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Prenatal phthalate exposure and reproductive function in young men

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ABSTRACT

Background: Prenatal exposure to phthalates is suggested to negatively impact male reproductive function, but human data are lacking.
Objectives: To study associations between prenatal exposure to diethylhexyl phthalate (DEHP) and diisononyl phthalate (DiNP), and reproductive parameters of adolescent men.
Methods: Using linear regression models adjusted for potential confounders, we studied associations between levels of DEHP- and DiNP metabolites in maternal sera from mean 12 weeks of pregnancy, and testicular size, semen quality and reproductive hormones in 112 adolescent sons, recruited from the general population.
Results: Men in the highest exposure tertile of one DiNP metabolite [mono-(carboxy-iso-octyl) phthalate], compared with men in the lowest tertile had: 4.3 mL (95% CI: 0.89, 7.6 mL; $p < 0.001$) lower total testicular volume; 30% (95% CI: 3.6, 63%; $p = 0.02$) higher levels of follicle-stimulating hormone; and 0.87 mL (95% CI: 0.28, 1.5 mL; $p = 0.004$) lower semen volume. Men in the highest exposure tertile of one DEHP metabolite [mono-(2-ethyl-5-hydroxylhexyl) phthalate] had 0.70 mL (95% CI: 0.090, 1.3 mL; $p = 0.03$) lower semen volume than men in the lowest exposure tertile. The levels of two DiNP metabolites [mono-(hydroxy-iso-nonyl) phthalate and mono-(oxo-iso-nonyl) phthalate] were linearly associated with luteinizing hormone ($p < 0.01$).
Conclusion: Prenatal levels of some metabolites of DEHP and DiNP seemed negatively associated with reproductive function of adolescent men.

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1. Introduction

Phthalates are constituents of consumer products which makes humans continuously exposed (Wittassek et al., 2011). Exposure to phthalates during fetal life has been suggested to cause reproductive disorders in men through endocrine disruption (Sharpe and Skakkebaek, 2008).

In male rats, prenatal exposure to diethylhexyl phthalate (DEHP) and diisononyl phthalate (DiNP) induce genital malformations (Gray et al., 2000). This is suggested to be due to a reduced fetal testosterone (T) production (Hu et al., 2009), which is reported for both DEHP and DiNP (Borch et al., 2004; Hannas et al., 2011).

Human studies have reported that prenatal phthalate exposure was associated with a shorter anogenital distance in boys, indicating a reduced masculinization (Bornehag et al., 2015; Bustamante-Montes et al., 2013; Suzuki et al., 2012; Swan et al., 2005) and perhaps a reduced future fertility (Dean and Sharpe, 2013). Similar exposure has additionally been associated with cryptorchidism (Swan, 2008; Wagner-Mahler et al., 2011), altered reproductive hormone levels (Araki et al., 2014; Main et al., 2006), and with an increased risk of hypospadias (Ormond et al., 2009). However, other studies were inconsistent with those above (Chevrier et al., 2012; Huang et al., 2009; Jensen et al., 2015; Lin et al., 2011a; Main et al., 2006) and only two studies (Bornehag et al., 2015; Huang et al., 2009) have focused on exposure in early pregnancy, considered as the most sensitive period for the developing male reproductive organs (Welsh et al., 2008). In addition, the so called secondary metabolites, which are suggested as the best exposure markers (Wittassek et al., 2011) were only measured in some of these studies.

Abbreviations: DEHP, diethylhexyl phthalate; DFI, DNA fragmentation index; DiNP, diisononyl phthalate; FSH, follicle-stimulating hormone; HDS, High DNA stainability; LC/MS/MS, liquid chromatography–tandem mass spectrometry; LH, luteinizing hormone; LOD, limit of detection; MECPP, mono-(2-ethyl-5-carboxypentyl) phthalate; MCiOP, mono-(carboxy-iso-octyl) phthalate; MEHHP, mono-(2-ethyl-5-hydroxylhexyl) phthalate; MHiNP, mono-(hydroxy-iso-nonyl) phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl) phthalate; MOiNP, mono-(oxo-iso-nonyl) phthalate; MPW, masculinization programming window; SHBG, sex hormone-binding globulin; T, testosterone

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In male rats, the development and size of the reproductive organs are programmed in an early masculinization programming window (MPW) (Macleod et al., 2010; Welsh et al., 2008) during which exposure to the compound dibutyl phthalate negatively affects the future reproductive organ size (Macleod et al., 2010).

From a previous study of reproductive function in men from the general population (Axelsson et al., 2013), we had access to maternal serum samples from early pregnancy through a Swedish screening program for rubella. Most of these samples were collected within the suggested corresponding MPW in humans, between the 8th and 14th gestational week (Welsh et al., 2008).

Our aim was to study associations between prenatal exposure to DEHP and DiNP, and male reproductive parameters, by measuring secondary metabolites in maternal samples.

2. Subjects and methods

This work was carried out in accordance with The Code of Ethics of the World Medical Association (WMA, 2013) and the Uniform Requirements for Manuscripts Submitted to Biomedical Journals. International Committee of Medical Journal Editors (1997).

