

Di(*n*-butyl) phthalate has no effect on the rat prepubertal testis despite its estrogenic activity *in vitro*

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Abstract: The aim of this study was to assess the impact of di(*n*-butyl) phthalate (DBP) on the rat's prepubertal testis. Male Wistar rats were given daily subcutaneous injections with DBP (20 or 200 μg) or a vehicle from the 5th to the 15th postnatal day (pd). On the 16th pd, the rats were euthanized, and the testes were dissected, weighed, and paraffin embedded. The blood was collected to determine the serum levels of testosterone (T), estradiol (E) and FSH. The following parameters were assessed in the testis sections: diameter and length of seminiferous tubules (st), numbers of spermatogonia A + intermediate + B (A/In/B), preleptotene spermatocytes (PL), leptotene + zygotene + pachytene spermatocytes (L/Z/PA) and Sertoli cells per testis, percentage of st containing gonocytes or pachytene spermatocytes or lumen. An estrogenicity *in vitro* test was performed by means of a transgenic yeast strain expressing human estrogen receptor alpha. At both doses, DBP had no influence on testis and seminal vesicle weight, st diameter and length, number of germ and Sertoli cells per testis, percentage of st containing gonocytes or pachytene spermatocytes or lumen. DBP did not change E, T or FSH serum levels. The *in vitro* yeast screen showed that DBP was a weak estrogenic compound, approximately six to seven orders of magnitude less potent than 17 β -estradiol. In conclusion, exposure of a rat to DBP in doses 100 or 1,000-fold higher than a Tolerable Daily Intake for humans had no effect on its testicular development. (*Folia Histochemica et Cytobiologica* 2011; Vol. 49, No. 4, pp. 685–689)

Key words: DBP, rat, spermatogenesis, testis, yeast estrogen screen

Introduction

Phthalates are chemicals ubiquitously employed in industry. They are commonly used as plasticizers in polyvinyl chloride (PCV), fixatives, detergents, lubricants, and solvents. Thanks to such a wide range of applications, phthalates can be found in inks, lacquers, food packaging and food products, cosmetics, and a range

of plastic products such as medical equipment and toys. Di(*n*-butyl) phthalate (DBP) is a compound extensively used in the cosmetics industry and can be found in such products as perfumes, nail varnish and hairsprays. DBP, diethyl phthalate and benzyl butyl phthalate metabolites are found in the urine in higher amounts than other xenoestrogens, suggesting that exposure to these three phthalates is more significant [1]. Phthalate exposure is possible through different ways, including the inhalation, dermal, oral and intravenous routes.

Numerous studies on DBP's influence performed on male rats during the prenatal period of life have demonstrated the negative effect of DBP on the testis and male reproductive tract. For example, DBP exposure *in utero* leads to the suppression of intratesticular testosterone level, Leydig cell aggregation and

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hyperplasia, an increased number of gonocytes, and the presence of multinucleated gonocytes in fetal testis, which is resolved in early postnatal life [2–4]. On the other hand, Barlow and Foster [3] showed that degeneration of the seminiferous epithelium after *in utero* DBP treatment progressed towards maturity. Additionally, fetal DBP treatment caused hypospadias, cryptorchidism, prostate, epididymis and vas deferens agenesis, decreased anogenital distance and delayed preputial separation, suggesting diminished testosterone action in the fetal period of life [5].

The only study to have examined DBP's influence on the prepubertal rat testis, performed by Kim et al. [6], showed that a daily dose of 20 mg/animal administered from the 5th to the 14th day of life caused reduction of testis weight and the disruption of androgen receptor (AR) and estrogen receptor (ER) concentration in the testes of rats aged 31 days and 42 days. The authors attributed the antiandrogenic activity to the negative influence of DBP rather than to the positive influence of estrogen.

Interestingly, Ge et al. [7] reported that another phthalate (diethylhexyl phthalate, DEHP) had a biphasic effect on pubertal male rats. A high DEHP dose (750 mg/kg b.w.) decreased serum testosterone levels and delayed the onset of puberty, while low DEHP doses (10 mg/kg b.w.) caused the opposite effect, increasing the serum testosterone level and advancing the onset of puberty. Estrogenic compounds are known to possess a similar biphasic effect. Although the high doses of estrogenic substances have a negative impact on testis development and initiation of spermatogenesis [8–10], lower doses have a stimulatory effect [10–12]. The stimulatory effect of estradiol on spermatogonia number/proliferation has been confirmed by recent studies [13, 14]. According to studies performed by Jobling et al. [15], DEHP is slightly estrogenic, which may explain its stimulatory effect on spermatogonia. However, DEHP estrogenicity has not been confirmed by other studies [16, 17]. Similarly, some studies have confirmed DBP estrogenicity [15–17], while others have not [18].

