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Prenatal di(2-ethylhexyl) phthalate exposure and disruption of adrenal androgens and glucocorticoids levels in cord blood: The Hokkaido Study

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Abstract

Di(2-ethylhexyl) phthalate (DEHP) is known for its endocrine disrupting properties. We previously demonstrated that prenatal DEHP exposure is associated with decreased progesterone levels and testosterone/estradiol ratio in the cord blood. However, evidence of the effects of prenatal DEHP exposure on adrenal androgen and glucocorticoids in infants is scarce. Thus, the objectives of this study were to investigate the association between prenatal DEHP exposure and adrenal androgen and glucocorticoids, and to discuss its effects on steroid hormone profiles in infants. This is part of a birth cohort study: The Hokkaido Study on Environment and Children's Health, Sapporo Cohort. Among the 514 participants, 202 mother-infant pairs with available data on maternal mono(2-ethylhexyl) phthalate (MEHP), adrenal androgen (dehydroepiandrosterone [DHEA] and androstenedione) and glucocorticoid (cortisol and cortisone) cord blood levels were included in this study. After adjusting for potential confounders, a linear regression analysis showed that maternal MEHP levels were associated with reduced cortisol and cortisone levels and glucocorticoid/adrenal androgen ratio, whereas increased DHEA levels and DHEA/androstenedione ratio. In a quartile model, when comparing the adjusted least square means in the 4th quartile of MEHP with those in the 1st quartile, cortisol and cortisone levels and glucocorticoid/adrenal androgen ratio decreased, whereas DHEA/androstenedione and cortisol/cortisone ratios increased. Significant p-value trends for cortisol and cortisone levels, cortisol/cortisone ratio, and glucocorticoid/adrenal androgen ratio were observed. In combination with the previous results of reduced progesterone levels and testosterone/estradiol

ratio, prenatal exposure to DEHP altered the steroid hormone profiles of infants. Further studies investigating the long-term effects of DEHP exposure on growth, neurodevelopment, and gonad and reproductive function are required.

Key Words: Di(2-ethylhexyl) phthalate (DEHP); mono(2-ethylhexyl) phthalate (MEHP); adrenal androgen; glucocorticoid; prenatal exposure; fetal blood

Abbreviations:

CI, confidence interval

CYP11A1, cytochrome P450 family 11 subfamily A member 1

CYP11B1, cytochrome P450 family 11 subfamily B member 1

CYP17A1, cytochrome P450 family 17 subfamily A member 1

CYP19A1, cytochrome P450 family 19 subfamily A member 1

CYP21A2, cytochrome P450 family 21 subfamily A member 2

CV, coefficient of variation

DBP, dibutyl phthalate

DEHP, di(2-ethylhexyl) phthalate

DHEA, dehydroepiandrosterone

- 73 DHEA-S, DHEA sulfate
- 74 HSD11B2, hydroxysteroid 11-beta dehydrogenase 2
- 75 HSD17B1, hydroxysteroid 17-beta dehydrogenase 1
- 76 HSD3B1, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
- 77 IQR, interquartile range
- 78 LSM, least square means
- 79 MEHP, mono(2-ethylhexyl) phthalate
- 80 PFOS, perfluorooctane sulfonate
- 81 MBP, Monobutyl phthalate
- 82 PPAR, peroxisome proliferator-activated receptor
- 83 SULT2A1, DHEA sulfotransferase family 2A member 1
- 84 T/E2, testosterone/estradiol
- 85

Introduction

Phthalate diesters (phthalates) have been used as plasticizers for various plastic products, including toys, food containers, furniture, personal care products, medical devices, and housing materials. According to a report from the Japan Plasticizer Industry Association and Ministry of Economy, Trade and Industry in 2012, di(2-ethylhexyl) phthalate (DEHP) constitutes >50% of the phthalates used in production in Japan. Phthalates are not chemically bonded to polyvinyl chloride in plastic products; thus, they can leach into the air, dust, foodstuffs, and other materials. Consequently, humans are constantly exposed to phthalates, and biomonitoring studies have demonstrated the widespread exposure of the general population to these chemicals (Ait Bamai et al., 2015; Fromme et al., 2007; Jensen et al., 2015; Koch et al., 2004; Wittassek et al., 2007).

