Title:

Phthalate exposure in pregnant women and their children in central Taiwan

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Abstract

Phthalate exposure has been found to be associated with endocrine disruption, respiratory effects, and reproductive and developmental toxicity. The intensive use of plastics may be increasing the exposure to phthalates in the Taiwanese population, particularly for young children.

We studied phthalate metabolites in pregnant women and their newborns in a general population in Central Taiwan. A total of 430 pregnant women agreed to participate and one hundred maternal urine samples and thirty paired cord blood and milk samples were randomly selected from those participants. Eleven phthalate metabolites (MEHP, 5OH-MEHP, 2cx-MEHP, 5cx-MEPP, 5oxo-MEHP, MiBP, MnBP, MBzP, OH-MiNP, oxo-MiNP, and cx-MiNP) representing exposure to five commonly used phthalates (DEHP, DiBP, DnBP, BBP, DiNP) were measured in the urine of pregnant women, cord serum and breast milk after delivery, and in the urine of their children. Exposure was estimated based on excretion factors and correlation among metabolites of the same parental compound. Thirty and fifty-nine urinary samples from 2 and 5 years-old children, respectively, were randomly selected from the 185 children who were followed successfully.

The total urinary phthalate metabolite concentration (geometric mean, μg/L) was found to be higher in 2-year-olds (398.6) and 5-year-olds (333.7) than in pregnant women

(205.2). Metabolites in urine are mainly from DEHP. The proportion of DiNP metabolites was higher in children's urine (4.39 and 8.31%, ages 2 and 5) than in that of adults (0.83%) ($p<0.01$). When compared with urinary levels, phthalate metabolite levels were low in cord blood (37.45) and milk (14.90). DEHP metabolite levels in women's urine and their corresponding cord blood were significantly correlated. When compared to other populations in the world, DEHP derived metabolites in maternal urine in Taiwan were higher, while phthalate metabolite levels in milk and cord blood were similar. The levels of phthalate metabolites in milk and cord blood were comparable to those found in other populations. Further studies of the effects on health related to DEHP and DiNP exposure are necessary.

Keywords:

Phthalate; environmental exposure; Taiwan; cord blood

Introduction

Phthalates are chemicals widely used in commercial products such as plastic softeners and solvents in personal care products, lubricants and insect repellents (Fay et al., 1999; Koo and Lee, 2004; Lee et al., 2005). Potential sources of exposure for di(2-ethylhexyl)phthalate (DEHP) include polyvinylchloride containing medical devices, food packaging, plastic toys, furniture, and car upholstery. Di-n-butyl phtyalate (DnBP) is present in medicines, cosmetics,

cellulose acetate plastics, latex adhesives, nail polish and other cosmetic products; butyl benzyl phthalate (BBP) is found in vinyl flooring, adhesives, sealants, food packaging, furniture upholstery, vinyl tile, carpet tiles, artificial leather, and di-isononyl phthalate (DiNP) is widely used in children's toys (Sathyanarayana, 2008). Recent studies suggest that the intensive use of plastic material in Taiwan may be increasing the exposure to DEHP in the Taiwanese population (Chen et al., 2008).

According to some epidemiological studies, phthalate exposure is associated with adverse health outcomes, such as shorter anogenital distances at birth (Swan, 2006), respiratory effects (Hoppin et al.,2004; Jaakkola et al., 1999; Jaakkola et al., 2000), and increased waist circumference and insulin resistance (Stahlhut et al., 2007). Exposure to MnBP, mono-benzyl phthalate (MBzP), and Mono-2-ethylhexyl phthalate (MEHP) is associated with an overall pattern of decline in sperm motility (Duty et al., 2004).

Pregnant and lactating women represent a population of special concern because of the potential impact of their exposure to phthalates on the fetus and nursing infant. I Exposure data for children under age 6 are scarce (Jahnke et al., 2005; McKee, 2004; NTP-CERHR, 2003a). Metabolites of DEHP, DBP and BBP have been monitored in children aged 2-6 (Koch et al., 2004; Koch et al., 2005). A decrease in the anogenital distance in male infants has been found to be associated with phthalate exposure, as determined by urinary MEP, MBP, MBzP and MiBP levels (Swan et al 2005). In another study, MBP in maternal urine

and amniotic fluid was found to be associated with a shorter anogenital distance only in female infants (Huang et al., 2009). In pregnant women, urinary MBP is negatively correlated with thyroxine, free thyroxine and FT4 levels (Huang et al., 2007).

