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Developmental effects of prenatal di-*n*-hexyl phthalate and dicyclohexyl phthalate exposure on reproductive tract of male rats: Postnatal outcomes

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ABSTRACT

The present study is to investigate the effects of *in utero* di-*n*-hexyl phthalate (DHP) and dicyclohexyl phthalate exposure (DCHP) on the development of male reproductive tract at prepubertal, pubertal and adult stages. Pregnant rats were exposed to DHP and DCHP at doses of 0, 20, 100 and 500 mg/kg/day, by gavage, on gestational days (GD) 6–19. Testosterone (T) levels of prepubertal rats diminished at high dose DHP and middle dose DCHP groups. MIS/AMH levels elevated in DHP and DCHP groups. T levels of pubertal rats decreased in low and high dose DHP and DCHP groups. Inhibin B levels of adult rats diminished in DCHP groups. Atrophic and amorphous tubules, spermatogenic cell debris, apoptotic cells, adherent tubules, Sertoli cell vacuolisation, prostatic atrophic tubules and prostatic intraepithelial neoplasia (PIN) were observed in the reproductive organs of treated animals at all developmental stages. There was an increase in immunoexpression of MIS/AMH in testes of treated rats. There were no changes in sperm head count but percentages of abnormal sperms increased. The diameters of seminiferous and epididymal tubules in treatment groups were significantly lower. This study shows that DHP and DCHP may have antiandrogenic effects on male reproductive development before and after birth.

1. Introduction

There is an increasing concern focusing on the potential of environmental toxicants to impair the human fertility because adverse developmental and reproductive effects were observed in laboratory animals and wildlife after exposure (Akingbemi et al., 2001; Foster et al., 2001). Phthalate esters have attracted considerable public attention due to their high production volume and use as plasticizers in commercial products, such as plastic food wraps, children's toys, blood transfusion and dialysis bags, and catheters, to make the plastic products more flexible (Bhattacharya et al., 2005; Zhang et al., 2004). However, phthalate esters are not covalently bound to the plastic material and are released into the environment after a while. These esters are frequently detected in outdoor and indoor air (Saito et al., 2001). In recent studies, exposures to some phthalates have shown to cause serious and irreversible changes in the development of reproductive tract, especially in males (Foster et al., 2001; Sharpe, 2001). They also have shown to have permanent effects on the male reproductive development if exposures occur during the critical period of sexual differentiation (i.e. late in the gestation) by interfering with androgen signaling pathway (Kavlock et al., 2002; Latini et al., 2006; Mylchreest et al., 1998, 1999, 2000).

Di-*n*-hexyl phthalate (DHP) and dicyclohexyl phthalate (DCHP) belong to the family of phthalic acid diesters which are produced in large quantities, and are primarily used as softeners and plasticizers in commonly used plastics (Kavlock et al., 2002). DCHP is a plasticizer used for production of nitrocellulose, ethyl cellulose, vinyl acetate, polyvinyl chloride, and resins (HSDB, 2008). DHP is often found as a minor component (less than 1%) of C6–C10-phthalate mixtures; it may also be an isomer in mixtures of diisohexyl phthalates (DIHP) at levels of 25% or lower (Kavlock et al., 2002). DHP and DCHP were found in food and in indoor environments (Rakkestad et al., 2007). DCHP was measured at a concentration of 0.07 μg m^{-3} in samples of indoor air from houses in Japan (Otake et al., 2004) and its monoester metabolite (i.e. monocyclohexyl phthalate) in urine of adults of the US general population (Blount et al., 2000).

DCHP and DHP were found to show both ERα-agonistic and ERβ- and AR-antagonistic activities (Nakai et al., 1999; Takeuchi et al., 2005). Okubo et al. (2003) demonstrated that DCHP had

Abbreviations: 3β-HSD, 3-beta-hydroxysteroid dehydrogenase; AR, androgen receptor; DBP, dibutyl phthalate; DCHP, dicyclohexyl phthatale; DEHP, di-(2-ethylhexyl) phthalate; DHP, di-*n*-hexyl phthalate; ER, estrogen receptor; FSH, follicle-stimulating hormone; GD, gestational day; LH, luteinizing hormone; MIS/AMH, Mullerian inhibiting substance/antiMullerian hormone; PIN, prostatic intraepithelial neoplasia; PND, postnatal day; T, testosterone.

