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Gestational and lactational exposure to di-isobutyl phthalate via diet in maternal mice decreases testosterone levels in male offspring

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HIGHLIGHTS

- Maternal exposure to DiBP decreased offspring serum and testis testosterone levels.
- DiBP decreased expression of 3β-HSD and P450scc in PD21 pups and PD80 adults.
- Pre- and postnatal DiBP exposure impaired adult sperm concentration and motility.

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ABSTRACT

Phthalates are a large family of ubiquitous environmental chemicals suspected of being endocrine disruptors, with exposure to these chemicals during prenatal and postnatal development possibly resulting in reproductive disorders. Di-isobutyl phthalate (DiBP) is widely used in consumer and industrial products, and although its exposure in the general population has increased in recent years, the mechanisms behind DiBP-induced reproductive disorders in male offspring remain unclear. Here, pregnant mice were exposed to 0 or 450 mg/kg bw/day DiBP via diet from gestation day (GD) 0 to GD21. Until postnatal day 21 (PD21), half of the exposed pups were also exposed to DiBP by lactation (TT), while the rest were not (TC). Half of each group were sacrificed on PD21, with the remaining mice fed a normal diet until PD80 (TCC and TTC, respectively). Reproductive toxicological parameters such as relative organ weights and testosterone levels were determined in male offspring on PD21 and PD80 and sperm quality was tested on PD80. Maternal exposure (pregnancy and lactation) led to decreased serum and testis testosterone concentrations, accompanied by decreased expression of 3β-hydroxysteroid dehydrogenase (3β-HSD) and cytochrome P450 family 11 subfamily A member 1 (CYP11A1) in PD21 pups and PD80 adults. Furthermore, the TTC group showed decreased epididymis sperm concentration and motility. Taken together, DiBP exposure in early life (prenatal and postnatal) impaired male reproductive function in later life, possibly by interfering with testosterone levels and CYP11A1, which might be a major steroidogenic enzyme targeted by DiBP or other phthalates.

1. Introduction

Both human and animal reproductive disorders and diseases can begin during prenatal and postnatal development (Kalb et al., 2016). For example, bisphenol A exposure is suggested to have endocrine- and neural-disrupting effects in weanlings, even at very low doses (Kabuto et al., 2004; Doerge et al., 2010; Prins et al., 2011; Kalb et al., 2016). Disruption of the hormone balance might be caused by exposure to endocrine-disrupting chemicals, which are biologically active compounds capable of mimicking or antagonizing the effects of endogenous hormones (Gore, 2010), leading to altered gene expression possibly undetectable until puberty or adulthood.

Members of the large phthalate family are potential endocrine disruptors, and include di-isobutyl phthalate (DiBP), a branched isomer of di-*n*-butyl phthalate (DBP). Due to its heat stability and high volatility, DiBP is often used as a gelling aid together with other plasticizers, or combined with other high molecular phthalates (e.g., *n*-butyl cyclohexyl, cyclohexyl, *n*-butyl-2-ethylhexyl, isooctyl, isodecyl, and di-(butoxyethyl) phthalate) to form nitrocellulose, cellulose ether, and polyacrylate dispersions (ECHA, 2009; U.S. EPA, 2009). It is also used in personal care products

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(e.g., nail polish, cosmetics), adhesives, food packaging, building materials, and medications (ECHA, 2009). Global production of both DBP and DiBP is estimated to be 450,000 tons/year, with 10,000 to 50,000 tons/year of DiBP manufactured and used in Europe (ECHA, 2009).