2.1. Study population

Between 2008 and 2010, we invited 1681 men presenting for the military health board. Among them, 241 (14%) accepted participation. In order to expand the participant number, we included another 73 men through announcement in schools, giving initially 314 participants (Axelsson et al., 2013). Inclusion criteria were: living within 60 km from the city of Malmö in southern Sweden; 17–20 years of age; participant and mother born and raised in Sweden. The men signed an informed consent, filled in questionnaires regarding height and weight, previous genital diseases, current smoking and paternal smoking during pregnancy, and finally delivered semen and blood samples. They were paid 500 SEK (55 EURO) for participation. The study was approved by the regional ethical review board.

The extent of maternal smoking during pregnancy was assessed through cotinine levels in serum (see Section 2.3). Forty-nine of the 314 initially included men were excluded since they or their mother declined further participation, leaving 265 men for search of maternal samples.

2.2. Maternal sampling

In Sweden, screening for rubella antibodies in serum is routinely done in early pregnancy. Unless the woman declines, part of the sample is stored in a biobank. We retrieved maternal samples of 112 men born 1989–1992, on whom this study was based. There was no difference in reproductive parameters between these men and the remaining 153 for whom no maternal samples were found (data not shown). The samples were obtained from the 6th to the 35th week of pregnancy (mean 12 weeks). Seventy-seven men (69%) had mothers sampled between 8 and 14 completed gestational weeks.

2.3. Analyses of exposure markers

Phthalate metabolites and the nicotine metabolite cotinine were analyzed in maternal serum by liquid chromatography–tandem mass spectrometry (LC/MS/MS). The analyzed phthalate metabolites were the secondary metabolites of DEHP: mono-(2-ethyl-5-hydroxylhexyl) phthalate (MEHHP), mono-(2-ethyl-5-oxohexyl) phthalate (MEOHP) and mono-(2-ethyl-5-carboxypentyl) phthalate (MECPP) and of DiNP: mono-(hydroxy-iso-nonyl) phthalate (MHiNP), mono-(oxo-iso-nonyl) phthalat (MOiNP) and mono-(carboxy-iso-octyl) phthalate (MCiOP). For the analysis, aliquots of 100 μl serum were added with isotopically labeled internal standards for all evaluated compounds. The samples were digested with glucuronidase, and proteins were precipitated using acetonitrile. The samples were prepared in 96-well plates and analyzed using a triple quadrupole linear ion trap mass spectrometer (QTRAP 5500; AB Sciex, Foster City, CA, USA) coupled to a liquid chromatography system (UFLCXR, Shimadzu Corporation, Kyoto, Japan; LC/MS/MS). The analysis was performed in negative ion mode. All data acquisition was performed using Analyst 1.6.1 software, and data was processed using Multiquant 2.1 (AB Sciex).

Limits of detection (LOD) for metabolite analyses was defined as the concentration corresponding to three times the standard deviation of the ratio of the peak at the same retention time as the analyzed compounds and the corresponding internal standards determined in the chemical blank samples. Coefficients of variation (CV) of a quality control sample were analyzed in all sample batches. A more detailed description of the method and the validation can be found in the Supplementary data.

2.4. Genital examination

All men were genitally examined by a physician for total testicular volume using Prader's orchidometer.

2.5. Semen analysis

We asked the men to keep 48–72 h of abstinence (which 42% fulfilled) but registered the actual length. Semen was analyzed for sperm concentration, total sperm count, progressive sperm motility and proportion of morphologically normal sperm according to WHO guidelines (World Health Organization, 1999). We additionally analyzed sperm DNA fragmentation index (DFI) and High DNA stainability (HDS) using the Sperm Chromatin Structure Assay (Evenson et al., 2002).

2.6. Analyses of reproductive hormones

Serum samples obtained from the men before noon, were analyzed at the laboratory of clinical chemistry, Skåne University Hospital, Sweden. Levels of T, follicle-stimulating hormone (FSH), luteinizing hormone (LH) and sex hormone-binding globulin (SHBG) were analyzed with ElectroChemiLuminiscenceImmunoassay (Roche Cobas), and estradiol by use of an immunofluorometric method (Delfia, Perkin-Elmer).

For T, CV was 3.8% at 3.2 nmol/L and 1.6% at 25 nmol/L, and LOD 0.087 nmol/L; FSH had CV 5.5% at 5.0 IU/L and LOD 0.10 IU/L; LH had CV 3.2% at 7.0 IU/L and LOD 0.10 IU/L; SHBG had CV 1.2% at 16 nmol/L, 1.4% at 34 nmol/L, and LOD 0.35 nmol/L; and estradiol had CV 20% at 30 pmol/L, 10% at 280 pmol/L, and LOD 8 pmol/L.

Concentration of free T was calculated according to Vermeulen et al. (1999).

2.7. Statistics

We used SPSS v 20-22 for statistical analyses, and defined $p < 0.05$ as statistically significant. Correlations between phthalate metabolites were studied using Spearman's rank correlation test.

To better fulfill model assumptions regarding normal distribution of residuals, we transformed DFI, HDS, T, free T, LH, FSH and SHBG by the natural logarithm, and sperm concentration and total sperm count by the cubic root. Likewise, we transformed phthalate metabolite concentrations and fetal age by the natural logarithm to increase statistical prediction (Tabachnick and Fidell, 2013). We

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