Dietary exposure to di-isobutyl phthalate increases urinary 5-methyl-2′-deoxycytidine level and affects reproductive function in adult male mice

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Abstract

Phthalates are a large family of ubiquitous environmental pollutants suspected of being endocrine disruptors. Epidemiological studies have associated phthalate metabolites with decreased reproductive parameters and linked phthalate exposure with the level of urinary 5-methyl-2′-deoxycytidine (5mdC, a product of methylated DNA). In this study, adult male mice were exposed to 450 mg di-isobutyl phthalate (DiBP)/(kg·day) via dietary exposure for 28 days. Mono-isobutyl phthalate (MiBP, the urinary metabolite) and reproductive function parameters were determined. The levels of 5mdC and 5-hydroxymethyl-2′-deoxycytidine (5hmdC) were measured in urine to evaluate if their contents were also altered by DiBP exposure in this animal model. Results showed that DiBP exposure led to a significant increase in the urinary 5mdC level and significant decreases in sperm concentration and motility in the epididymis, accompanied with reduced testosterone levels and downregulation of the P450 cholesterol side-chain cleavage enzyme (P450scc) gene in the mice testes. Our findings indicated that exposure to DiBP increased the urinary 5mdC levels, which supported our recent epidemiological study about the associations of urinary 5mdC with phthalate exposure in the male human population. In addition, DiBP exposure impaired male reproductive function, possibly by disturbing testosterone levels; P450scc might be a major steroidogenic enzyme targeted by DiBP or other phthalates.

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Introduction

Di-isobutyl phthalate (DiBP), a branched isomer of di-n-butyl phthalate (DBP), belongs to a large family of phthalates. Because of its heat stability and low volatility, it is often used as a gelling aid together with other plasticizers, or combined with other high molecular weight phthalates (e.g., n-butyl cyclohexyl, cyclohexyl, n-butyl-2-ethylhexyl, isoocetyl, isodecyl, and di-(butoxyethyl) phthalate) for nitrocellulose, cellulose ether, and polyacrylate dispersions (ECHA, 2009; US EPA, 2009). It is also used in personal care products (e.g., nail polish, cosmetics), adhesives, food packaging, building materials, and medications (ECHA, 2009). Global production of DBP and DiBP has been estimated to be at 450,000 tons/year, with the quantity of DiBP manufactured and used in Europe in the range of 10,000 to 50,000 tons/year (ECHA, 2009). The information regarding the

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annual production and usage in China is scarce, but the environmental occurrence of DiBP has been widely detected in Chinese water (mean 2.83 μg/L; Li et al., 2017), sediments (0.601 μg/g; Kang et al., 2016), and house dust (27.3 μg/g; Li et al., 2016). In Lake Chaohu, the fifth largest freshwater lake in China, DiBP has become one of the dominant phthalates, and its level has increased year by year, suggesting an increasing usage of DiBP in Eastern China (Kang et al., 2016).

Humans may be exposed to phthalates via ingestion, dermal contact, and inhalation. In vivo, diester phthalates can be metabolized into monoester metabolites and excreted in urine (Wittassek et al., 2011). Many studies have used urinary metabolites of phthalates as biomarkers of human exposure (Adibi et al., 2008; CDC, 2012; Fromme et al., 2007; Hines et al., 2009; Marsee et al., 2006; Swan, 2005; Wittassek and Angerer, 2008; Wittassek et al., 2009; Ye et al., 2009). Mono-isobutyl phthalate (MiBP), a major metabolite of DiBP, has been detected in various biological fluids (e.g., urine, amniotic fluid, and breast milk) (Hogberg et al., 2008; Latini et al., 2009). Over the last decade, the levels of urinary phthalate metabolites, including di(2-ethylhexyl) phthalate (DEHP) and DBP, have decreased, whereas exposure to replacement and unregulated phthalates, such as DiBP and di-isononyl phthalate (DiNP), has increased in the US population (Zota et al., 2014). In China, MiBP was found to be the second highest detected urinary phthalate metabolite (geometric mean: 34.2 μg/g creatinine in adult men (Pan et al., 2015); 75.1 μg/g creatinine in children (Wang et al., 2015)). Epidemiological studies have suggested that MI and MiBP are inversely associated with sex hormone levels (e.g., testosterone and luteinizing hormone) and semen parameters, including sperm concentration, morphology, and acrosin activity (Hauser et al., 2006; Joensen et al., 2012; Meeker et al., 2009; Pan et al., 2006). In rodents, after prenatal exposure to DBP, anti-androgenic effects have been observed, including reduced testis, epididymis, and seminal vesicle weight, and increased malformation, suggesting DiBP might be a subchronic toxicant (Ge et al., 2007; Saillenfait et al., 2008). DiBP and its metabolites may also exhibit potential in vitro estrogenic effects. The relative estrogenic potencies of phthalates have been reported in descending order as: butyl benzyl phthalate (BBP) > DBP > DiBP > diethyl phthalate (DEP) > DiNP (Harris et al., 1997).