Phthalates can be released into the environment as they are not covalently bound to plastics. For humans, the primary causes of diester phthalate exposure include ingestion, inhalation, or dermal contact, and following exposure, phthalates are biotransformed into monoester metabolites and discharged via urine (Wittassek et al., 2011). Several studies have used urinary phthalate metabolites as biomarkers of human exposure (Swan, 2005; Marsee et al., 2006; Fromme et al., 2007; Adibi et al., 2008; Wittassek and Angerer, 2008; Hines et al., 2009; Wittassek et al., 2009; Ye et al., 2009; CDC, 2012). Monoisobutyl phthalate (MiBP), a major metabolite of DiBP, has been detected in various biological fluids (e.g., urine, amniotic fluid, breast milk) (Hogberg et al., 2008; Latini et al., 2009). In the last decade, urinary phthalate metabolite levels, including DBP and di(2-ethylhexyl) phthalate (DEHP), of the US population have decreased, whereas exposure to replacement and unregulated phthalates, such as DiBP and di-isononyl phthalate (DiNP), has increased (Zota et al., 2014). To date, MiBP is the second highest urinary phthalate metabolite to be detected in the Chinese population (adult men: geometric mean: 34.2 μg/g creatinine; children: 75.1 μg/g creatinine) (Pan et al., 2015; Wang et al., 2015). Human epidemiological research has indicated that MBP and MiBP are negatively correlated with luteinizing hormone and serum testosterone levels, sperm morphology, as well as sperm acrosin activity (Hauser et al., 2006; Pan et al., 2006; Meeker et al., 2009; Joensen et al., 2012). Furthermore, research on the exposure of pregnant women and newborns has detected DiBP metabolites in maternal urine, cord blood, and breast milk, indicating the potential for DiBP to be transferred to offspring (Fromme et al., 2011; Lin et al., 2011). Adverse health effects, especially anti-androgenic effects on male reproductive development, have been observed in rodents after prenatal exposure to DiBP (Ge et al., 2007). Exposure to DiBP at higher doses (125 mg/kg/day) has also been found to decrease testis, seminal vesicle, and epididymis weights, and increase the incidence of male rat external reproductive tract malformation (Saillenfait et al., 2008), suggesting that DiBP might be a subchronic toxicant. The *in vitro* estrogenic potential of a few phthalate compounds and metabolites have also been reported. The relative potencies of these phthalate compounds are proposed to be butyl benzyl phthalate (BBP) > DBP > DiBP > diethyl phthalate (DEP) > DiNP ((Harris et al., 1997). However, there is no *in vivo* estrogenic potential of DiBP in female rats (Sedha et al., 2015). Taken together, there is increasing evidence of an association between DiBP and adverse health effects such as estrogenic potency and male reproductive malfunction; however, current toxicological data are insufficient and the involved mechanisms remain unclear. Further investigation into the mechanisms behind DiBP-induced reproductive impairment is required.

We hypothesized that DiBP can be transferred to offspring through cord blood and breast milk, disturbing the hormonal balance in the development of organs and increasing offspring vulnerability to the adverse effects of this contaminant. Thus, the effects of gestational and lactational DiBP exposure on testis function in the weanling and adult stages of male mice were investigated. Specific hormone levels in serum and testes were determined. The mRNA and protein levels involved in steroidogenesis were also investigated to evaluate the adverse effects of DiBP on testicular function in offspring at the weanling and adult stages.

2. Materials and methods

2.1. Chemicals

Di-isobutyl phthalate (DiBP, purity 99%) was provided by Sigma-Aldrich (St Louis, MO, USA), TRIZOL from Ambion (Life Technologies, Carlsbad, USA), oligo-$(dT)_{15}$ primer and ribonuclease inhibitor from TaKaRa Bio (Otsu, Japan), M-MLV reverse transcriptase from Promega (Madison, WI, USA), and RIPA buffer from Applygen Technologies Inc. (Beijing, China).

2.2. Animals and treatment

Fifteen individuals of each sex (male and virgin female, ICR mice, 8 weeks old) provided by the Weitong Lihua Experimental Animal Center (Beijing, China) were housed in a mass air displacement room with a controlled environment (12:12 h light:dark cycle, 20–26 °C, and 40–60% relative humidity). After one week of acclimatization, male and female mice cohabitated at a rate of 1:1 at night, and during the day were separated. The indicator for successful mating was a vaginal plug found in the morning, and pregnant mice were caged alone until parturition. In this study, the day a positive vaginal smear was found was defined as gestational day 0 (GD0). Female mice from GD0 to GD21 were administered concentrations of 0 or 2.8 g DiBP/kg diet (dry weight) after a 14-d food intake preliminary experiment, which determined the food intake for one mouse (weight 30 g) to be 4.5–5.0 g per day. Dosed diets were prepared by mixing DiBP into an appropriate amount of feed and the formulated diets were stored frozen. Prior to use, the formulated feed was homogeneously mixed with blank feed using a V-Shell Blender from Beijing HFK Bioscience Co., Ltd. (China). The same method was used to prepare the control diets, except that no DiBP was added. The DiBP dose was chosen according to earlier toxicological research, which showed reduced fetal weight after exposure to 500 mg/kg and higher DiBP doses (Saillenfait et al., 2006). Based on the food intake measured from GD4 to GD16 in the formal experiment, the final DiBP exposure dosage was translated from 2.8 g DiBP/kg diet to 450 mg/kg/day (Fig. S1). On the day of delivery, the mothers exposed to DiBP were divided into two subgroups of equal size. The first group was treated with DiBP for 21 days (pups exposed both prenatally and postnatally, TT group) and the second group received no treatment (pups exposed prenatally only, TC group). In addition, 24–48 h following delivery, the size of each litter was equalized by allocating pups from large litters to smaller litters to ensure each litter had eight pups (n = 8). Half of the male pups in each group were sacrificed at PD21 and the remaining half were fostered with a normal diet (no DiBP exposure) until PD80. This resulted in pups exposed postnatally (TCC group) and pups exposed prenatally and postnatally (TTC group) (Fig. 1). When treatment ended, mice from each group were first weighed, then bled via retro-orbital puncture, and finally sacrificed via cervical dislocation. Following sacrifice, the testes and epididymides were immediately removed and then weighed. One testicle from each mouse was frozen in liquid nitrogen and stored at −80 °C for further use, while the epididymis was immersed in T6 solution until further analysis. To collect the serum, blood was clotted by incubation for 1 h at room temperature, then centrifuged at 5000 rpm for 5 min at room temperature. The collected serum was stored at −80 °C until further use. All animal care and treatment protocols were approved by the Committee on the Ethics of Animal Experiments from the Institute of Zoology, Chinese Academy of Sciences.