DEHP is a potential endocrine-disrupting chemical, and multiple adverse effects on human health due to DEHP exposure in early life were found. Phthalate exposure has been reported to shorten the anogenital distance of infants (Bornehag et al., 2015; Swan et al., 2005; Swan et al., 2015). The associations between phthalates exposure and neurodevelopment and childhood obesity were also investigated (Ejaredar et al., 2015; Kim and Park, 2014). The underlying mechanisms of their effects were not clearly understood; however, disruption of steroidogenesis could be one of the contributing factors because steroid hormones play an important role in homeostasis. Sex steroid hormones including testosterone, progesterone, and estradiol, have effects predominantly in the gonads; and dehydroepiandrosterone (DHEA) and androstenedione, which are weak adrenal steroid precursors, are

activated to form androgens and estrogens that have important roles in sex differentiation and maturation (Labrie et al., 2001). Glucocorticoids, including cortisol, and cortisone are synthesized within the adrenal cortex, and are involved in a wide range of physiological processes. Glucocorticoids are essential for regulating and/or modulating homeostasis in metabolism, growth, neurodevelopment, and the immune system (Braun et al., 2013; Reynolds, 2010). As a whole, steroid hormones in early life play important roles in reproductive growth and neurodevelopment for later life (Hollier et al., 2014; Quinn et al., 2016).

In previous experimental studies in rat, Chinese rare minnow, and zebrafish, DEHP and/or its primary metabolite, mono (2-ethylhexyl) phthalate (MEHP), were reported to upregulate and/or downregulate several enzymes in the steroidogenesis pathway (Akingbemi et al., 2001, 2004; Lehmann et al., 2004; Sekaran and Jagadeesan, 2015; Thompson et al., 2005; Zhu et al., 2016a; Zhu et al., 2016b). Exposure to DEHP could modify steroidogenesis and disrupt both hypothalamic-pituitary-gonad and hypothalamic-pituitary-adrenal axes. One animal study demonstrate that dibythol phthalate initiated a rapid and dynamic change in gene expression unique to the fetal testis while in the adrenal was unaffected (Thompson et al., 2005). However, there are fundamental regulation differences of steroidogenesis in the fetal testis between rodent and human (Scott et al., 2009). In addition to species differences, exposure timing, duration, and dosage variations between studies are not relevant for the human exposure scenario. Thus, results from animal studies are limited in predicting the impact of phthalates exposure on adrenal steroid

production in human.

Despite the importance of DEHP properties on steroidogenesis, epidemiological studies on DEHP exposure, especially during early life, and its effects on adrenal androgen and glucocorticoid modulation are limited. In birth cohort studies in Mexico, the DHEA sulfate (DHEA-S) levels were increased and decreased following prenatal phthalate exposure in pubescent boys and girls, respectively (Ferguson et al., 2014; Watkins et al., 2014). In a Danish cohort, urinary phthalate metabolites in children were measured every 6 months from the baseline (aged 5.9 years) to pubertal age; girls with levels of monobutyl phthalate (MBP) and DEHP metabolites that were above the geometric group mean had lower levels of DHEA-S and androstenedione, whereas boys with higher MBP levels had lower DHEA-S levels (Mouritsen et al., 2013). Currently, only one study has investigated the effects of phthalate exposure on androstenedione and glucocorticoid levels in amniotic fluid, and the authors did not identify a significant association between phthalate and steroid hormone levels, including glucocorticoids (Jensen et al., 2015).

We recently demonstrated that prenatal DEHP exposure resulted in reduced progesterone levels and a reduced testosterone/estradiol (T/E2) ratio in cord blood (Araki et al., 2014). The aims of this study were to examine the effects of prenatal DEHP exposure on adrenal androgens (DHEA and androstenedione) and glucocorticoids (cortisol and cortisone), and to discuss its effects on the steroid hormone profile of infants, including adrenal androgens and glucocorticoids, and sex hormones (progesterone, testosterone, and estradiol) as reported by Araki et al. (2014).