Taken together, there is increasing evidence of an association between DiBP and adverse health effects such as estrogenic activity, embryonic development, and sematic cell reprogramming (Gao et al., 2013; Ito et al., 2010; Koh et al., 2011).

Studies have discovered that exposure to environmental chemicals, such as phthalates, can alter the extent of methylation and hydroxymethylation in the genome or certain genes of mammals (Dolinoy et al., 2007; Tellez-Plaza et al., 2014). Animal research has also shown that phthalate exposure might induce aberrant methylation of certain genes, and that alterations in DNA methylation might contribute to the testicular toxicity of phthalates (Prados et al., 2015; Wu et al., 2019). Epidemiological studies suggest that DNA methylation in specific genes of the human placenta as well as in repetitive DNA sequences is associated with phthalate exposure (LaRocca et al., 2014; Zhao et al., 2015). Recently, measurements of methylated (5mdC) and hydroxymethylated deoxynucleosides (5hmdC) in urine were established (Hu et al., 2012; Yin et al., 2015). Several biological processes (e.g., apoptosis) are responsible for DNA degradation to single deoxynucleosides (Nagata et al., 2003). In addition, the process of DNA demethylation involves cellular excision repair (Zhu, 2009). Earlier studies suggest that the resulting products of DNA degradation or excision repair could be excreted into urine (Hu et al., 2012; Yin et al., 2015). Recently, we reported on the contents of phthalate metabolites, 5mdC, and 5hmdC in urine as well as semen quality in 562 Chinese adult men (Pan et al., 2016). The results showed that urinary 5mdC and 5hmdC were positively associated with phthalate metabolite levels, and higher contents of 5mdC and/or 5hmdC in urine were associated with below-reference semen quality. Thus, we conducted an animal exposure study to gain a better understanding of the associations among the levels of phthalate metabolites, 5mdC, 5hmdC, and semen quality.

Based on the wide usage, production, and reported subchronic toxicity, DiBP was selected to test in vivo reproductive toxicity using a mouse model. The present study aimed (1) to evaluate testicular function after 28 day of DiBP exposure and (2) to assess any association between DiBP and urinary 5mdC and 5hmdC levels following exposure by measuring urinary phthalate metabolites, deoxycytidine (dC), 5mdC, and 5hmdC.

1. Materials and methods

1.1. Chemicals

We purchased DiBP (CAS No. 84-69-5, purity 99%) from Sigma-Aldrich (St Louis, MO, USA). β-Glucuronidase (Escherichia coli K12) was obtained from Roche Biomedical (Mannheim, Germany). 2′-Deoxyctydine (dC), 5mdC, 5hmdC, and their isotope-labeled internal standards 2′-deoxyctydine-13C,15N2, 5mdC-13C,15N2, 5-methyl-2′-deoxyctydine-d3 (5mdC-d3), and 5-hydroxymethyl-2′-deoxyctydine-d3 (5hmdC-d3) were purchased from Toronto Chemical Research (Toronto, Canada). The native standard of MiBP was obtained from Cambridge Isotope Laboratories (Andover, MA, USA). The d4 mass-labeled internal standard of MiBP was purchased from Institute for Dünnschichttechnologie and Mikrosensorik (Teltow, Germany). TRIzol reagent was purchased from Ambion (Life Technologies, Carlsbad, USA). Oligo-(dT)15 primer and ribonuclease inhibitor were purchased from TaKaRa Bio (Otsu, Japan). Moloney murine
leukemia virus reverse transcriptase (M-MLV-RT) was purchased from Promega (Madison, WI, USA). The radioimmuno- precipitation assay (RIPA) buffer was purchased from Applygen Technologies Inc. (Beijing, China).