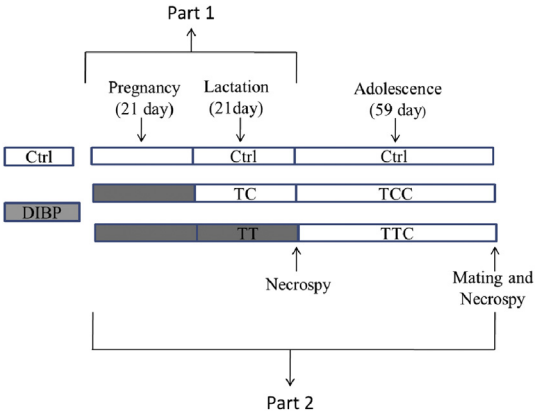

Fig. 1. Brief schematic of the experimental design and sample collection.

2.3. Sperm quality and hormone level analyses

Fresh cauda epididymides from 10 mice (PD80 F1) in each group were weighed and immersed in T6 solution. Subsequently, pricks were made in each cauda using a 1 mL syringe, and sperm were released into the media for 15 min. For sperm count, the sperm suspension was diluted a total of five times using 0.01 M phosphate-buffered saline (PBS), and a hemocytometer under an optical microscope was used to count the sperm. In this paper, the sperm count results are expressed as 10^6 sperm per cauda epididymis. For sperm motility analysis, a computer assisted sperm analysis (CASA) system (Version 12 CEROS, Hamilton Thorne Research, Beverly, MA, USA) was applied, and the following settings were used for cell detection: minimal contrast, 50; minimal cell size, 4 pixels; 60 frames at a frame rate of 60 Hz. For each specimen, no less than 200 tracks were measured at 37 °C with a Slide Warmer (#720230, Hamilton Thorne Research, Beverly, MA, USA) present. After dilution with 0.01 M PBS to a proper concentration, the sperm in the T6 solution were fixed on slides to observe malformation rates.

Serum estradiol, testosterone, and luteinizing hormone, and testis testosterone were analyzed using commercial radioimmunoassay (RIA) kits (Beijing North Institute of Biological Technology, China). Testicular testosterone concentration was normalized to testis protein concentration. The detecting limits for hormone analysis were: testosterone 0.1–20 ng/mL; estradiol 10–1000 pg/mL; luteinizing hormone (LH) 5–200 mIU/mL.

2.4. Real-time PCR and protein expression level analysis

Total RNA of the mice testes (n = 6) were isolated using TRIzol (Life Technologies-Invitrogen, Carlsbad, CA, USA). The isolated RNA purity was measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). Following RNA isolation, complimentary DNA (cDNA) synthesis was carried out, and mRNA levels of selected genes were then analyzed.

Reverse transcription was performed using high purity dNTPs, oligo-$(dT)_{15}$ primer, M-MLV reverse transcriptase, and ribonuclease inhibitor, and incubation at 42 °C for 60 min was followed by 95 °C for 5 min. The Stratagene Mx3000p qPCR system (Agilent, USA) was used to perform real-time PCR, and quantification of gene expression was achieved using the SuperReal PreMix Plus (SYBR green) kit (Tiangen, Beijing, China). Supplementary Material Table S1 shows the designed primers for the mouse genes of interest that were used for qPCR, and in this study the internal control was the housekeeping gene β-actin. The program settings were: 15 min at 95 °C, 40 cycles for 10 s at 95 °C, 20 s at 58 °C, and 30 s at 72 °C. The relative expression ratio of a target gene was calculated according to previous study (Arocho et al., 2006).

Protein extraction from testes frozen at −80 °C was carried out by homogenizing the samples for 5 min in RIPA buffer, with the lysates then centrifuged at 12,000 *g* for 20 min. The protein content was measured using a bicinchoninic acid protein assay kit (Tiangen, Beijing, China). Each sample was run on a 10% SDS-PAGE, then transferred onto a polyvinylidene difluoride (PVDF) transfer membrane (GE Healthcare Bio-Sciences AB, Sweden). The membrane was blocked for 1 h using 5% milk in TBST at room temperature, followed by incubation with primary antibodies for anti-AR (Abcam, ab74272), anti-StAR (Santa Cruz, FL-285), anti-CYP17a (Abcam, ab125022), anti-LHR (Proteintech, 19968-1-AP), anti-CYP11A1 (Millipore, ABS235), and anti-3β-HSD (Abcam, ab150384) overnight at 4 °C. After washing with TBST, the membrane was incubated with an appropriate secondary IgG-HRP antibody. Protein visualization was performed using an Image-Quant LAS 4000 (GE Healthcare Bio-Sciences AB, Sweden).