Many experimental studies using different laboratory animals (primarily rats) have examined the reproductive toxicity, developmental toxicity, endocrine disruption, and genotoxicity that might be induced by phthalic acids. For example, anti-androgenic effects including delayed puberty in F_0, decreased sperm production and fecundity in F_1, malformations in F_1 reproductive organs, and decreased F_2 litter size, were reported for DBP (NTP-CERHR, 2003b). DBP's metabolite, MBP, is responsible for the toxic effects associated with DBP exposure. These include increased prenatal mortality, decreased fetal weight, cleft palate, fused sternebrae, reduced anogenital distance in males, cryptorchidism, hypospadias, and agenesis of the epididymides or seminal vesicles (NTP-CERHR, 2003b). High doses of DiNP caused an increase in liver weight, peroxisomal proliferation, skeletal variations and renal toxicity in a one-generation and a two-generation toxicity study (Moorman et al., 2000; NTP-CERHR, 2003a). In rats, BBP exposure was associated with decreased testicular weight, reduced ano-genital distance, increased incidence of nipple retention and decreased birth weight in both sexes of the first filial generation (Gray et al., 2000; Parks et al., 2000). Treatment with DEHP was also associated with altered ano-genital distance and nipple retention (NTP-CERHR, 2005).

In vitro studies help in understanding the possible mechanisms of toxicity. Phthalates and their metabolites can bind to several nuclear receptors and act as endocrine disruptors or metabolic disruptors (Desvergne et al., 2009). In a series of reporter gene assays, DBP, MBP and DEHP have been found to have both anti-androgenic and androgenic activities at different concentrations. These compounds also showed thyroid receptor (TR) antagonistic activity. Only DBP was reported to have estrogenic activity (Shen et al., 2009). BBP has an affinity for binding to estrogen receptors (ER) (Blair et al., 2000; Hashimoto et al., 2000; Matthews et al., 2000; Zacharewski et al., 1998), and activates ER-mediated transcription (Coldham et al., 1997; Harris et al., 1997; Hashimoto et al., 2000; Nishihara et al., 2000; Zacharewski et al., 1998). DEHP has a weak agonistic activity at aryl hydrocarbon receptors (AhR) (Kruger et al., 2008), constitutive androstane receptors (CAR, Nr1i3) (Eveillard et al., 2009), and Pregnane X nuclear receptors (PXR, Nr1i2) (Cooper et al., 2008; Hurst and Waxman, 2004). Phthalates, especially MEHP, interfere with steroid production, particularly estradiol production and aromatase expression. A possible mechanism for this interference is through mediation at peroxisome proliferator-activated receptors (PPAR) (Lovekamp-Swan et al., 2003; Lovekamp and Davis, 2001). MEHP is a true ligand for all three PPAR isotypes and a selective modulator of PPAR gamma (Desvergne et al., 2009). BBP does not activate progesterone receptor-mediated transcription (Tran et al., 1996) or AR-mediated transcription (Sohoni and Sumpter, 1998). While BBP alone did not show a significant agonistic AhR

137 effect, it enhanced TCDD induced AhR activity in a dose-dependent manner (Kruger et al.,
138 2008). BBP exposure in female rats is also associated with a significant increase in liver
139 Ethoxyresorufin-O-deethylation (EROD) activity (Singletary et al., 1997). BBP also induces
140 human breast cancer cell proliferation (Harris et al., 1997; Korner et al., 1998; Soto et al.,
141 1997).