1.2. Animal treatment

Male ICR (Institute of Cancer Research) mice (age 6–8 weeks) were obtained from the Weitong Lihua Experimental Animal Center (Beijing, China). Mice were housed in an environmentally controlled (12:12 hr light:dark cycle, 20–26°C, and 40%–60% relative humidity) mass air displacement room. Food and water were provided ad libitum throughout the study. After one week of acclimatization, 40 mice were randomly divided into two groups of equal size and administered either 0 or 2.8 g DiBP/(kg diet) (dry weight) for 28 days. The dosed diets were prepared by mixing DiBP into food, which was then stored frozen. The dosed feed was then mixed homogeneously with blank feed in a V-Shell Blender by Beijing HFK Bioscience Co., Ltd. Control diets without DiBP were prepared in the same manner. Once every four days, mouse body weight was recorded, and the food intake was estimated by removing feces carefully and re-weighing the cage with food residue. The daily DiBP intake was calculated as follows: DiBP daily intake (mg/(kg·day)) = DiBP concentration in diet (g/kg) × food intake (g/day) / mouse body weight (g). Fresh urine was immediately collected with a 1.5 mL Eppendorf tube and stored at −80°C until further use, whereas matrix recovery was validated by spiking 10 ng of MiBP standard to-noise ratio greater than 10, and was 0.2 ng/mL for MiBP.

1.5. Measurements of mice urinary deoxynucleosides

The contents of de, SmdC, and ShmdC in urine were analyzed following previous studies (Pan et al., 2016; Yin et al., 2015). In brief, thawed urine was centrifuged at 5000 r/min for 5 min at 37°C, with the supernatant (200 μL) then spiked with mass-labeled internal standards, then extracted using Bond Elut PCX SPE cartridges (3 mL, 60 mg, Agilent Technologies, USA). The contents of de, SmdC, and ShmdC were measured using the same instrument and chromatographic column mentioned above. 2.0 mmol/L NH4HCO3 in water (A) and methanol (B) were used as mobile phases. The MRM transitions were as follows: 228 → 112 for de, 242 → 126 for SmdC, 258 → 142 for ShmdC, 231 → 115 for de-C15N2, 245 → 129 for SmdC-d3, and 261 → 145 for ShmdC-d3. The LOQs were 0.2 nmol/L for both de and SmdC and 0.1 nmol/L for ShmdC. Matrix recoveries were all >94%. The inter-day precisions were all <8% (Pan et al., 2016).

1.6. Real-time polymerase chain reaction (PCR) and western blot analysis

Total ribonucleic acid (RNA) was isolated from testes collected from 6 individuals in each group (n = 6) using TRIzol solvent. Real-time PCR was performed as described previously (Wang et al., 2017). Mice-specific primers were designed for the genes of interest and are given in Appendix A, Table S2.
expression ratios (R) of target genes were calculated based on previous study (Arocho et al., 2006).

Testis protein was extracted by homogenization in RIPA buffer followed by centrifugation at 12,000 × g for 20 min at 4°C. After the determination of protein concentration using the bicinchoninic acid (BCA) protein assay kit (Tiangen, Beijing, China), Western blot analysis was performed. The primary antibodies were as follows: anti-androgen receptor (AR) (Abcam, ab74272), anti-steroidogenic acute regulatory protein (StAR) (Santa Cruz, FL-285), anti-cytochrome P450 cholesterol side-chain cleavage enzyme (P450scc) (Millipore, ABS235), anti-cytochrome P450 family 17 subfamily A (CYP17a) (Abcam, ab125022), and anti-3β-hydroxysteroid dehydrogenase (HSD) (Abcam, ab150384).

1.7. Statistical analysis

Statistical analysis was conducted with PASW software 18.0 (IBM, USA). The Shapiro–Wilk test was conducted to test the normality of data. Differences between two groups were determined by Student’s t-tests; the significance level was set at 0.05.