2.5. Statistical analysis

Statistical analysis was performed using PASW software (version 18.0, IBM, USA). Differences in gene expression levels between two individual groups were determined using two-way ANOVA (factors: age, dosage, and gene/protein expression) followed by Duncan's multiple range test. Differences in body weight, organ weight, and testosterone levels between two individual groups were determined using one-way ANOVA followed by Duncan's multiple range test.

3. Results

3.1. Exposure to DiBP during gestation and lactation and the effect on PD21 F1 male offspring

3.1.1. Effect of DiBP on body and relative organ weights

Maternal weight gain during pregnancy was not affected by exposure to DiBP, and no differences in litter size or fetal viability were observed between DiBP-treated and control groups (data not shown). No differences were observed in testis weight, relative testis weight, and anogenital distance between the control and the gestational exposure group (TC) (Table 1). In addition, no differences were observed in testis weight, relative testis weight, and anogenital distance between the control and the gestational and lactational exposure group (TT) (Table 1). These results show that DiBP did not significantly affect testis weight or anogenital distance in PD21 F1 male offspring, and did not cause disfiguration of

Table 1
Body weight, liver weight, testis weight, and anogenital distance of PD21 male mice offspring. Data are means ± SE (n = 15–18). Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

	Body weight (g)	Liver weight (g)	Testis weight (mg)	Anogenital distance (mm)	Relative liver weight (%)	Relative testis weight (‰)	Relative anogenital distance (mm/g)
Ctrl	17.61 ± 0.25^a	0.785 ± 0.029^a	48.1 ± 1.1^a	11.70 ± 0.18^a	4.43 ± 0.12^a	2.74 ± 0.06^a	0.67 ± 0.01^a
TC	16.89 ± 0.28^a	0.767 ± 0.020^a	44.0 ± 1.4^b	11.60 ± 0.21^a	4.53 ± 0.05^{ab}	2.61 ± 0.09^a	0.69 ± 0.02^a
TT	17.32 ± 0.35^a	0.828 ± 0.026^a	45.3 ± 1.7^{ab}	11.71 ± 0.22^a	4.77 ± 0.07^b	2.62 ± 0.07^a	0.68 ± 0.01^a

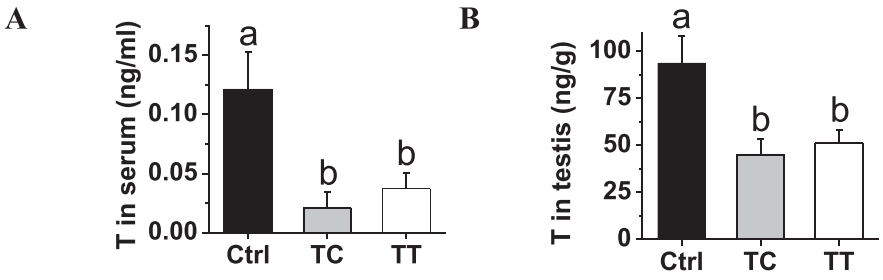

Fig. 2. Concentration of testosterone (T) in serum (A) or testes (B) of PD21 F1 male mice. Results are means ± SE (n = 10). Ctrl group: unexposed control, TC group: pups exposed prenatally, TT group: pups exposed both prenatally and postnatally. Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

external or internal genitalia.

3.1.2. Effect of DiBP on serum and testicular testosterone levels in mice

Maternal exposure to DiBP caused a significant reduction of testosterone levels in the serum and testes of PD21 F1 male offspring (Fig. 2). Furthermore, both serum and testis testosterone levels showed a slight, though non-significant decrease in the TC group compared with the TT group. As for serum estradiol and luteinizing hormone levels, no significant differences were found among the three groups (Fig. S1). These results indicate that exposure to DiBP during the gestational period was the main cause of testosterone decrease in PD21 F1 male offspring.

3.1.3. Effect of DiBP on transcriptional and protein levels of genes involved in testosterone synthesis in the testis

Quantitative PCR showed that DiBP exposure significantly affected the expression levels of testosterone synthesis related genes in the testis tissue of PD21 F1 male offspring, with *Cyp11a1*, *Cyp17a1*, and *3β-HSD* in the TT group and *Cyp11a1* in the TC group significantly downregulated compared with that in the control (Fig. 3A).

DiBP affected the amount of proteins in the relationship with testosterone function, with the levels of CYP11A1 and 3β-HSD both significantly lower than those in the control group, as obtained by Western blot analysis (Fig. 3B).