142 We monitored eleven phthalate metabolites (MEHP, 5OH-MEHP, 2cx-MEHP,
143 5cx-MEPP, 5oxo-MEHP, MiBP, MnBP, MBzP, OH-MiNP, oxo-MiNP, and cx-MiNP) in
144 pregnant women (urine, serum and milk), their newborns (cord blood) and prospectively in
145 their children at ages 2-3 and 5-6 (urine) from a general population in Central Taiwan. These
146 eleven metabolites were derived from exposure to five commonly used phthalates: DEHP
147 (MEHP, 5OH-MEHP, 2cx-MEHP, 5cx-MEPP, and 5oxo-MEHP), DiBP (MiBP), DnBP
148 (MnBP), BBP (MnBP and MBzP), and DiNP (OH-MiNP, oxo-MiNP, and cx-MiNP).
149 Exposure to phthalic acids was estimated based on the 95% confidence interval for the level
150 of each measured urinary metabolite, and excretion fraction published in the literature.
151 Correlations among metabolites of the same parental compounds and among different types
152 of samples from pregnant women and their corresponding children were also tested.
153
154 **Methods**
155

Participants, specimen and data collection

The subjects were pregnant women from Central Taiwan, aged between 25 and 35 and without clinical complications. We invited all pregnant women visiting the local medical center between December 2000 and November 2001 to participate in this study. A total of 610 women were approached, and 430 (participation rate: 75%) agreed to be interviewed. To the best of our knowledge, those who refused to participate did not differ in age or social status from those who did participate. All of the participants completed a questionnaire concerning maternal age, parity, baby's weight, educational level, disease history, dietary and smoking habits, and breast-feeding history. Maternal urine was collected from subjects during the third trimester of pregnancy (28-36 weeks), and umbilical-cord serum was collected upon delivery. Women who agreed to collect samples of breast milk were trained in collection procedures in order to minimize the risk of contamination. A total of 175 participants provided adequate breast-milk (>60 ml) samples. Newborns were followed again when they were 2-3 years old (185 subjects, in 2003-2004) and 5-6 years old (185 subjects, in 2006-2007). For newborns and children aged 2-3, spot urine samples were collected in a pediatric urine bag with the assistance of a parent at the hospital. For children aged 5-6, urine samples were collected in a glass beaker. Immediately after collection, urine samples were transferred into amber glass bottles and stored at -20°C for analyses of phthalate monoesters and creatinine. One hundred maternal urine samples, fifty-nine urine samples from children

175 aged 5-6 , 30 urine samples from children aged 2-3 , and 30 paired cord blood samples and
176 milk samples were randomly retrieved and sent for analysis. The difference in the number of
177 children's samples was due to freshness and budget limitations, and the fact that one of the
178 samples had insufficient volume for creatinine analysis.
179
180 ***Analysis of phthalate metabolites***
181 The concentrations of eleven phthalate metabolites (MEHP, 5OH-MEHP, 2cx-MEHP,
182 5cx-MEPP, 5oxo-MEHP, MiBP, MnBP, MBzP, OH-MiNP, oxo-MiNP, and cx-MiNP) in
183 urine, cord serum and breast milk were determined with LC-MS-MS methods as described in
184 previous publications (Koch et al., 2003; Preuss et al., 2005) by Dr Jürgen Angerer's lab at
185 the University of Erlangen, Germany. Metabolite concentrations are expressed as "μg/L" or
186 "μg/g creatinine". "Total metabolites" refers to the sum of metabolites calculated by adding
187 the concentrations of all metabolites.
188
189 ***Determination of creatinine levels in urine***
190 Urinary creatinine levels were measured by Kaohsiung Medical University Chung-Ho
191 Memorial Hospital, using the spectrophotometric method, with picric acid as the reactive
192 agent, and read at 520nm.
193

194 ***Statistical methods***

195 We verified the distribution of data for phthalate metabolites for normality. As the
196 data were generally skewed slightly to the right, log transformations of phthalate metabolite
197 values and geometric means were applied in parametric statistical tests. Metabolite levels
198 under the limits of detection (<LOD) were recorded as half the LOD value. Samples from
199 male and female children were considered both separately and together to determine gender
200 differences. Pearson's correlation tests (r = Pearson correlation coefficient) were used to
201 check for correlations among values of metabolites from the same parental compound in
202 different matrices. The metabolite profile of the urine samples from each subject was
203 expressed as a percentage. The geometric means of the groups were used for parametric tests
204 and medians were used for non parametric tests. The Statistical Package for Social Science
205 (version 15.0; SPSS, Chicago, IL, USA) was used for statistical analysis.

206

207 ***Estimation of parental compound levels***

208 The amount of parental phthalate to which each subject might have been exposed was
209 estimated using excretion fractions reported in the literature (Wittassek et al., 2007). We
210 calculated the range of possible original parental compound levels, based on the 95%
211 confidence interval for the level of each urinary metabolite.