Methods

Participants

This study was based on the Sapporo Cohort of the Hokkaido Study on Environment and Children's Health. Details of this study, regarding the population, data collection, sampling of the biological specimens, and contents of the questionnaire, have been previously described (Kishi et al., 2013; Kishi et al., 2011). Briefly, native Japanese women at an obstetrics and gynecology hospital in Sapporo (Hokkaido, Japan), who lived in Sapporo City or surrounding areas, were enrolled in the study at 23–35 weeks of gestation between July 2002 and October 2005. Among the 1796 pregnant women approached, 25% were excluded as they were enrolled in the Japanese Cord Blood Bank or planned to deliver the baby at another hospital. Eventually, 514 pregnant women (28.6%) were enrolled in this study. Selection of participants included in the analysis was described in our previous study (Araki et al., 2014). Briefly, of the 514 participants, 10 were excluded from the study due to miscarriage, stillbirth, relocation, or voluntary withdrawal prior to delivery. A total of 493 maternal blood samples were available for MEHP measurements. However, maternal blood samples collected during hospitalization after delivery were excluded from this analysis due to the relatively short biological half-life of DEHP. Steroid hormone measurements were available for 295 infant cord blood samples. Finally, 202 samples with available MEHP and steroid hormone levels were included in the statistical analysis.

MEHP measurement

Maternal blood samples were obtained at the time of their hospital examination following recruitment. If a blood sample could not be taken during pregnancy due to maternal anemia, a blood sample was collected during hospitalization, within a week after delivery. All samples were stored at -80°C until analysis. MEHP, which is the primary metabolite of DEHP, was measured by gas chromatography-mass spectrometry at Nagoya University. The methods for the preparation of samples and standard solutions, and the instrumental analysis have been described previously (Araki et al., 2014; Jia et al., 2015). The detection limit was 0.278 ng/mL (1 pmol/mL). MEHP levels in a tube containing the same medium as the reaction vial were measured to determine background levels. To exclude the possibility of environmental contamination of DEHP, glassware used for MEHP measurements was heated at 200°C for 2 h. A total of 493 maternal blood samples were analyzed for MEHP levels. The coefficient of variation (CV) of MEHP measurements taken within a single day was 2.0%–7.8% for 6 days, and the day-to-day CV for 6 days was 6.2%, at 5 pmol/mL of concentration, as described previously (Jia et al., 2015).

Measurement of steroid hormones

At the time of delivery, a blood sample was collected from the umbilical cord and stored at -80°C

until analysis. Cord blood samples were available only from the children born vaginally. The concentrations of adrenal androgens and glucocorticoids were measured using liquid chromatography–tandem mass spectrometry (LC-MS/MS; Aska Pharma Medical Co., Ltd., Kanagawa, Japan). The methods for the preparation of samples, standard solutions, and instrumental analysis were described previously (Yamashita et al., 2007a; Yamashita et al., 2007b). The mean intra-assay CVs for steroid hormone measurements were as follows: DHEA, 2.1%–5.2%; androstenedione, 2.3%–6.8%; cortisol, 3.9%–10.9%; and cortisone, 1.3%–9.9%. The mean inter-assay CVs for steroid hormones were: DHEA, 3.3%–4.6%; androstenedione, 6.6%–7.1%; cortisol, 7.6%–11.3%; and cortisone: 7.8%–9.3% (Mitsui et al., 2016; Mitsui et al., 2015).

Questionnaire and medical record

The participants completed a self-administered questionnaire regarding information on maternal age, education level, household income, smoking and alcohol consumption during the first trimester, and medical history. Medical records were obtained at delivery for information regarding pre-pregnancy body mass index, pregnancy complications, parity, gestational age, infant sex, infant size, Apgar score, and congenital anomalies, including hypospadias and cryptorchidism, .