3.2. Exposure to DiBP during gestation and lactation and the effect on adult (PD80) F1 male offspring

3.2.1. Effect of DiBP on body and relative organ weight

Compared with the control, the liver, relative liver, testis, and relative testis weights of the gestational exposure group (TCC) were significantly higher (Table 2). In addition, the liver, relative liver,

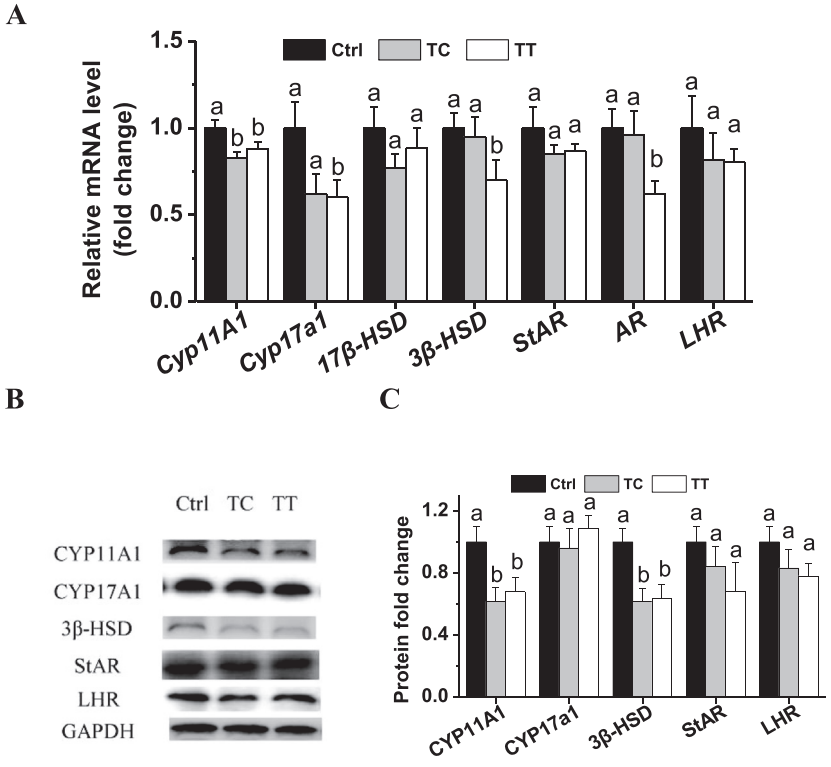

Fig. 3. Expressions of testosterone synthesis and related genes in testes from PD21 F1 mice. Quantitative real-time PCR analysis (A). Western blot analysis of testosterone synthesis and related proteins in testes, representative Western blots are shown in the left panels (B), and results from densitometry analysis are shown in the right panels (C). Data are means ± SE (n = 6). Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

and testis weights of the gestational and lactational exposure group (TTC) were significantly higher than those of the control (Table 2), although relative testis weight was not significantly different (Table 2).

3.2.2. Effect of DiBP on mice serum and testicular testosterone level

Compared with the control group, significant reductions in both serum and testicular testosterone levels in the TTC group were observed (Fig. 4). In addition, serum and testis testosterone levels were also decreased in the TCC group, though not significantly (Fig. 4).

3.2.3. Effect of DiBP on sperm quality

Analysis of adult (PD80) F1 male mice sperm quality showed significantly lower sperm concentration and motility in the TTC group compared with the control group, whereas there were no significant differences in the TCC group (Fig. 5). Moreover, spermatozoa smears showed no significant differences in the sperm malformation rates among the three groups (data not shown).

3.2.4. Effect of DiBP on transcriptional and protein levels of genes related to testosterone function in the testis

DiBP exposure significantly affected the expressions of genes involved in testosterone synthesis, steroid transportation, and hormone receptor synthesis in the testis tissue of adult (PD80) F1 male offspring. In the TCC group, *androgen receptor* (*AR*), *Cyp11a1*, *Cyp17a1*, *17β-HSD*, *3β-HSD*, and *luteinizing hormone receptor* (*LHR*) were all significantly downregulated (Fig. 6A). This was also observed in the TTC group, except for *Cyp17a1*, which did not show significant alteration (Fig. 6A). Compared with the PD21 groups, no significant differences were observed (Table S1).

Through extraction of testis tissue proteins from adult (PD80) F1 male offspring, followed by Western blot analysis, DiBP was found to affect testosterone synthesis and function related protein levels. CYP11A1 levels in the TCC and TTC groups were significantly lower compared with those in the control; 3β-HSD and AR levels were significantly lower; and LHR levels were significantly higher in the TTC group than those in the control (Fig. 6B). In addition, the expression proteins in these groups showed no significant differences when compared with those of the PD21 groups (Table S1).

4. Discussion

Recognizing that developing organisms are uniquely sensitive to perturbation by environmental pollutants with endocrine disrupting activity, this study aimed to determine if exposure to DiBP during critical developmental windows could alter hormone programming of the reproductive organs and functions in adult life.

Phthalates exhibit xenoestrogenic activity and are considered endocrine disruptors (Schug et al., 2011). Toxicological studies on model animals have demonstrated that some phthalates are testicular toxicants that can cause reduced Leydig cell steroid hormone biosynthesis (Akingbemi et al., 2001; Fisher et al., 2003).