212

(1) Parental compound = metabolite concentration × excretion fraction

We also calculated the daily intake (Estimated Daily Intake, EDI) for these parental compounds, taking into account an average body weight of 55 kg for Taiwanese women, 16.5 kg for children aged 2-3, and 20 kg for children aged 5-6 (DOH 2000). A daily urine excretion of 0.8-2.2L was calculated for pregnant women, 0.6L for children aged 2-3 and 0.7 L for children aged 5-6 (Fleisher et al., 2002).

(2) Estimated daily intake = estimated parental compound concentration × daily urine excretion × average body weight

As an example, to estimate the maximum exposure to BBP for children aged 5 and 6, we used the MnBP concentration in their urine (GM max= 4.86), and an excretion rate of 73%, as reported by Wittassek et al 2007; therefore, the parental compounds from which this metabolite originated should be 4.86×100÷73＝6.66μg/L. For the estimation of daily intake, a daily urinary excretion of 0.7L urine/day for children this age was multiplied by the estimated parental compound concentration and the result divided by the average body weight of 20 kg. The estimated daily intake in this example would be 6.66×0.7=0.23 μg/kg bw/day.

Results

General characteristics of the population

Table 1 shows the general characteristics of participating subjects, including maternal age, maternal education level, and breast feeding patterns. The average age of the mothers was 29, and the average pre-pregnant BMI was 21. Forty-one percent of the breast-feeding mothers were taking supplements: vitamins, calcium, folic acid or Chinese herbs. Forty-six percent of the infant subjects were male. Mean body weights at birth for the 2-3-year-old cohort and the 5-6-year-old cohort were 3290±460g and 3240±450g, respectively.

Urinary metabolite levels

Urinary phthalate metabolite levels in children at ages 2 and 5 and pregnant women were compared using values with and without creatinine adjustment (Table 2). Generally, metabolite levels were higher in children than in pregnant women. This could be observed whether creatinine adjustment was applied or not. Total phthalate metabolite concentrations without creatinine adjustment were found to be higher in 2-year-old children (GM = 398.6μg/L, 282.6–562.3) and 5-year-old children (333.7μg/L, 251.8–442.2) than in pregnant women (205.2μg/L, 172.7–243.8). When creatinine adjustment was applied, children appeared to have even higher phthalate metabolite concentrations; however, we should take

252 into account the fact that the level of creatinine in pregnant women's urine was higher than
253 that in children (PW: 76.60μg/L; 5Y: 59.53μg/L; and 2Y: 62.28μg/L).
254 Analysis of the proportion of metabolites in urine helps identify the parental
255 compound and source of exposure. Phthalate metabolites in urine were mainly those from
256 DEHP, followed by metabolites from DnBP or BBP and those from DiNP. The sums of
257 urinary DEHP metabolites, with a GM of 102.2 μg/L for pregnant women, 152.3μg/L for
258 5-year old children and 200.3μg/L for 2-year old children, were proportionally higher. The
259 second most abundant metabolite was MnBP (Pw: 72.29μg/L; 5y: 75.09μg/L; 2y:
260 100.44μg/L). It was followed by MiBP (Pw: 12.49μg/L; 5y: 25.24μg/L; 2y: 17.21μg/L), and
261 MBzP (Pw: 0.96μg/L; 5y: 3.61μg/L; 2y: 3.40μg/L). The ratio of DiNP metabolites/Total
262 phthalate metabolites observed in children's urine samples was higher than that in adult
263 samples. The total DiNP metabolite concentration was 1.71μg/L for pregnant women,
264 27.73μg/L for 5-year old children and 17.46μg/L for 2-year old children. The proportion of
265 each metabolite over total phthalate metabolite is shown in Table 3. No gender difference
266 was found at any age, either for the total concentration or for any particular metabolite.
267
268 ***Phthalate metabolites in cord sera and milk samples***
269 Only MEHP, MiNP and MnBP were detected in some of the breast milk samples
270 (Table 4). MEHP was detected in 73% of the samples. When compared to urine, the

concentrations of phthalate metabolites were much lower in milk and cord blood samples. The compositions of the phthalate metabolites found in these matrices were also very different. The most abundant metabolite in cord blood serum samples was MEHP, rather than oxidized metabolites of DEHP (Table 4).