2. Results

2.1. Effect of DiBP on body and organ weights

During an exposure of 28 day, the body weight of ICR mice in the treatment group increased from 34.8 to 40.8 g. However, no significant difference was observed compared to that in the control group (Fig. 1a). The absolute and relative weights of testes and epididymides are listed in Appendix A, Table S1; no significant differences were observed between the treatment and control groups.

2.2. MiBP content in urine

The food intake per day during the exposure period is presented in Fig. 1b. Based on the values of food intake and the body weight, DiBP daily intake was estimated, and the value was basically stable at a mean level of 450 mg/(kg·day) (Fig. 1c), comparable to that of an earlier toxicological study (Saillenfait et al., 2006). The average concentrations of MiBP in the urine were found to be 0.14 and 639 μg/mL in the control and treatment groups, respectively (Fig. 1d). The results from the control group also showed the ubiquitous occurrence of phthalate in the environment.

2.3. Urinary dC, 5mdC, and 5hmdC contents

Urinary dC, 5mdC, and 5hmdC concentrations in the control and DiBP treatment groups are given in Fig. 2. In the control group, urinary dC, 5mdC, and 5hmdC levels from day 3 to day 27 were relatively stable (mean levels: 0.82, 1.08, and 0.58 μmol/L, respectively); no significant differences were observed. In contrast, the levels of urinary dC and 5mdC in the treatment group increased significantly during the exposure period by 89% and 75%, respectively, compared with the first day of

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Fig. 1 – The body weight (a), daily food intake (b), di-isobutyl phthalate (DiBP) daily intake (c), and urinary mono-isobutyl phthalate (MiBP) level (d) for adult male ICR (Institute of Cancer Research) mice during DiBP exposure for 28 day via food (n = 3). Values are means ± standard error (SE). Ctrl: control.
measurement (day 3, \( p < 0.05 \)); and by 66% and 67%, respectively, on day 27 compared with the control group on the same day (\( p < 0.05 \)). Additionally, the content of urinary ShmdC on day 27 increased by 42%, with marginal significance (\( p = 0.08 \)) compared with the control group.

2.4. Sperm quality and hormone levels in serum and testis

Compared with the control group, epididymal sperm concentration and sperm motility and progressiveness were significantly lower in the DiBP treatment group (Fig. 3a and b), whereas the sperm malformation rate was significantly elevated over that in the control group (Fig. 3c). The hormone contents in mice serum and testes are given in Fig. 4; levels of serum FSH, serum testosterone, and testicular testosterone in the DiBP-treated group were significantly decreased compared with those in the control (Fig. 4a, c and e), whereas the levels of serum E2 and LH between the DiBP-treated and control groups showed no significant differences (Fig. 4b and d).

2.5. Expression level of genes involved in steroidogenesis

The transcriptional levels of genes related to steroidogenesis in mice after DiBP treatment were analyzed (Fig. 5). Compared with the control, the levels of steroidogenic acute regulatory protein (StAR) and P450scc were significantly lower in the DiBP treatment group. In addition, compared with the control group, significantly lower mRNA levels were observed for AR, 3β-HSD, and CYP17a in the DiBP treatment group. However, no significant differences in the expression of scavenger receptor class B member 1 (SR-B1), follicle stimulating hormone receptor (FSHR), and estrogen receptor beta (ERβ) (Fig. S2) were observed between the DiBP-treated and control groups.

The protein levels of genes related to steroidogenesis (StAR, P450scc, and 3β-HSD) were markedly lower in the DiBP treatment group (Fig. 6). However, the protein levels of CYP17a and AR exhibited no significant differences between the control and DiBP treatment groups.

3. Discussion

Phthalates are currently suspected of being endocrine disruptors (Schug et al., 2011). Exposure of the US general population to unregulated DiBP and DiNP has increased over the last decade due to their substitution for DBP and DEHP (Zota et al., 2014). DiBP was shown to be the second highest phthalate in dietary intake, and consequently, the urinary level of MiBP was the second highest of phthalates detected in Chinese populations (e.g., adult males, children) (Pan et al., 2015; Wang et al., 2015). Animal studies have indicated that some phthalates can reduce steroid hormone biosynthesis in Leydig cells. Phthalates with different side-chains might disrupt the synthesis of hormones via different mechanisms (Akingbemi et al., 2001; Fisher et al., 2003). Our previous study indicated that the contents of MBP and MiBP in urine are...
negatively associated with the levels of testosterone in Chinese adult men (Pan et al., 2015). In addition, acrosin activity, sperm motility, and semen morphology are also negatively associated with MBP and MiBP (Pan et al., 2015). However, the biological mechanism for the effects of phthalate exposure on sperm quality remains unclear.