Testosterone plays an important role in germ cell survival and development (Walker, 2009). Phthalate metabolites can reduce testis testosterone secretion, which, in turn, can cause spermatogenesis dysfunction because the adult testis requires a relatively high testosterone concentration to maintain and restore spermatogenesis (Barreto et al., 2007). In the current study, impaired semen quality, defined by decreased sperm concentration and motility, of adult male offspring was observed after maternal exposure to DiBP during the gestational and lactational stages, indicating that DiBP might disrupt Leydig cell testosterone production, ultimately leading to decreased sperm quality in the epididymis via reduced sperm motility.

Previous research has reported reduced anogenital distance in fetal rat testes exposed to 600 mg DiBP/kg bw/day from GD7 to GD20/21 (Borch et al., 2006). In the present study, however, maternal exposure to DiBP did not result in significant differences in the relative testis weights or anogenital distances or in the external or internal genitalia of PD21 male offspring. Reductions in testicular testosterone content, accompanied with declines in the protein levels of Cyp11a1 reported previously (Borch et al., 2006), were observed in our study. The significant decreases in serum and testicular testosterone levels occurred in both the gestational only (TC) and gestational and lactational exposure (TT) groups, implying that exposure to DiBP during gestation might be a key factor leading to decreased testosterone levels, and could be enhanced by lactational exposure. A similar decreasing trend in testosterone levels was observed in the PD80 TCC and TTC groups, which were not exposed to DiBP after PD21, indicating an unrecoverable injury to testosterone biosynthesis induced by DiBP exposure during gestation and lactation. The reduction in testosterone levels was also accompanied by decreased sperm concentration and motility in the TTC group. These short-term and long-lasting effects of DiBP on testosterone biosynthesis are consistent with those of DEHP and DBP on pregnant rats (Jiang et al., 2007; Culty et al., 2008). In the testes, testosterone is essential for the survival of germ cells as well as sperm development (Walker, 2009). It is synthesized from cholesterol as a substrate in Leydig cells (Payne and Hales, 2004). The scavenger receptor B1 (SR-B1) pathway facilitates the uptake of cholesterol from HDL as the predominant source of cholesterol for adrenal steroidogenesis (Martinez-Arguelles and Papadopoulos, 2015). After this, cholesterol is imported into the mitochondria, which is the rate-limiting step in steroid biosynthesis (Rone et al., 2009; Martinez-Arguelles and Papadopoulos, 2015). It has been reported that DEHP/MEHP might be associated with the storage and import of cholesterol after acute exposure (Martinez-Arguelles and Papadopoulos, 2015). In our present study, however, the cholesterol levels in the testes were not significantly increased in the two PD21 groups or two PD80 groups compared with each control group (data not shown). StAR plays an important role in the transportation of cholesterol from the outer mitochondria to the inner mitochondrial membrane in Leydig cells (Manna and Stocco, 2005). In the DiBP treatment group, StAR mRNA and protein levels were lower than those found in the control, though with no statistical significance. Similar observations have been noted in earlier research, in which exposure to DBP was reported to decrease testosterone biosynthesis and reduce the levels of key proteins controlling cholesterol transport (SR-B1 and StAR) (Borch et al.,

Table 2
Body weight, liver weight, and testis weight of adult (PD80) F1 mice. Data are means ± SE (n = 15–17). Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

	Body weight (g)	Liver weight (g)	Testis weight (mg)	Epididymis weight (mg)	Relative liver weight (%)	Relative testis weight (%)	Relative epididymis weight (‰)
Ctrl	38.37 ± 0.82^{a}	1.66 ± 0.07^{a}	119.5 ± 3.4^{a}	31.0 ± 0.6^{a}	4.32 ± 0.11^{a}	0.31 ± 0.01^{a}	0.8 ± 0.02^{a}
TCC	41.10 ± 0.89^{b}	1.90 ± 0.04^{b}	152.0 ± 3.0^{b}	32.8 ± 1.5^{a}	4.63 ± 0.09^{b}	0.37 ± 0.01^{b}	0.8 ± 0.04^{a}
TTC	40.22 ± 0.71^{ab}	1.86 ± 0.03^{b}	134.0 ± 4.9^{b}	30.0 ± 1.2^{a}	4.63 ± 0.12^{b}	0.33 ± 0.01^{a}	0.7 ± 0.03^{a}

