)	−0.045 (−0.202, 0.112)	−0.013 (−0.175, 0.148)
RMR_{A2}	**−0.236** (−0.398, −0.073)**	**−0.282*** (−0.441, −0.124)**	−0.141 (−0.304, 0.023)

[a] unit, nmol/g · creatinine; *p-value <0.05, **p-value <0.01, ***p-value <0.001
Bold, significant differences at *p*-value < 0.05

related to childhood obesity, based on our findings that MEHHP and MEOHP were associated with BMI percentiles.

Our study has some potential limitations, which we attempted to address. Our study used only first morning spot urine, so the metabolite levels should be corrected for the concentration of urinary creatinine. However, the excretion of urinary creatinine has been reported to be affected by BMI as well as age and gender (Correia-Sá et al. 2018; Watkins et al. 2014). Therefore, for obesity-related issues, it may be necessary to collect a 24-h urine sample that does not require correction for urinary creatinine. We had the advantage of using RMRs to compare urinary metabolites so that metabolite levels do not need to be corrected for creatinine concentrations. In studies that rely on spot urine to assess exposure to highly variable compounds (such as DEHP), a small sample size (such as ours) may weaken the statistical explanation of the results. Therefore, further studies should use larger samples, to achieve the statistical power required to analyze the effects of DEHP exposure. We did not include this as a variable in statistical analysis due to the lack of questionnaire information on the lifestyle factors of individuals. However, our study aimed to assess the potential of RMRs as an additional tool in evaluating DEHP exposure.

In conclusion, the three weight groups did not differ significantly ($p > 0.05$) in their levels of DEHP metabolites, while RMRs showed a significant difference. And we observed that the ratio between secondary metabolites of DEHP among RMRs was negatively associated with childhood obesity. Therefore, we suggest that analyzing RMRs, which reflect the DEHP metabolism, might be more useful than analyzing absolute metabolite concentrations, in assessing the risks of DEHP to human health.

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Declarations

Ethics approval and consent to participate The experimental protocol was approved by the Institutional Review Board of Inje University Hospital (SGPAIK 2015-01-001-001), and informed written consent was obtained from all volunteers or their parents before enrollment.

Consent for publication Not applicable

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