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In utero combined di-(2-ethylhexyl) phthalate and diethyl phthalate exposure cumulatively impairs rat fetal Leydig cell development

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ABSTRACT

Phthalate diesters, including di-(2-ethylhexyl) phthalate (DEHP) and diethyl phthalate (DEP), are chemicals to which humans are ubiquitously exposed. Humans are exposed simultaneously to multiple environmental chemicals, including DEHP and DEP. There is little information available about how each chemical may interact to each other if they were exposed at same time. The present study investigated effects of the combinational exposure of rats to DEP and DEHP on fetal Leydig cell development. The results showed that the gestational (GD12-20) exposure of DEP + DEHP resulted in synergistic and/or dose-additive effects on the development of fetal Leydig cell. The lowest observed adverse-effect levels (LOAEL) for fetal Leydig cell (aggregation and cell size), and StAR expressions were of 10 mg/kg and, lower than when these chemicals were exposed alone. Also, mathematical modeling the response curves supports the dose-addition model over integrated-addition model. Overall, these data demonstrate that individual phthalate with a similar mechanism of action can elicit cumulative, dose additive, and sometimes synergistic, effects on the development of male reproductive system when administered as a mixture.

1. Introduction

Phthalates are the most widely used plasticizers and are found in a wide range of the consumer products, from food packaging to medical devices and pharmaceuticals (Doyle et al., 2013). Humans and animals are exposed to phthalates via multiple routes, including ingestion, inhalation, absorption by dermal contact, and the parenteral routes (placenta and milk) (Shelby, 2006). Numerous studies have shown that *in utero* exposure to certain phthalate compounds, such as diethylhexyl phthalate (DEHP), can disrupt the development of the reproductive system in male offspring (Lin et al., 2008). Many of these effects have been attributed to a reduction in testosterone by the fetal testis through reduced expressions of steroidogenic genes in Leydig cells (Barlow et al., 2003; Hannas et al., 2012; Lehmann et al., 2004; Liu et al., 2005; Parks et al., 2000).

Diethyl phthalate (DEP) consists of a benzene ring with two carboxylic acid ethyl esters attached to it in the ortho (1,2) pattern. DEP has been found in a large number of cosmetics and personal care products in adults (Duty et al., 2005) and young children (Sathyanarayana, 2008). A previous study found that phthalate esters with ester side chains four to six carbons in length in the ortho configuration such as DEHP would be anti-androgenic *in utero*, while DEP (C2-ortho position) would be inactive (Gray et al., 2000). However, studies examining the impact of DEP exposure during gestation found reduced testosterone concentrations in offspring in one rodent study, but not with offspring reproductive tract defects (Fujii et al., 2005; Howdeshell et al., 2008a,b). As high levels of MEP, the monoester of DEP, have been detected in human urine (Blount et al., 2000), studies about DEP are necessary because the mechanism on testosterone reduction is still unclear and experts are divided about its effects.

There were significant levels of DEP and DEHP in pregnant mothers and fetuses. Our previous investigation showed that the median levels of DEP, DEHP and its metabolite mono-ethylhexyl phthalate (MEHP) were 1.6–2.9, 0.3–1.2, and 1.2–2.1 mg/L in 113 control human maternal blood samples and 0.9–2.4, 0.1–0.9, and 0.9–1.7 mg/L in the control human neonatal cord blood samples (Zhang et al., 2009). The levels of phthalates were significantly higher in 88 human low-birth weight cohorts, with the median levels of DEP, DEHP, and MEHP of

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1.6–2.7, 0.5–1.3, and 1.8–3.5 mg/L in the human maternal blood samples and 1.3–2.0, 0.3–1.0, and 1.6–3.4 mg/L in low-birth weight human neonatal cord blood samples (Zhang et al., 2009). Phthalate levels in infertile semen in Chinese men were also significantly higher, with the mean levels of DEP of 5.66 μg/L in 94 control human semen samples and 10.61 μg/L in 94 infertile human samples and DEHP of 13.68 μg/L in human controls and 27.66 μg/L in infertile human samples (Wang et al., 2015).

Reports from health advocacy groups have emphasized the need for testing combinations of phthalates to better assess the health risks of human exposure to multiple sources of the chemicals (DiGangi et al., 2002; Purvis and Gibson, 2005). To date, there are a few published studies available that have tested the effects of prenatal exposure to combinations of different phthalates esters. In the present study, we hypothesized that prenatal exposure of male rats to a mixture of DEP and DEHP with the same mechanism of action, but different active metabolites, would act in a cumulative, dose-additive fashion to inhibit fetal testicular steroid hormone production and gene expression profiles and to increase Leydig cell aggregation. To address this hypothesis, we studied male rats that prenatally exposed to DEP or DEHP or a combination of the two during the fetal period (gestation days [GD] 12–21). The results indicated that individual phthalate compounds with a similar mechanism of action, like DEP and DEHP, can elicit cumulative, dose additive, and sometimes even synergistic, effects on the development of fetal Leydig cells. Synergistic effect refers to the effect caused when exposure to two or more chemicals at as time results in health effects that are greater than the sum of the effects of the individual chemicals.