Statistical analyses

Distribution of adrenal androgen and glucocorticoid levels among boys and girls were

200 compared using the Mann-Whitney U test. Associations between maternal MEHP concentrations and
201 infant adrenal androgen and glucocorticoid levels were initially calculated using the Spearman's rank
202 correlation coefficient, followed by multivariate linear regression analysis. MEHP levels and adrenal
203 androgen and glucocorticoid concentrations were converted to a log10 scale because they did not fall
204 into a normal distribution. To evaluate whether the association between hormone and MEHP levels
205 differed between sexes, a multivariate linear regression model for all study participants was first
206 constructed with the interaction terms of hormone levels to sex and MEHP interaction. Since the
207 MEHP and sex interaction was not significant for any of the hormones ($P_{\text{interaction}} > 0.05$), a further
208 analysis was performed, in which both sexes were included in the same model. The interquartile range
209 (IQR) for MEHP concentrations and the least square means (LSM) and 95% confidence intervals
210 (CIs) for hormone levels were calculated. To calculate a p-value for the trend, linear contrast
211 coefficients of -3, -1, +1, and +3 were assigned to 1st, 2nd, 3rd, and 4th quartiles, respectively (Goudarzi
212 et al., 2016; Itoh et al., 2016). The 1st quartile was compared to the 2nd, 3rd, and 4th quartile MEHP, and
213 the p-values were adjusted using the Bonferroni correction. When the levels were below the detection
214 limits, half of their values were used for individual hormones. Inclusion of covariates was based on
215 biological considerations, and included maternal age (continuous), maternal smoking during
216 pregnancy (yes or no), maternal alcohol consumption during pregnancy (yes or no), gestational age
217 (continuous), the week of gestation at which blood samples were taken (continuous), infant sex (boy
218 or girl), and Apgar score (ordinal variable). A previous report from our cohort population

demonstrated that prenatal perfluorooctane sulfonate (PFOS) levels were associated with steroid hormone levels (Goudarzi et al., 2016; Itoh et al., 2016). Therefore, PFOS was also included in the adjusted model. All statistical analyses were performed using JMP Clinical 5.0 software (SAS Institute Inc., NC, USA).

Ethical approval

This study was approved by the Institutional Ethical Review Board for Epidemiological Studies at Hokkaido University Graduate School of Medicine, Hokkaido University Center for Environmental and Health Sciences, and Nagoya University Graduate School of Medicine, in accordance with the principles of the Declaration of Helsinki. All participants provided written informed consents.

Results

The characteristics of the participants included in this study are shown in Supplemental Table S1. Briefly, mean $\pm$ standard deviation maternal age at delivery (years) was 29.8 ± 4.9 , and 54.5% were primiparous. For infants, the proportion of the boys was 46.0%, mean gestational age was 39.5 ± 1.0 weeks, and birth weight was 3138.6 ± 331.3 g. Apgar score at one minute after birth was nine for 82.7% children. Concentrations of MEHP in all samples were above the detection limit, and the median (IQR) concentration was 10.4 ng/mL (5.88–15.3 ng/mL). The concentration of MEHP was not significantly associated with any maternal or infant characteristics.

The adrenal androgen and glucocorticoid levels in infants are shown in Table 1. DHEA levels were significantly higher in girls than in boys. Correlations between MEHP levels and adrenal androgen and glucocorticoid concentrations are shown in Table 2, and the results of the linear regression after adjusting for potential confounders are shown in Table 3. MEHP levels were inversely associated with cortisol and cortisone levels and glucocorticoid/adrenal androgen ratio, but positively associated with DHEA levels and DHEA/androstenedione ratios.

The associations between MEHP and adrenal androgen and cortisol levels were analyzed for potential non-linear relationships. The LSM for each hormone in each MEHP quartile is shown in Figure 1, with details of the data in Supplemental Table S2. The adjusted LSM adrenal androgen and cortisol levels in relation to the MEHP quartile showed a significant p-value trend for cortisol and cortisone levels, cortisol/cortisone ratio, and glucocorticoid/adrenal androgen ratio. When comparing the LSM in the 4th quartile of MEHP with those in the 1st quartile, cortisol and cortisone concentrations and glucocorticoid/adrenal androgen ratio were decreased, whereas DHEA/androstenedione and cortisol/cortisone ratios were increased.