The aim of our study was to investigate whether low doses of DBP can have a positive impact on testis development, i.e. the opposite to the previously reported negative influence of high doses [19].

Material and methods

Experimental set-up. Newborn male Wistar rats were used in this study. The animal experiment was approved by the Bioethics Committee of the Medical University of Lodz. Animals were maintained at a stable temperature (22°C) and a 12 hour light/12 hour dark cycle. During the acclimatization period (five days before mating), pregnancy and after parturition, mothers had access to water and soy-free

food (Agropol, Poland) *ad libitum*. A soy-free diet is important because soya is known to be the source of estrogenic substances. DBP was dissolved in dimethylsulfoxide (DMSO) and olive oil. Newborn rats were given daily subcutaneous injections with DBP 20 µg (DBP20) or DBP 200 µg (DBP200) or vehicles (C), from the 5th to the 15th post-natal day (pd). The volume of each injection was 0.05 ml. All the injected substances were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). The autopsy was performed on the 16th pd to observe the possible disturbances caused by DBP within a short time following treatment. Such rapid disruptive actions have previously been observed for different xenoestrogens [8] and hormones [20]. All the sacrificed animals were weighed. Both testes and seminal vesicles were dissected, weighed, fixed in Bouin's fluid and embedded in paraffin. Blood was collected by cardiac puncture, centrifuged and stored at –80°C for hormone analysis. The experimental groups contained 20, 18, and 14 newborn animals, respectively for C, DBP200 and DBP20 group.

Quantitative analysis of seminiferous tubules. Seminiferous tubule examinations were performed as described previously to assess the diameter, length and number of germ and Sertoli cells per testis [8]. The percentage of seminiferous tubules containing gonocytes (G) or spermatocytes in the pachytene stage of first meiotic division (ScPa) or lumen was obtained by counting 100 randomly-selected round seminiferous tubules. A tubule was counted as positive for containing lumen if the lumen was $\geq 10 \mu\text{m}$ in diameter. G and ScPa were distinguished based on their morphology and location within the seminiferous epithelium. The tubule was counted as positive for G or ScPa if it contained at least one G or ScPa, respectively.

Hormonal determinations. FSH, testosterone and estradiol levels were measured in defrosted serum samples. Enzyme Linked Immunosorbent Assay, Rat FSH Elisa (Boicodex-Hycel, Belgium) was used for the determination of FSH with a sensitivity of 0.2 ng/ml. Concentrations of testosterone and estradiol were determined by the competitive immunoassay technique (Ortho-Clinical Diagnostic, Amersham, UK). The sensitivities were 0.03 nmol/l for testosterone and 10.0 pmol/l for estradiol.

Yeast Estrogen Screen. Yeast Estrogen Screen (YES) is an *in vitro* assay which employs a genetically modified strain of *Saccharomyces cerevisiae* to assess the estrogenic potential of compounds and their ability to interact with human estrogen receptor α (hER α). Normally, yeasts do not contain ER. This strain carries an hER α gene inserted into the chromosome. Moreover, it carries *lacZ* reporter gene integrated into multicopy plasmid. *lacZ* is under the control of estrogen responsive elements (EREs) and encodes the enzyme β -galactosidase. The YES assay is based on the binding of test compounds to hER α . This binding activates EREs and induces the *lacZ* reporter gene. β -galactosidase is se-

creted into the growth medium where it metabolizes the yellow-colored substrate, CPRG (chlorophenol red-β-D-galactopyranoside) (Sigma Aldrich). The absorbance of the product is measured at 540 nm.

For the purpose of this study, the test was performed according to the modified protocol published elsewhere [21]. 10 μl aliquots of 17β-estradiol and DBP dissolved in DMSO at a range of concentrations were mixed with 190 μl of assay medium in microtiter plate wells. The yeast strain for the YES assay was received from the Sub-Department of Toxicology at Wageningen University in the Netherlands.

Statistics. Distribution of data was assessed according to the Shapiro–Wilk’s test. One-way ANOVA followed by a *post hoc* test was applied to assess the statistical significance of the data. The Newman–Keuls test was chosen as the *post hoc* test for normally distributed data (body, testis and seminal vesicles weights, seminiferous tubule diameter and length, number of germ and Sertoli cells per testis), while the Kruskal–Wallis test was chosen for not normally distributed data (percentage of seminiferous tubules containing G, ScPa and lumen, serum levels of FSH, testosterone and estradiol). Differences were considered significant at *p* < 0.05.