In the present study, DiBP was selected to test in vivo reproductive toxicity using the mouse model. This is the first report on the content of 5mdC and 5hmC in mice urine following DiBP exposure. The contents of 5mC and 5hmC in urine were both significantly increased with DiBP exposure, consistent with the results of our earlier epidemiological study on the association between urinary 5mdC and 5hmC content with phthalate exposure in the male population (Pan et al., 2016). The present study provides evidence for the effect of environmental contaminants on the contents of urinary 5mdC and 5hmC in an animal model.

The molar percentage ratios of 5mdC/dC and 5hmC/dC in genomic DNA (% 5mdC and % 5hmC) are considered useful biomarkers for evaluating global DNA methylation and hydroxymethylation levels (Tellez-Plaza et al., 2014). It is important to investigate the ratios in urine and how these ratios are correlated in the genome. We could not know what the pattern was like without the detection of the non-methylated deoxycytidine. Therefore, we first measured urinary dC in adult male mice to obtain the ratios of % 5mdC and % 5hmC. The level of dC (mean value 0.82 μmol/L in control group) in urine was rather low (even lower than urinary 5mdC; mean 1.08 μmol/L in the control group). The levels of 5mC and 5hmC are known to be much lower than that of the precursor cytosine in mammalian tissues, with 5mC accounting for 2%–8% of cytosine (Zhu, 2009) and 5hmC accounting for 0.7%–16.3% of 5mC in mice (Globisch et al., 2010; Rusyn et al., 2001; Wu and Zhang, 2011). Compared with these tissue values, the % 5mdC and % 5hmC ratios in the urine of control mice (132% and 71%, respectively) were very high, though no significant differences in % 5mdC or % 5hmC were found between the control and treatment groups. Thus, it is unlikely that urinary % 5mdC or % 5hmC were correlated with global DNA methylation or hydroxymethylation levels. The reasons for the higher ratios of 5mdC and 5hmC in urine than in the genome are unknown. One possible explanation is that deoxynucleosides in urine appear as metabolic byproducts of genomic DNA, and turnover of modified cytosine (5mC and 5hmC) is more rapid than cytosine in genomic DNA (Yin et al., 2015).

In this study, elevated levels of dC, 5mC, and 5hmC were observed in the DiBP treatment group (Fig. 2). However, the detailed mechanism for the disrupted metabolism of urinary deoxynucleotides after DiBP treatment is elusive. A previous study reported on increased production of reactive oxygen species after phthalate exposure, and induced oxidative stress causing DNA lesions or apoptosis, leading to high levels of DNA degradation (Tetz et al., 2013). Thus, we speculate that DiBP treatment increased the release of excessive products (dC, 5mC, and 5hmC) into urine by inducing oxidative stress.

A relatively high level of testis testosterone is required for the maintenance and restoration of spermatogenesis. Phthalate metabolites reportedly reduce the secreted content of testosterone in the testes, resulting in spermatogenesis dysfunction (Barreto et al., 2007). In the current study, impaired semen quality (decrease in sperm concentration and motility) of adult male mice was observed after 28 day of DiBP exposure. Significant decreases were observed in serum levels of testosterone (T), luteinizing hormone (LH), follicle-stimulating hormone (FSH), and estradiol (E2) in serum and concentrations of testosterone in the testis in control and DiBP treatment groups. Results are means ± SE (n = 6), * p < 0.05 compared with the control.