2. Materials and methods

2.1. Animal treatment

Adult male and female Sprague-Dawley rats were purchased from Shanghai Laboratory Animal Center (Shanghai, China). After adjustment for one week, male and female rats were mated. When the pregnancy was confirmed, the female rat was individually housed (23 ± 2 °C, relative humidity 55 ± 5%), in a 12-h light-dark cycle environment. The animals were housed in IVC cages (one rat per cage) on soft chip bedding and provided pellet chow (Shanghai Laboratory Animal Center). This study was approved by the Wenzhou Medical University Laboratory Animal Ethics Committee, and all procedures were performed in accordance with the policies. The investigation conformed to the procedures described in the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996). DEP and DEHP were purchased from Sigma (St. Louis, MO, USA) and suspended in the corn oil (vehicle control) for gavage. The pregnant dams were divided into 15 groups: DEP 0 (control, corn oil), 10, 100, 500 or 1000 mg/kg body weight, DEHP 0 (control, corn oil), 10, 100, 500 or 1000 mg/kg body weight, and DEP + DEHP (1:1, w/w) 0 (control, corn oil), 10, 100, 500 or 1000 mg/kg body weight. All dams were gavaged daily from GD 12 to GD 21 (n = 6 for each group). Pregnant dams gave birth at GD 21.5. Following measurement of the pup body weight, the pups were put into the airtight IVC cages, and were sacrificed by asphyxiation with CO_2 at GD 21.5 (postnatal day 1). Three sets of randomly selected fetal testes (at least one testis per rat) were immediately removed, frozen in liquid nitrogen and stored at −80 °C. The other remaining testis was fixed by Bouin's solution for one day for the histochemical staining.

2.2. Testicular testosterone assay

Testicular steroids were extracted from the testes of DEP, DEHP, and DEP + DEHP-exposed pups (n = 6 control; n = 6 DEP, DEHP, and DEP + DEHP per dose) as described (Lin et al., 2008). Testicular testosterone concentrations were measured by a tritium-based RIA validated for use with rat serum, as described (Akingbemi et al., 2001).

2.3. Tissue array and 3β-hydroxysteroid dehydrogenase (3β-HSD) enzymatic staining

3β-HSD is the biomarker of fetal Leydig cell. Enzymological staining for 3β-HSD in the frozen section of the fetal testis was performed as described previously (Lin et al., 2008). In brief, frozen testes from 15 groups (0, 10, 100, 500, and 1000 mg/kg DEP, DEHP, and DEP + DEHP) were embedded in the same blocks as a set of tissue array. Ten-micrometer-thick cryostat sections were prepared at −20 °C. The incubation solution contained 0.4 mM etiocholanolone as substrate and 2 mM NAD^+ as a cofactor. In addition, the incubation medium contained tetranitroblue tetrazolium as the H^+ acceptor, 0.1 M phosphate buffer at pH 7.2. The sections were incubated for 30 min at 37 °C in a humidified chamber in darkness. Additional sections were incubated without substrate as negative controls in order to determine nonspecific dehydrogenase effect. The sections were washed using PBS twice, and fixed using 4% PBS buffered paraformaldehyde solution. After twice washes, the sections were contrastained using 4′6-diamidino-2-phenylindole (DAPI, Sigma). The sections were covered with 50% glycerol and covered with a coverslip. The morphology of the testis was observed using a fluorescence microscope (Olympus BH Series) at excitation wavelength 350 nm combined with bright-field photography. To enumerate fetal Leydig cell numbers, testicular tissues were sampled according to the Fractionator technique as described (Akingbemi et al., 2004). The total number of fetal Leydig cells per testis was calculated by multiplying the number of fetal Leydig cells counted in a known fraction of the testis by the inverse of the sampling probability, and average fetal Leydig cell numbers per testis per treatment group were determined. Testis volume per testis was calculated by the accumulation of sections as described (Lin et al., 2008). Numbers of fetal Leydig cell clusters were counted, and frequency distributions were calculated.

2.4. Tissue array and immunohistochemical staining of 3β-HSD

One testis from each rat was collected and used for immunohistochemical staining. Five testes per group were used and testis samples were prepared and then embedded in paraffin as a tissue array. Four micrometer-thick transverse sections were cut and mounted on the glass slides. Avidin-biotin immunostaining was conducted following manufacturer's instructions (Vector, Burlingame, CA, USA). Antigen retrieval was performed by microwave irradiation for 10 min in 10 mM (pH 6.0) of citrate buffer, after which endogenous peroxidase was blocked with 0.5% of H_2O_2 in methanol for 30 min. Sections were then incubated with an 3β-HSD rabbit polyclonal antibody (Abcam, Cambridge, UK) diluted 1:200 for 1 h at room temperature. Diaminobenzidine was used for visualizing the antibody-antigen complexes, positively labeling Leydig cells by brown cytoplasmic staining. Mayer hematoxylin was applied as the counterstaining. The sections were then dehydrated in graded concentrations of alcohol and coverslipped with resin (Permount, SP15-100; Fisher Scientific, Thermo Fisher Scientific, Waltham, UK). Non-immune rabbit IgG (Sigma-Aldrich, St. Louis, MO) was used in the incubation of the negative control sections at the working dilution the same as the primary antibody. The cells with positive stainings of 3β-HSD were counted. Cell size and nuclear size were measured using image analysis software as below.

2.5. Computer-assisted image analysis

Eight randomly selected fields in each of three nonadjacent sections per testis were captured using a BX53 Olympus microscope (Tokyo, Japan) equipped with digital camera interfaced to a computer. The