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the most potent estrogenic activity among phthalate esters examined, but its estrogenic potency was 1,700,000 times less than that of 17β-estradiol.

Hoshino et al. (2005) investigated the effects of DCHP in a two-generation study on rats. They found that there were no effects on reproductive endpoints such as fertility, mating, gestation and birth index, however, decreases in sperm head counts and focal seminiferous tubule atrophy were observed at 80–107 mg/kg bw/day in F1 males. In a short-term study by Foster et al. (1980), a single dose of 2400 mg/kg bw/day DHP was given by gavage in corn oil to a group of 12 pubertal Sprague–Dawley rats (4-week old) for 4 days. Marked effects on testis weight (65% of control value) were noted in the absence of body weight effects. Histological examination revealed that marked seminiferous tubular atrophy with the majority of tubules showing few spermatogonia and Sertoli cells, but normal Leydig cell morphology. In another study, dietary exposure to DHP at dose of 0, 380, 800 or 1670 mg/kg bw/day resulted in dose-related adverse effects on fertility. Saillenfait et al. (2009a) administered DHP or DEHP pregnant Sprague–Dawley rats by gavage on gestation days 12–21, at doses of 0, 50, 125, 250, or 500 mg DnHP/kg-d. They found that male offspring displayed shorter anogenital distance on PND 1 and testicular pathology, consisting in seminiferous tubular atrophy, was also observed in mature animals. They observed the adverse effects appeared at 125 mg DHP/kg-d, and were more pronounced at 250 and 500 mg DHP/kg-d.

The hormonal status is crucial for the development of male reproductive tract. The production of testosterone is critical for the normal masculinization of the male reproductive tract and a reduction in testosterone is a factor in the occurrence of hypospadias and cryptorchidism observed after phthalate treatment (Parks et al., 2000). At high concentration of AMH expression in Sertoli cells in adulthood may indicate failure of maturation of Sertoli cells, though it can also reflect deficiencies in androgen action. Both FSH and T are essential for normal spermatogenesis. Prepubertal serum inhibin B is a marker for the presence and integrity of testicular tissue (Pierik et al., 2003).

The aim of this study is to investigate the effects of *in utero* di-*n*-hexyl phthalate (DHP) and dicyclohexyl phthalate (DCHP) exposures on the development of male reproductive tract. Pregnant Wistar rats were exposed to DHP and DCHP at doses of 0, 20, 100 and 500 mg/kg/day, by gavage, on gestational days (GD) 6–19, and the effects of these phthalates on morphology, hormonal, histopathologic changes and sperm quality were observed at different postnatal developmental stages as prepubertal (postnatal day (PND) 20), pubertal (PND 32) and adult (PND 90) stages.

2. Materials and methods

2.1. Chemicals

Di-*n*-hexyl phthalate (CAS No. 84-75-3) with purity of 97% and dicyclohexyl phthalate (CAS No. 84-61-7) with purity of 99% were supplied by Alfa Aesar and Aldrich Chemistry (Ankara, Turkey), respectively, and dissolved in corn oil (vehicle).

2.2. Animals treatments

Pregnant time-mated female Wistar albino rats on gestational day 0 (GD0; the day when sperm was detected in the vaginal lavage) were purchased from the Experimental Animals Production Center, Hacettepe University in Ankara, Turkey. All rats were housed in polycarbonate cages with stainless steel covers, and in a room maintained 12 h light/dark cycle with a temperature of 22 ± 2 °C and a relative humidity of 50 ± 5, and given standard rat diet (Korkutelim Feed Factory, Afyon, Turkey) and tap water *ad libitum*. The pregnant rats were distributed on a random basis into control (vehicle) and treatment groups ($N = 10$) and housed individually.

2.3. Treatments

The pregnant rats were administered DHP and DCHP in corn oil by gavage at dose of 0 (vehicle), 20, 100 and 500 mg/kg/day from GD 6 to 19. The solutions were prepared fresh daily according to dams' weights. The dosing volume was 0.25 ml in all groups. The rats in the vehicle control group received corn oil in equal amounts as in experimental groups. After delivery, all pups were allowed to grow with dam for 1 month. Then, male pups were separated from female dams and pups. Male rats were housed at four per cage and allowed free access for standard rat diet and tap water *ad libitum*. The rats were allowed to grow up until prepubertal (postnatal day (PD) 20), pubertal (PD 32) and adult (PD 90) stages. At necropsy, the animals were weighed and sacrificed under ether anesthesia followed by decapitation, and tissues (testes, epididymis, ventral prostate and seminal vesicle) were excised immediately. The tissues were dissected and weighed in order to calculate the organ/body weight ratios for each animal. The organ weight was considered as absolute organ weight, whereas organ/body weight ratio was considered as relative organ weight. All experimental procedures and animal use were confirmed as the Approval of Ethics Committee of Hacettepe University.