Since there were significant differences in DHEA levels in the cord blood with respect to sex, the associations between MEHP levels and DHEA concentrations were examined separately in boys and girls for both, linear and quartile models. However, there was no association found between MEHP and DHEA levels in either boys or girls (data not shown).

Discussion

We have investigated the associations between maternal MEHP levels and cord blood adrenal androgens (DHEA and androstenedione) and glucocorticoids (cortisol and cortisone) in mother-infant pairs of a prospective birth cohort. Our results demonstrated a significant association between increased maternal MEHP levels and decreased levels of cortisol and cortisone levels and glucocorticoid/adrenal androgen ratio, and increased DHEA/androstenedione ratio in linear models. In quartile models, p-values for trends were statistically significant for cortisol and cortisone levels, and cortisol/cortisone and glucocorticoid/adrenal/androgen ratios. However, in the 4th quartile of MEHP, cortisol and cortisone levels, glucocorticoid/adrenal androgen ratio were significantly lower, and DHEA/androstenedione and cortisol/cortisone ratios were significantly higher compared with the 1st quartile of MEHP. We have previously reported that increased maternal MEHP levels decreased progesterone levels and testosterone/estradiol (T/E2) ratio in the cord blood (Araki et al., 2014). In combination, the study provided novel evidence regarding the effects of prenatal DEHP exposure on steroidogenesis disruption in infants, and a threshold for such effects was found.

In previous studies, we demonstrated that PFOS levels were inversely associated with progesterone, T/E2 ratio, cortisol, and cortisone, and positively associated with DHEA (Goudarzi et al., 2016; Itoh et al., 2016). Nuclear receptors, including peroxisome proliferator-activated receptor (PPAR) alpha and gamma, are essential regulators of steroidogenesis, and both PFOS and DEHP are known ligands of these receptors (Lovekamp-Swan et al., 2003; Vanden Heuvel et al., 2006). Thus,

276 the effects of PFOS and DEHP exposure on steroidogenesis could overlap. In addition, there was a
277 statistically significance correlation between PFOS and MEHP (Spearman's $\rho = 0.453$, $P < 0.001$).
278 Therefore, we performed a mutual adjustment with PFOS so that the changes of steroid hormone
279 levels by MEHP in this study were independent of the effect of PFOS. For further assessment, as an
280 indicator of infant stress at birth, we have included the Apgar score (one minute after birth, ordinal
281 variable) into the adjusted model and the results remained consistent (Supplemental Table S3).
282 Therefore, stress of the birth process cannot be a confounder in our study.

283 Previous studies examining the effects of exposure to DEHP on adrenal androgen and
284 glucocorticoids in humans are limited. A Danish pregnancy-screening biobank study, which consisted
285 of cases of cryptorchidism and hypospadias and control boys, reported that increased levels of mono
286 (2-ethyl-5-carboxypentyl) phthalate, another metabolite of DEHP, was associated with higher levels of
287 androstenedione and cortisol in the second trimester amniotic fluid (Jensen et al., 2015). Further, a
288 statistically significant association between DEHP metabolites and hormones seemed more strongly in
289 cases than controls (Jensen et al., 2015). However, increased MEHP levels in the present study were
290 associated with reduced androstenedione and cortisol levels, although the p-values did not reach
291 statistically significant levels. Steroid hormone levels shift during the gestational period, such that the
292 inconsistent results could be due to the timing of the steroid measurements (Kuijper et al., 2013). In
293 addition, there were no cases of cryptorchidism or hypospadias in present study. Thus, difference of
294 the study participants could be another reason for the inconsistencies of the results.