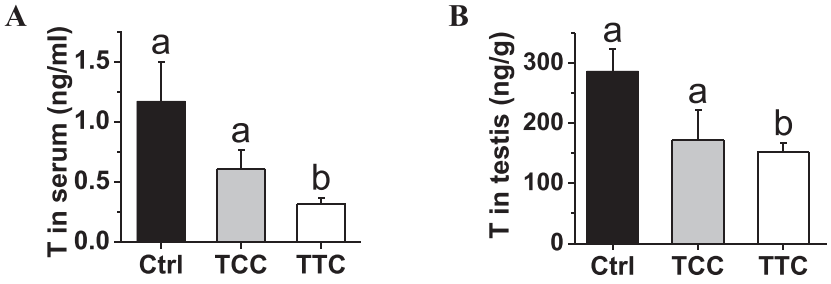

Fig. 4. Concentration of testosterone (T) in serum (A) or testes (B) of PD80 F1 male mice. Results are means ± SE (n = 10). Ctrl group: unexposed control, TCC group: pups exposed prenatally, TTC group: pups exposed both prenatally and postnatally. Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

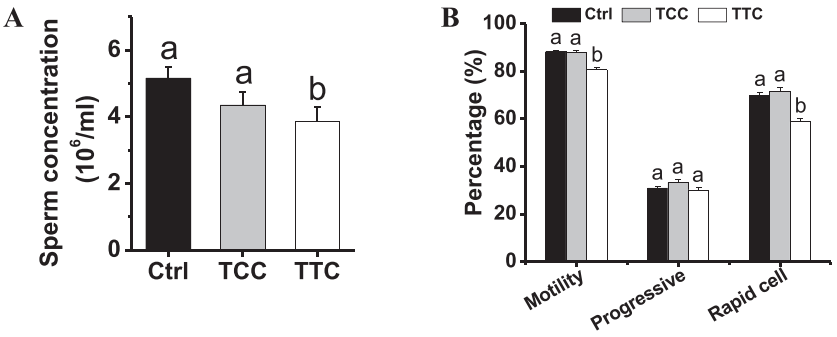

Fig. 5. Sperm concentration (A) and motility (B) of PD80 F1 male mice. Results are means ± SE (n = 10). Ctrl group: unexposed control, TCC group: pups exposed prenatally, TTC group: pups exposed both prenatally and postnatally. Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

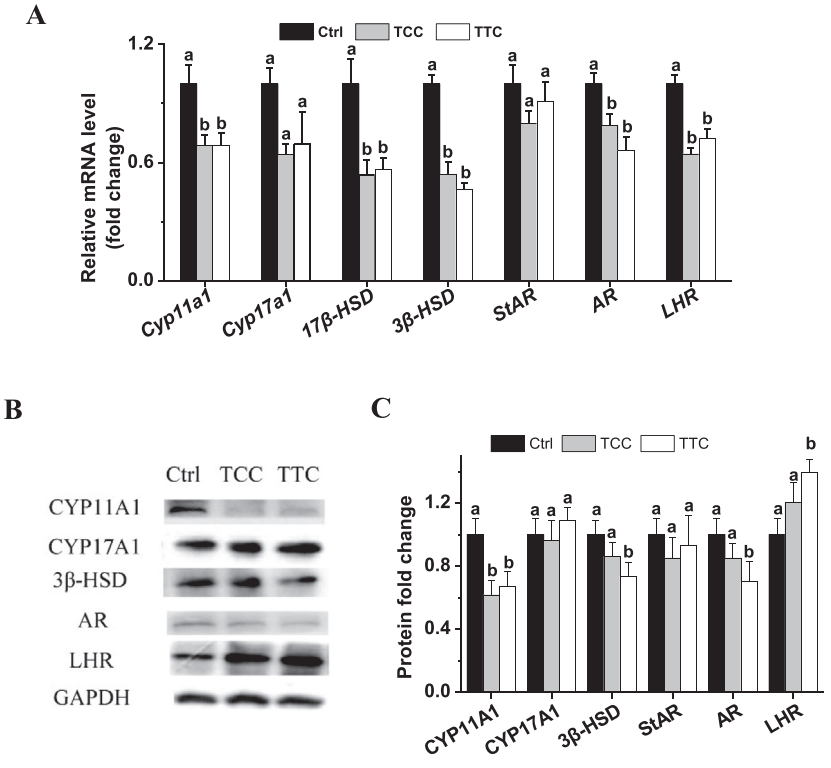

Fig. 6. Expressions of testosterone synthesis and related genes in testes from PD80 F1 mice. Quantitative real-time PCR analysis (A). Western blot analysis of testosterone synthesis and related proteins in testes, representative Western blots are shown in the left panels (B), and results from densitometry analysis are shown in the right panels (C). Data are means ± SE (n = 6). Ctrl group: unexposed control, TCC group: pups exposed prenatally, TTC group: pups exposed both prenatally and postnatally. Values not sharing a common superscript differ significantly at $p < 0.05$ by ANOVA and Duncan's multiple range tests.

2006). In addition, decreases in StAR mRNA levels have also been observed in rats exposed to DEHP, DINP, DIBP, and DIHP *in utero* (Hannas et al., 2011), suggesting interference effects of DiBP on cholesterol transportation, and possible disturbance of testosterone biosynthesis.