![Fig. 4](image-url)
and testis testosterone levels between the treatment and control groups. The protein levels of genes involved in testosterone production showed significant decreases in the DiBP treatment group, indicating that DiBP might reduce the content of testosterone in the testis, and ultimately cause a decrease in sperm quality. Testosterone is necessary for germ cell survival and development in the testis (Walker, 2009). It is synthesized in Leydig cells through a series of reactions catalyzed by four enzymes (i.e., P450scc, 3β-HSD, CYP17A1, and 17β-HSD). The mRNA and protein levels of P450scc and 3β-HSD in mice testes decreased significantly after DiBP treatment. The expression of CYP17A1 was also significantly decreased at the mRNA level, while not at the protein level (Figs. 5 and 6). It was possible that although the gene expression in individual Leydig cells may decrease with phthalate exposure, the number of Leydig cells in testes may increase at the same time (Corton and Lapinskas, 2005), consequently leading to a different expression extent in protein levels. An earlier animal study also showed that DiBP exposure reduces the content of testicular testosterone, similar to the effects of DEHP, DBP, and DinP on rat fetal testicular testosterone production (Borch et al., 2006).

Cholesterol is a substrate for testosterone biosynthesis. Several potential sources of cholesterol can be employed for steroidogenesis in Leydig cells. In rodents, high density lipoprotein (HDL)-derived selective uptake of cholesteryl esters, via scavenger receptor B1 (SR-B1), provides most cholesterol for steroidogenesis. StAR is responsible for cholesterol transport to the inner mitochondrial membrane. In the DiBP treatment group, StAR was decreased at the mRNA and protein levels. Similar observations were noted in a mouse DBP exposure study, which indicated that exposure decreases testosterone biosynthesis, accompanied by a decrease in key genes involved in cholesterol transport (SR-B1 and StAR) (Borch et al., 2006).

Testosterone levels are precisely regulated via negative feedback control of the hypothalamic–pituitary–testicular axis and primary LH acting on steroidogenic enzymes and steroid transport proteins. For example, StAR expression can be provoked via LH binding to its receptor (LHR) and the cAMP-dependent pathway (Dufau, 1988; Zirkin and Chen, 2000). Our results showed decreased STAR mRNA and protein levels in the testes of the DiBP treatment group. No observable changes were observed in the serum LH levels. The mRNA levels of LHR in the testes of the DiBP treatment group did not change, suggesting that the decrease in steroidogenesis in male mice due to DiBP might be independent of the hypothalamic–pituitary–gonadal (HPG) axis.

AR plays an important role in testosterone signaling, which is necessary for germ cell development and survival. In the absence of AR in Sertoli cells in mice, spermatogenesis does not progress beyond the pachytene or diplotene stages of meiosis (De Gendt et al., 2004; Tsai et al., 2006), and the integrity of junctional complexes in the blood-testis barrier is disturbed (Meng et al., 2005; Wang et al., 2006). In the current study, the mRNA levels of AR were decreased, suggesting that AR might be involved in DiBP-induced testicular toxicity.

Taken together, our results showed an anti-androgenic effect by DiBP on adult male mice. Leydig cells might be a main target for DiBP exposure, and the secretion of testosterone was inhibited, possibly due to the down-regulation of several important genes responsible for testosterone biosynthesis in Leydig cells (e.g., StAR, P450scc, and 3β-HSD). Further, the reduction of testosterone may affect spermatogenesis in the testis, consequently resulting in poor semen quality. Although the dosage was higher than environmentally relevant levels, the present study provided possible mechanisms for DiBP-induced male reproductive function impairment, and may fill the information gap on the toxicity of DiBP to some extent.

4. Conclusions

In summary, DiBP exposure resulted in significantly lower sperm concentration and motility and significantly higher sperm malformation rates in the epididymis. The low semen quality might be due to disruption of testosterone synthesis,
and Leydig cells might be an important target of DiBP or other phthalates. This is the first report on the levels of urinary 5mC and 5hmC in mice following DiBP exposure. The level of 5mC in urine was significantly increased with DiBP exposure, supporting our earlier study about the association between urinary 5mC content and phthalate metabolite concentrations in the male population. The present study may improve our understanding on the epidemiological associations among the phthalate exposure, urinary deoxynucleosides and reproductive function parameters. Future study is still warranted to further explore the underlying mechanism involved.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2017.04.036.

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via diet in maternal mice decreases testosterone levels in male offspring. Chemosphere 172, 260–267.