295 This is the first study that examined MEHP exposure and DHEA levels at birth, and reported
296 that MEHP levels were inversely associated with DHEA levels in a linear model. In birth cohort
297 studies in Mexico, DHEA-S levels were increased and decreased in pubescent boys and girls,
298 respectively, following prenatal phthalate exposure (Ferguson et al., 2014; Watkins et al., 2014).
299 Mouritsen et al.(2013) reported the results of a prospective cohort study in Denmark, in which
300 phthalate levels in the urine of children were inversely associated with the DHEA-S levels in both
301 sexes (Mouritsen et al., 2013). These studies in Mexico and Denmark measured hormone levels in
302 children at puberty, whereas the present study measured hormones at birth. Another explanation for
303 the inconsistencies between the results of this study and those of previous studies is that DHEA-S,
304 instead of DHEA, was measured in the Mexico and Denmark studies. The levels of a DHEA
305 sulfotransferase family 2A member 1 (SULT2A1), which converts DHEA to DHEA-S, were reported
306 to be downregulated by phthalates in a previous study (Harris et al., 2007). Therefore, the effects of
307 DEHP exposure on the levels of DHEA and DHEA-S could differ.

308 The findings of this study could be explained by previous experimental studies as shown in
309 Figure 2. Decreased levels of progesterone following DEHP exposure likely resulted from the
310 downregulation of hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1
311 (HSD3B1). Similarly, increases in the DHEA levels and DHEA/androstenedione ratio could also
312 result from the downregulation of HSD3B1. Increased cortisol/cortisone ratios could be due to
313 DEHP-mediated downregulation of hydroxysteroid 11-beta dehydrogenase 2 (HSD11B2). Finally,

314 decreased levels of cortisol and cortisone, together with a reduced glucocorticoid/adrenal androgen
315 ratio, could be due to a shift of steroidogenesis from glucocorticoids to adrenal androgen by
316 downregulation of HSD3B1 and hydroxysteroid 17-beta dehydrogenase 1 (HSD17B1). Additionally,
317 cholesterol is a substrate of whole steroid hormones. Thus, changes in the cholesterol profile
318 following exposure to DEHP may exist as demonstrated in a previous study of elderly patients in
319 which reduced levels of cholesterol were detected following DEHP exposure (Olsén et al., 2012).

320 Previous animal and in vitro studies have provided evidence that phthalates, including DEHP
321 and/or MEHP, modify the expression of genes encoding steroidogenesis enzymes. MEHP exposure
322 decreased HSD11B2, cytochrome P450 family 11 subfamily A member 1 (CYP11A1), HSD3B1, and
323 HSD17B1 (Akingbemi et al., 2001; Hong et al., 2009; Lehmann et al., 2004; Sekaran and Jagadeesan,
324 2015; Thompson et al., 2005; Zhao et al., 2010; Zhu et al., 2016a; Zhu et al., 2016b). Although one
325 experimental study suggested upregulation of CYP17A1 (Wang et al., 2013), other studies found
326 DEHP and/or MEHP exposure induced inhibition of cytochrome P450 family 17 subfamily A
327 member 1 (CYP17A1) (Akingbemi et al., 2001; Chauvigne et al., 2011; Lehmann et al., 2004). Thus,
328 increased levels of DHEA in this study may be better explained by inhibition of SULT2A1, HSD3B1,
329 and HSD17B1 than activation of CYP17A1. As for cytochrome P450 family 19 subfamily A member
330 1 (CYP19A1), Wang et al. suggested that direction of up/downregulation differ between sex (Wang et
331 al., 2013). Nevertheless, many of the in vitro and animal studies were conducted with concentrations
332 and doses that are not relevant for the human exposure scenario. Moreover, the changes in gene

expression of steroidogenic enzymes may vary significantly depending on the experimental conditions, such as cell lines, species, doses, timing, and duration of exposure. (Akingbemin et al., 2001, 2004; Ge et al., 2007).

As we described in our previous study (Araki et al., 2014), the levels of MEHP in this cohort were slightly higher than those in American adults (NHANES 1999–2000), elderly Swedish subjects, and pregnant women in Australia (Hart et al., 2014; Lind et al., 2012; Silva et al., 2004). Slightly higher MEHP levels in this study can be considered acceptable as the levels of DEHP in house dust in Sapporo, Japan were higher compared with the studies from other countries, and DEHP intake in the Japanese population was higher than that of most other studies (Ait Bamai et al., 2014; Ait Bamai et al., 2015). On the other hand, as for the first limitation, it should be also noted that MEHP in blood was measured as urine samples were not available in this study. All samples were handled carefully to avoid *ex vivo* hydrolysis of DEHP; therefore, measurement errors due to contamination were minimal (Araki et al., 2014). Nevertheless, mono (2-ethyl-5-carboxypentyl) phthalate and mono (2-ethyl-5-hydroxyhexyl) phthalate have previously been detected in maternal serum (Hart et al., 2014); thus, these secondary metabolites should be measured in future studies.