Correlational studies

We found that DEHP metabolite concentrations in urine samples of pregnant women (without creatinine adjustment) and their corresponding cord blood samples were significantly correlated for two of the metabolites, 5cxMEPP (r=0.53, p<0.01) and 2cxMMHP (r=0.44, p<0.01) (Table 5). This correlation was still observed when the creatinine adjustment was applied to the urine samples (data not shown). Oxo-MiNP concentrations in urinary samples of children at both ages were well correlated (r =0.41, p<0.05).

Concentrations of different metabolites derived from DEHP and DiNP within the same sample were well correlated as were the sum of metabolites from the same parental compound (Pearson's correlation between 0.7 and 0.988). DBzP-derived metabolites are MBzP and MnBP. We observed no correlation between the concentrations of these two metabolites (data not shown). DnBP is another source for MnBP. This lack of DBzP derivation suggests that DnBP is the main contributing source for the formation of MnBP.

290

291 ***Estimation of parental compound exposure***

292 The estimated parental contribution to actual phthalate metabolite levels in urinary
293 samples and the estimated daily intake of these compounds suggested that children were
294 more exposed to phthalate than were pregnant women. Children aged 2-3 seem to have been
295 exposed to more total phthalate, particularly to DEHP and DnBP at that younger age than
296 when they were 5-6 years old. An estimated daily intake of each parental compound and a
297 tolerable daily intake (TDI) reference are shown in Table 6. The exposure of the participants
298 in this project did not exceed tolerable daily intake as determined by the European Food
299 Safety Authority (2005).

300

301 **Discussion**

302 Compared to a CDC study of a population aged 6 and older in the USA (EPA, 2005),
303 the phthalate metabolite levels in the current study (as a geometric mean) were higher for
304 MEHP, 5OH MEHP, 5oxoMEHP, 5cxMEPP and MnBP, and lower for MBzP. When
305 compared to a German study of nursery school children aged 2-6 (Koch et al., 2004), the
306 level of MEHP in our study (as a median) was higher, while the levels of 5OH MEHP, 5oxo
307 MEHP, MnBP and MBzP were lower. When compared to levels in children aged 3-5 in the
308 GerES IV study (Becker et al 2009), only the MEHP level in our study was higher. The levels

309 of 5OH MEHP, 5oxo MEHP and MnBP in our study were lower than those in a Japanese
310 study performed exclusively on pregnant women (Suzuki et al., 2009). As stated in previous
311 reports about human DEHP metabolism, the urinary DEHP metabolites are oxidized products,
312 rather than monoesters, and the simple monoester MEHP was the dominant metabolite in
313 blood serum (Wittassek et al., 2007). The MEHP concentration was also found to be higher
314 than that of other oxidized metabolites in our cord serum samples (Table 4). Although
315 phthalate metabolite levels in milk and cord sera were low, cord blood metabolite levels were
316 well correlated with maternal urinary metabolite levels (Table 5); therefore, maternal urinary
317 metabolite levels may be useful in prenatal exposure studies.

318 The phthalate metabolites detected in breast milk in this study were mainly MEHP
319 and MnBP (Table 4). The levels of MEHP in milk were lower than those reported from the
320 USA (Calafat et al 2004), Denmark (Mortensen et al 2005, Main et al 2006), Finland (Main
321 et al 2006) and Italy (Latini et al 2009). Levels of MnBP were second highest in the studies in
322 Finland and Italy Concentrations of phthalate metabolites in milk, blood and serum were
323 close to the limit of detection; therefore, phthalate metabolites in urine maybe more
324 informative than those in milk or serum (Hogberg et al., 2008).