Results

Neither DBP200 nor DBP20 had any influence on the body or testis or seminal vesicle weights, total seminiferous tubule length or diameter (Table 1). Similarly, none of the DBP doses had any influence on the number of germ cells or Sertoli cells per testis, percentage of seminiferous tubules containing G, ScPa and lumen.

Testosterone and estradiol serum levels were not influenced by DBP treatment (Table 2). FSH in the group exposed to DBP20 was not changed in comparison with the C group. Interestingly, blood FSH level was slightly elevated in the group exposed to the higher DBP dose, although this result was not statistically significant.

The Yeast Estrogen Screen showed that DBP activated the ER-mediated response in the YES assay and stimulated the expression of β-galactosidase in the transgenic yeasts. DBP was approximately six to seven orders of magnitude less potent than 17β-estradiol (Figure 1).

Discussion

In the presented study, DBP had no effect on the analyzed parameters of testicular development. Spermatogenesis advancement was not influenced by administered DBP doses. Neither testis weight and morphology, nor testosterone, estradiol or FSH serum levels were affected. In the same study model, 17β-estradiol, diethylstilbestrol (DES) and zearalenone (ZEA) showed a negative impact on testicular development even when administered in lower doses [8].

Table 1. Influence of DBP 20 μg or DBP 200 μg/animal/day administered from the 5th to 15th postnatal day on testicular parameters in rats aged 16 days in comparison with the control group. Results are expressed as means ± standard deviation for variables with normal distribution and as median (min-max) for variables with not-normal distribution

| | Weights of | | | Seminiferous tubules' | | | Seminiferous tubules with | | | Number of | | | |
|---------|------------|--------------|-----------------------|-----------------------|----------------|------------|---------------------------|------------|----------------------------|--------------------|----------------------------|-----------------------------|--|
| | Body [g] | Testes [mg] | Seminal vesicles [mg] | Diameter [μm] | Length [cm] | Lumen (%) | Gonocytes (%) | ScPa (%) | SgA + In + B (mill/testis) | ScPI (mill/testis) | ScL + Z + Pa (mill/testis) | Sertoli cells (mill/testis) | |
| Control | 29.6 ± 3.8 | 100.3 ± 14.9 | 7.7 ± 1.8 | 82.5 ± 3.7 | 1588.6 ± 258.4 | 2 (0–17) | 4 (2–8) | 39 (19–53) | 9.7 ± 3.0 | 12.3 ± 5.4 | 12.2 ± 4.0 | 69.4 ± 10.5 | |
| DBP20 | 30.4 ± 3.4 | 100.8 ± 9.4 | 8.2 ± 1.5 | 83.7 ± 4.0 | 1596.7 ± 198.6 | 1.5 (0–14) | 5 (1–10) | 37 (22–50) | 8.7 ± 1.4 | 15.5 ± 4.4 | 8.6 ± 4.5 | 67.8 ± 7.5 | |
| DBP200 | 28.3 ± 4.1 | 94.9 ± 11.7 | 9.7 ± 3.9 | 82.5 ± 2.9 | 1484.8 ± 141.9 | 0 (0–3) | 6 (2–13) | 27 (16–42) | 9.0 ± 1.6 | 13.2 ± 2.8 | 7.3 ± 5.0 | 68.1 ± 7.3 | |

DBP — di(*n*-butyl) phthalate; ScPa — pachytene spermatocytes; SgA — spermatogonia A; Sg In — spermatogonia intermedia; SgB — spermatogonia B; ScPI — preleptotene spermatocytes; ScL — leptotene spermatocytes; ScZ — zygotene spermatocytes, mill/testis — million per testis

Table 2. Results of hormonal determinations in the serum of rats aged 16 days, which were under the influence of di(*n*-butyl) phthalate (DBP) 20 μg or 200 μg /animal/day from the 5th to the 15th postnatal day in comparison with the control group

| | Estradiol [pg/ml] | Testosterone [ng/ml] | FSH [ng/ml] |
|---------|-------------------|----------------------|----------------|
| Control | 44.6 (32.9–64.5) | 1.8 (0.9–5.4) | 4.9 (0.9–11.0) |
| DBP20 | 44.1 (23.7–107.4) | 1.4 (0.9–4.4) | 5.4 (1.7–6.8) |
| DBP200 | 74.3 (22.9–86.2) | 1.9 (1.1–8.6) | 6.6 (3.3–8.9) |