Another limitation of this study was that the MEHP level was measured only once from second to third trimester. However, although there are contradicting discussions regarding this in the literature, several previous reports have found that single measurements can be useful (Hoppin et al., 2002; Townsend et al., 2013). In addition, the blood sampling week was adjusted to minimize the

effect of timing of exposure in this study. Although, we cannot rule out a possibility of the effect from acute exposure before delivery at the hospital, non-differential errors bias an effect towards the null. In this study, all participants who delivered vaginally with available cord blood samples were included in the analysis. Compared to the initial cohort population, the infants had an increased gestational age and heavier birth weight than those who were excluded from the study. As a result, healthier infants were included in the analysis; thus, the effects of MEHP may be underestimated in this study. In addition, the participation rate in our cohort study is rather low, which may limit the extrapolation of our results to the general population. Finally, the observation of only minimal reductions in the levels of cortisol in the linear model ($0.1 > P > 0.05$) may have been due to the limited sample size. Future studies with larger sample sizes are then required to confirm the results. A major strength of the present study is that this has a prospective birth cohort design, in which the effects of prenatal DEHP exposure on fetal adrenal androgen and glucocorticoids could be estimated. In addition, steroid hormones were measured by LC-MS/MS methods, which are considered more accurate than other methods, such as radioimmunoassay.

Conclusion

In summary, this study found significant associations between increased maternal MEHP levels and reduced cortisol and cortisone levels and glucocorticoid/adrenal androgen ratio and increased DHEA/androstenedione and cortisol/cortisone ratios. In combination with the previous results of

reduced progesterone levels (Araki et al., 2014), the present results suggested that prenatal DEHP exposure disrupted the steroid hormone profile of infants, despite the levels of DEHP in the general population being markedly lower than those in experimental studies. Disrupting the balance of steroid hormones may cause adverse effects on reproductive growth, development, and other health outcomes in later life. The clinical significance of these findings is unclear at present because it remains unknown whether these small hormonal alterations exert any effect on health.

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1 **Table 1.** Distribution of adrenal androgen and glucocorticoid concentrations

	Detection limit	>Detection limit (%)	Total (n = 202) Med (IQR)	Boys (n = 93) Med (IQR)	Girls (n = 109) Med (IQR)	^a P-value
Adrenal androgen						
DHEA (ng/mL)	0.010	100	2.31 (1.79, 3.07)	2.08 (1.59, 2.71)	2.62 (1.99, 3.42)	<0.001
Androstenedione (ng/mL)	0.010	100	0.46 (0.36, 0.58)	0.47 (0.38, 0.59)	0.45 (0.35, 0.58)	0.572
Glucocorticoid						
Cortisol (ng/mL)	0.250	96.5	40.5 (22.7, 66.7)	41.1 (22.3, 66.8)	39.3 (24.1, 66.8)	0.902
Cortisone (ng/mL)	0.100	93.6	96.9 (70.3, 124)	97.2 (72.5, 126)	96.0 (69.2, 125)	0.734

2 DHEA, dehydroepiandrosterone; IQR, the interquartile range

3 ^ap-values were calculated by Mann-Whitney U test comparing boys and girls

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5

11 **Table 3.** Adjusted linear regression coefficients of adrenal androgen and glucocorticoid in the cord blood in relation to MEHP