2.4. Hormone analysis

At the end of the study, the animals were weighed and sacrificed under ether anesthesia followed by decapitation. The blood was collected from heart and the serum samples were stored at −20 °C until hormone analysis. Testosterone, estradiol, FSH, LH (*Endocrinetech*, Newark, CA), inhibin B and MIS/AMH (Diagnostic System Laboratories, Webster, TX, USA) were measured by using commercially available EIA kits. The intra- and inter-assay coefficients of variations were less than 13%.

2.5. Histopathological analysis

After weighing, tissue samples (testes, epididymis, prostate and seminal vesicle) were fixed in Bouin's solution for 8 h. For adult testes, the tissues were cut into 3 pieces at 4th hour of fixation and then continued with fixation. The tissues were embedded in paraffin, cut at 4 μm thickness, stained with Harris hematoxylin and eosin. All slides were examined using Olympus BX51 system light microscope. The photographs were captured using Bs200prop software.

2.6. Immunohistochemical staining of 3β-HSD and MIS/AMH

Sections from testes tissue samples were cut at 4 μm and processed for immunohistochemical examination by the avidin–biotin–peroxidase method by using Vectastain Elite ABC kit (Vector Labs, Burlingame, CA). For immunohistochemistry staining, primary antibodies were used in the following dilution: mouse anti-goat 3β-HSD, 1:50; and mouse anti-goat MIS/AMH, 1:50. Briefly, the sections were deparaffinized and rehydrated in PBS for 10 min. Antigen retrieval was performed by heating slides in citrate buffer (pH 6) in water bath for 2 h at 98 °C and then cooled for 20 min. Then the slides were washed twice in phosphate buffer for 20 min. After that, endogenous peroxide activity was blocked with 30-min incubation in methanol containing 1% hydrogen peroxide for 15 min and washed twice in phosphate buffer solution (pH 7.2) for 5 min each. Tissue sections were incubated for 20 min with buffer containing 5% normal serum for protein blockage. Primary antibodies for anti-3β-HSD (1:50) and anti-MIS/AMH (1:50) and secondary antibodies were diluted in 5% normal serum. Afterwards, the sections were incubated with primary antibodies overnight at +4 °C. Sections were then incubated in goat anti-rabbit secondary antiserum (Vector Labs, Burlingame, CA) for 1 h followed by avidin–biotin–peroxidase reagents (Elite Vectastain kit, Vector Labs) for 1 h at room temperature. Nova Red (Vector Labs, Burlingame, CA) was used as the chromagen. The sections were counterstained using methyl green (Vector Labs, Burlingame, CA). Then, the sections were imaged using Olympus BX51 microscope with camera.

2.7. Sperm counts and morphology of epididymal sperm

The count and morphology of epididymal sperm were analyzed as described previously by Aydogan and Barlas (2006). The left epididymis was cut into caput and cauda epididymis. Weight of the caput epididymis was recorded so as to calculate sperm counts. The caput epididymis was put into 1 ml of 0.9% saline and minced with scissors. One hundred microliters aliquot of the mixture was diluted with 800 μl of 0.9% saline, and then sperm were put into stain for an hour after adding 100 μl of eosin-Y solution to the latter mixture. Sperm head number was counted by using a Neubauer's chamber. The data were expressed as the number of sperm per gram of caput epididymal tissue. With a view to demonstrating the sperm deformities, the left cauda epididymis was put into 1 ml of 0.9% saline and conserved at +4 °C overnight so as to release the sperm into the liquid. The day after, the samples were rinsed gently, and 100 μl aliquots of the samples were diluted with 900 μl of 0.9% saline. The sperm samples were transferred to microscope slide and fixed with alcohol. After fixation, the samples were stained with eosin-Y solution overnight, washed with distilled water, passed through increased alcohol