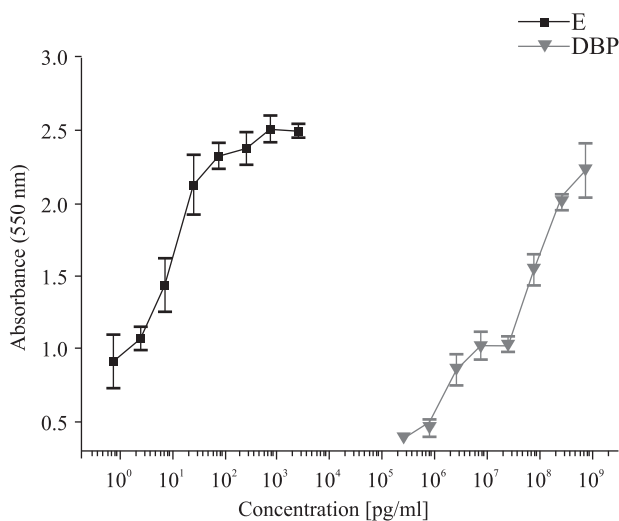


Figure 1. Results of the Yeast Estrogen Screen (YES) assay. β -galactosidase activity of yeast strain after treatment with 17 β -estradiol (E) and di(*n*-butyl) phthalate (DBP). Each value represents the mean of the three replicates

The majority of reports on DBP's influence on the male reproductive tract describe treatment with doses of around 500 mg/kg b.w. on pregnant females [3, 22, 23] or about 1 g/kg b.w. (20 mg/animal) on neonatal rats [6] to cause adverse effects. To the best of our knowledge, there have been no studies examining the influence of DBP in doses of 1 and 10 mg/kg b.w. (reflecting the dose of 20 and 200 μg /animal used in the presented study) on testicular development. Based on the previous studies where a DBP dose of 5 mg/animal did not exert any biological effect on analyzed parameters (testis weight, serum testosterone level, AR and ER β concentrations) [6], no negative impact of such low doses should be expected. However, a study by Ge et al. [7] suggested that besides the negative impact of high doses, the stimulatory effect of low phthalate doses on the onset of puberty can also be observed, as when rats have been treated with a dose of 10 mg/kg b.w. of DEHP, which can be compared with higher DBP dose used in the presented study. In our study, however, this treatment did not cause any effect.

On the other hand, DBP showed a slight estrogenic activity *in vitro*. This observation is in agreement with other studies [15–17], although some studies do not confirm DBP estrogenicity [18]. The pre-

sented study reveals that DBP has estrogenic potential *in vitro*, although its potential to induce β -galactosidase synthesis in the yeast model was very weak in comparison with 17 β -estradiol.

The doses of DBP which have been proved by other authors [3, 6, 22, 23] to have negative effects on reproductive parameters were extremely high, and exceeded the DBP estimated environmental exposure. The estimated daily exposure of an average person to DBP varies widely according to different studies; it approximates to 0.007–7 $\mu\text{g}/\text{kg}/\text{b.w.}$ for adults and 1.4–2.4 $\mu\text{g}/\text{kg}/\text{b.w.}$ for infants [24]. The primary source of DBP exposure is food. According to the Ministry of Agriculture, Fisheries and Food in Great Britain, the average adult DBP exposure is 0.013–0.031 mg/person/day (equivalent of 0.2 to 0.48 $\mu\text{g}/\text{kg}$ b.w./day if mean b.w. is 65 kg) [25]. In Denmark, average daily exposure was much higher and approximates 1.6 $\mu\text{g}/\text{kg}$ b.w. in adults, 8 $\mu\text{g}/\text{kg}$ b.w. in children aged 1–6 years and 3.5 $\mu\text{g}/\text{kg}$ b.w. in children aged 7–14 years [26]. According to Blount et al. [1], women aged 20–40 years old, who are at childbearing age, have higher estimated exposure.

A Tolerable Daily Intake (TDI) for DBP is 10 $\mu\text{g}/\text{kg}$ b.w. This has been determined by the European Food Safety Authority (EFSA), based on the lowest observed adverse effect level, (LOAEL), which was 2 mg/kg b.w./day [27]. It shows that the environmental exposure levels for DBP are below TDI. However, the exposure reported by Müller et al. [26] was in the same order of magnitude as TDI. Doses given to rats in this study were two (DBP20) or three (DBP200) orders of magnitude higher than TDI, but they had no effect on the developing testis or hormone levels. This could lead to the statement that DBP exposure from food, industrial products and the environment does not influence such parameters in humans or in animals.

Nonetheless, results from an experimental set-up on rats should not be directly extrapolated into environmental relationships. It should be kept in mind that other parameters which were not within the scope of this study might be affected by the abovementioned doses of DBP. Moreover, the environmental influence of compounds with estrogenic activity should not be regarded as the simple effect of one compound on the organism. For example, the mixture of DBP and 17 β -estradiol had approximately additive activity in

the *in vitro* yeast screen [16]. The additive influence of these compounds might be of importance even if the exposure levels of a particular compound are below the threshold of their activity.

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