	Crude model				Adjusted model			
	β	(95% CI)	p-value		β	(95% CI)	p-value	
DHEA	0.246	0.095	0.397	0.002	0.205	0.029	0.381	0.023
Androstenedione	-0.079	-0.199	0.042	0.198	-0.082	-0.224	0.059	0.252
DHEA/androstenedione	0.325	0.147	0.503	<0.001	0.287	0.073	0.501	0.009
Androstenedione/testosterone	0.002	0.000	0.003	0.082	0.001	-0.001	0.004	0.163
Cortisol	-0.527	-0.809	-0.245	<0.001	-0.412	-0.751	-0.072	0.018
Cortisone	-0.734	-1.111	-0.358	<0.001	-0.570	-1.026	-0.114	0.015
Cortisol/cortisone	0.207	0.040	0.375	0.015	0.158	-0.041	0.358	0.119
Glucocorticoid/adrenal	-0.828	-1.258	-0.398	<0.001	-0.649	-1.167	-0.132	0.014

12 ~~and~~ hormone levels and MEHP concentrations were log10-transformed and included in the model separately.

13 β for linear regression coefficients

14 Adjusted for maternal age, smoking and alcohol consumption during pregnancy, gestational age, blood sampling week, infant sex, and PFOS

15 95%CI, 95% confidence interval; DHEA, dehydroepiandrosterone; MEHP, mono(2-ethylhexyl)phthalate; PFOS, perfluorooctane sulfonate levels,

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17

18

Figure 1

Figure 2

Figure legend

Figure 1

X-axis shows the mono(2-ethylhexyl) phthalate (MEHP) quartiles, and Y-axis shows each hormone level. The adjusted least square means (LSMs) with 95% confidence intervals for each hormone in the cord blood in relation to the MEHP concentration quartile fit to all study participants are shown with a *p*-value trend for: (A) DHEA, (B) DHEA/androstenedione ratio, (C) cortisol, (D) cortisone, (E) cortisol/cortisone ratio, and (F) glucocorticoid (cortisol and cortisone)/adrenal androgen (DHEA and androstenedione) ratio. There was a significant and inverse association between MEHP levels and cortisol and cortisone levels, glucocorticoid/adrenal androgen ratio, but a positive association between MEHP levels and the cortisol/cortisone ratio. The 1st quartile (≤ 5.88 ng/mL) was compared with the 2nd (5.88–10.4 ng/mL), 3rd (10.4–15.3 ng/mL), and 4th (>15.3 ng/mL) quartile MEHP levels. Statistical significance of the *P* values were $*p < 0.017$ and $**P < 0.0033$ based on Bonferroni correction. When compared with the LSM of the 1st MEHP quartile, the 4th MEHP quartile of cortisol, cortisone, and glucocorticoid/adrenal androgen ratios were significantly decreased, whereas the DHEA/androstenedione ratio and cortisol/cortisone ratio were significantly increased. LSMs were adjusted for maternal age, smoking and alcohol consumption during pregnancy, gestational age, blood sampling week, infant sex, and perfluorooctane sulfonate level

Abbreviations: DHEA, dehydroepiandrosterone; LSM, least square means, MEHP, mono(2-ethylhexyl) phthalate

20 Figure 2

21 Steroid metabolic pathways and their disruption following mono(2-ethylhexyl) phthalate (MEHP)
22 exposure. Squares indicate the hormones measured in this study and our previous report. Black bold
23 arrows indicate the direction of shift following MEHP exposure. Black characters and arrows in gray
24 background and rounded rectangles indicate enzymes reported as upregulated or downregulated by di
25 (2-ethylhexyl) phthalate (DEHP) and/or MEHP in experimental studies.

26 Abbreviations: CYP11A1, cytochrome P450 family 11 subfamily A member 1; CYP11B1,
27 cytochrome P450 family 11 subfamily B member 1; CYP17A1, cytochrome P450 family 17 subfamily
28 A member 1; CYP19A1, cytochrome P450 family 19 subfamily A member 1; CYP21A2, cytochrome
29 P450 family 21 subfamily A member 2; HSD11B2, hydroxysteroid 11-beta dehydrogenase 2;
30 HSD17B1, hydroxysteroid 17-beta dehydrogenase 1; HSD3B1, hydroxy-delta-5-steroid
31 dehydrogenase, 3 beta- and steroid delta-isomerase 1; SULT2A1, DHEA sulfotransferase family 2A
32 member 1