325 We found no correlation among levels of metabolites in different matrices from the
326 same person, i.e., urine sample, milk sample, and cord blood. This observation is in
327 agreement with previous studies, where authors concluded that phthalate concentrations in

328 urine were higher than those in serum, milk or saliva, and did not reflect the concentrations of
329 oxidative metabolites in other body fluids, especially milk (Hines et al., 2009, Hogberg et al.,
330 2008). The composition of phthalate metabolites was very different in urine and milk samples.
331 Although it is not clear how phthalate metabolites are secreted into different body fluids or
332 travel across the placenta, they probably have different rates of secretion. An in vitro
333 experiment where the placenta was perfused with different phthalate monoesters showed
334 differential diffusion rates across the placenta for different phthalate metabolites (Mose et al
335 2007). This suggested that physical-chemical properties of the compounds may influence
336 the tissue distribution of the metabolites. This may also explain the lack of correlation among
337 different matrices in the same subject. In general, DEHP and DiNP derived metabolites better
338 represented the overall phthalate exposure as they were the most important contributors in
339 pregnant women and children, respectively.

340 The phthalate exposure profile suggests that the major health risks that might be
341 associated with this population could be anti-androgenic activity and tyrosine receptor
342 antagonistic activity related to MBP, DEHP, and DBP exposure. Mixtures of phthalate esters
343 exhibit cumulative, largely dose-additive effects on male rat reproductive tract development
344 when administered during sexual differentiation in utero (Howdeshell, Furr et al., 2008).
345 DBP+DEHP increased the incidence of many reproductive malformations including
346 epididymal agenesis and reduced androgen-dependent organ weights (Howdeshell et al 2007).

Mixtures including BBP, DBP, DEHP, DiBP and DPP reduced testosterone production in a dose-additive manner (Howdeshell, Wilson et al 2008).

DiNP is widely used in toys and the proportions of its metabolites differed among urine samples from 2-3 year-olds, 5-6 year-olds, and mothers. Our levels tended to be lower than those in Japanese (Suzuki et al., 2009), US (CDC, 2005; Swan et al., 2005), and German populations (Becker et al., 2009). Higher exposure to DiNP in children could be associated with renal toxicity or problems in skeletal development.

MnBP and MBzP are breakdown products of BBP. In an experiment where stable isotope-labelled BBP was administered to eight volunteers in a single dose, 73% was excreted as MBzP and 6% as MnBP on a molar basis (Anderson et al., 2001). We found that the concentration of MnBP was much larger than that of MBzP in our urine samples. Because MnBP can also be derived from DnBP, our results suggest that DnBP is probably the principal contributor of MnBP in this population.

Although estimated daily intake values were below the tolerable daily intake according to CSTEE 1998 (Koch et al, 2003) and EFSA (2005), MiBP and MnBP levels in maternal urine were higher than those reported for mothers who gave birth to newborns with shorter ano-genital distances (Swan, 2006; Swan et al., 2005), indicating a potential risk for anti-androgenic effects in this population.

Higher exposures to DEHP in general and to DiNP in children seem to characterize the phthalate exposure in this population. Although the daily intake values estimated from urinary metabolites were within the tolerable daily intake levels as published by the European Food Safety Authority (EFSA, 2005), it is worth noting that phthalate acids are currently banned for use in toys and school supplies and, therefore, TDI is no longer applicable. Further studies will look deeper into the health effects reported as a result of exposure to DEHP and DiNP, e.g., gender-related behaviour, obesity, liver function, bone density, and allergy, in the population we have been following for several years.

Conclusions

Eleven phthalate metabolites derived from exposure to five commonly used phthalates were monitored. Higher exposures to DEHP in general, to DiNP for children, and lower exposure to BBP are the characteristics of phthalate exposure for pregnant women and their children in Central Taiwan.

We found no association between metabolite levels in the mothers and in their children at ages 2 or 5. This may be due to differences in types of ingested food and habits. Metabolite levels in different matrices from the same person, i.e., urine sample, milk sample, and cord blood were not associated. Metabolites derived from the same parental compound, as in the cases of DEHP and DiNP, were well correlated. Although the metabolites derived

384 from a particular phthalic acid are inter-correlated, the additive effects and variability of the
385 secretion fraction suggest that the measurement of individual metabolites is necessary in
386 order to estimate the total exposure.

387 Compared to other populations in the world, the phthalate metabolite levels found in
388 this study were higher for DEHP derived metabolites in urine, whether or not the data was
389 adjusted by creatinine. The levels of phthalate metabolites in milk and cord blood were
390 comparable to those found in other populations. Further follow up studies on health effects
391 related to DEHP and DiNP exposure are necessary.

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