Cholesterol is converted to pregnenolone by Cyp11a1 in the mitochondria. Like cholesterol import, this conversion is also a rate-limiting step in steroid hormone synthesis (Hanukoglu, 1992). Studies on rats exposed to DEHP, DINP, DIBP, and DIHP have reported significant decreases in the gene expressions of Cyp11a1 and Cyp17a1 (only DEHP) (Culty et al., 2008; Hannas et al., 2011). After *in utero* exposure to DEHP, rats aged PND60 have shown a significant decrease in 3β-HSD gene expression (Culty et al., 2008). In the current study, the gene and protein expression levels of Cyp11A1 and 3β-HSD related to testosterone synthesis were significantly lower at the weanling stage (PD21) following maternal exposure to DiBP than they were in the control group. The decrease in Cyp11A1 and 3β-HSD observed at the adult stage (PD80) indicated the persistent effects of uterine and lactational DiBP exposure. Decreased testicular steroid production in the DiBP-treated groups suggests that the steroidogenesis function of the Leydig cells in the weanling male mice was disturbed by DiBP. These effects on testosterone biosynthesis remained after adolescence, even though the TCC and TTC groups were not exposed to DiBP after PD21.

The hypothalamic-pituitary-testicular axis strictly regulates testosterone production via negative feed-back control and the primary luteinizing hormone acting on steroidogenic enzymes and steroid transport proteins (Dufau, 1998; Lei et al., 2001). For example, StAR expression can be stimulated via luteinizing hormone (LH) binding to LHR and the cAMP-dependent pathway (Dufau, 1998; Zirkin and Chen, 2000). In this study, no obvious alternations in serum LH levels or LHR mRNA expression were observed. For the development of Leydig cells, Gestational Day 20 is a pivotal age in which cells will express LH receptors and become LH sensitive (Habert et al., 2001; Culty et al., 2008). For this reason, the gene expression levels of LHR in the PD21 TC and PD80 TCC groups decreased in the present study, as did other receptors and enzymes essential for testosterone production. Surprisingly, though the gene expression of LHR decreased significantly in the PD80 DiBP groups, the protein levels of LHR in the testes increased, which might reflect an increased number of Leydig cells in adult testes exposed *in utero* to DiBP rather than increased gene expression in individual Leydig cells; however, this remains uncertain. Moreover, with the decrease in testosterone, there might be a negative regulation to increase the LHR protein level.

Cholesterol is a substrate for testosterone biosynthesis. The endoplasmic reticulum of Leydig cells can employ different cholesterol sources for steroidogenesis and *de novo* synthesis, including cholesterol esters (stored within lipid droplets), circulating HDL via scavenger receptor B1 (SR-B1), and circulating LDL via receptor-mediated endocytosis. Most cholesterol for steroidogenesis in rodents is provided via HDL-derived selective uptake of cholesteryl esters through SR-B1. In the DiBP treatment group, StAR mRNA and protein levels were lower than those in the control. Similar observations were noted in earlier research, in which exposure to DBP decreased testosterone biosynthesis and reduced the levels of key proteins controlling cholesterol transport (SR-B1 and StAR) (Borch et al., 2006).

Testosterone signaling targets Sertoli cells. In normal spermatogenesis, Sertoli cell AR is critical, as in its absence mice spermatogenesis cannot progress beyond the pachytene or diplotene stages of meiosis (De Gendt et al., 2004; Tsai et al., 2006), and maintenance of junctional complex integrity, a key factor determining the strength of the blood-testis barrier, cannot be carried out (Meng et al., 2005; Wang et al., 2006). In this experiment, AR at the mRNA level was decreased in both the PD21 and PD80 groups compared with each control, consistent with the decrease in testosterone in both serum and testes. These decreases were not affected by age, suggesting persistent effects on testicular toxicity of DiBP exposure.

Recently, the EU banned the use of DEHP, DBP, and BBP in toys due to their reproductive toxicity, particularly their ability to cause atrophy or agenesis in the testis and epididymis and reduce semen quality in adult life. These detrimental reproductive effects might also be induced by DiBP exposure. The current study aimed to explore whether the effects of DiBP on testosterone synthesis in weanling and adult stages were comparable to the effects of DBP and other phthalates. Thus, only a single, relatively high DiBP dose was applied, with no-observed-adverse-effect-level (NOAEL) provided. However, the present study provides new evidence regarding DiBP reproductive toxicity in weanling and adult stages and highlights possible concern in regards to DiBP use as a substitute for DBP. Additional developmental, especially postnatal, studies are required for identification of possible reproductive effects of DiBP.

In summary, DiBP exposure during early life (prenatal and postnatal) resulted in significantly lower sperm concentration and motility in the epididymis of mice in later life. The low semen quality might be due to testosterone synthesis disruption, accompanied by downregulation of genes and proteins involved in testosterone synthesis, and thus Leydig cells might be an important target of DiBP and other phthalates. These results provide additional support for the hypothesis that DiBP can interfere with the hormone balance necessary for normal development.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.01.011.

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