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THE EFFECTS OF DI-*N*-BUTYL PHTHALATE ON THE GERM CELLS OF LABORATORY MICE

WPŁYW FTALANU DI-*N*-BUTYLU NA KOMÓRKI PŁCIOWE MYSZY LABORATORYJNYCH

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Słowa kluczowe: *ftalan di-n-butylu, uszkodzenia DNA, mikrojądra, pozostałości ftalanów, ilość i jakość plemników*

ABSTRACT

Phthalate are found in the environmental samples due to their wide use in the industry as plasticizers. Di-n-butyl phthalate (DBP) is mainly used in nitrocellulose and polyvinyl acetate products as well as in personal-care products.

This study was performed to investigate the influence of exposure to DBP on the quantity and quality (motility, morphology) and DNA damage (induction of micronuclei and DNA strand breaks) of male mice gametes. The estimation of DBP residues was also done. Eight weeks exposure to DBP (500 mg/kg bw and 2000 mg/kg bw) did not significantly affect testes and epididymes weights as well as sperm count. DBP clearly diminished sperm motility, enhanced frequency of abnormal sperm heads and not significantly increased DNA strand breaks in germ cells as well as frequency of micronuclei in spermatids. There were no bioacumulation of DBP in mice. Results suggest that DBP may affect the male mice germ cells.

STRESZCZENIE

Ftalany są powszechnie wykorzystywane w przemyśle jako plastyfikatory, dlatego też często występują w próbach środowiskowych. Ftalan di-n-butylu (DBP) jest składnikiem produktów nitrocelulozowych i poliwinylowych, jak również produktów do higieny osobistej.

Celem pracy było zbadanie wpływu podawania DBP na ilość i jakość gamet męskich myszy (ruchliwość, morfologia) oraz na występowanie uszkodzeń DNA (indukcja mikrojąder oraz pęknięć nici DNA). Zbadano także stężenie pozostałości DBP w gametach. Ośmiotygodniowe narażanie na DBP (500 mg/kg mc i 2000 mg/kg mc) nie powodowało istotnych zmian w ciężarze jąder i najądrzy ani w liczebności plemników. Narażenie na DBP, zwłaszcza w dawce 2000 mg/kg mc wpływało wyraźnie na zmniejszenie ruchliwości plemników i zwiększenie odsetka plemników o nieprawidłowej budowie morfologicznej. Powodowało też nieznaczne zwiększenie pęknięć nici DNA w gametach oraz zwiększenie częstości występowania mikrojąder w spermatydach. Nie wykazano bioakumulacji DBP w gonadach samców myszy. Uzyskane wyniki sugerują, że DBP może oddziaływać niekorzystnie na męskie komórki rozrodcze myszy.

INTRODUCTION

Phthalate esters (esters of 1,2-benzenedicarboxylic acid) belong to the large and diversified class of peroxisome proliferators which include herbicides, hypolipidemic drugs, and phthalate plasticizers. They are widely used in industry as plasticizers in many synthetic products, for example in food packaging, biomedical devices and toys. In some plastic goods phthalates constitute up to 50% of the total weight. They are used also in personal care products, e.g. soaps, shampoos and perfumes [46].

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Phthalates are found widespread in the environment because, they are not reversibly bound in the polymer matrix, and can migrate from the plastic to the external environment [30, 33]. Exposure to phthalates occurs primarily through ingestion and inhalation although dermal exposure may be important in overall exposure to these compounds. Occupational exposure to phthalates takes place during polymerization of polyvinyl chloride during manufacture of PCV products and in rubber industry. Non-occupational exposure to phthalates occurs due to use of personal-care products, paints, food contacting plastics, medical devices and pharmaceutics containing phthalates [3, 4].

Although, in general, acute toxicity of the most commonly used phthalates is low, some phthalates have been shown to be carcinogenic, causing reproductive toxicity at high doses. Chronic exposure can induce changes in germ cells. Some phthalates are blamed for causing damage to the testes, decreased sperm production and for xenoestrogenous or antiandrogenous activity [1, 19].

Of the most commonly used phthalates, dibutyl phthalate (DBP) is widely used in PCV, nitrocellulose and polyvinyl acetate products, in painting inks, and as lubricants for aerosol valves, a skin emollient and as a plasticizers in nail polish, fingernail elongator and hair spray [6]. For instance DBP was detected in 19 of the 21 nail polishes and in 11 of the 42 perfumes at the mean level from 444,567 to1671,139 μg/ml [28].

DBP and its metabolities may have significant potential toxicity, especially regarding long-term effects [9, 38].

DBP is known to be developmentally toxic in mice and rats. It induces fetotoxic effects in the absence of maternal toxicity. DBP is reported to be teratogenic at high doses in rodent [13, 43]. Reproductive tract malformations were found in male offspring of female rats exposed to DBP throughout pregnancy and lactation [55]. In animals exposed to dietary level of 1% of DBP reduction in the weight of prostate, testis and seminal vesicles and in daily sperm production were noted. Dibutyl phthalate has antiandrogenic properties and is suspected to be responsible for endocrine disruptor like effect [34, 47]. Majority of papers described the effects of DBP on the sperm production and quality of gametes. There are no papers about the relation between potential changes in genetic material and diminished sperm production and quality.

One of the aims of this paper was estimation of DBP effects on the male mice genetic material. This study was performed to investigate the influence of exposure of laboratory mice to DBP during 8-weeks, which covers full spermatogenesis cycle, on the quantity and quality of male's gametes as well as to detect DNA damage in haploid germ cells and induction of micronuclei in spermatids. The levels of concentrations of DBP in testes of exposed animals were also studied in order to elucidate the mechanisms of action laying behind the investigated effects.

MATERIALS AND METHODS

Pzh:Sfis outbread male mice weighting 25±1.95 g (35-39 days old) were kept in the study room and observed one week before the initiation of the experiment. The animals were housed in cages, in a room designed for control of temperature, humidity and light cycle. Tap water and rodent diet were available *ad libitum.* At the beginning of the experiment 42-46 days old male mice were assigned to two experimental and one control groups and exposed by gavage to olive oil (control group) or DBP (CAS No 84-74-2, Sigma) solution in olive oil for 8 weeks, 3 days per week. The doses of DBP were 500 mg/kg b.w. (1/16 LD_{50}) and 2000 mg/kg b.w. (1/4 LD_{50}) daily. Animals were weighed every week.

Five males per group were weighed and sacrificed after 4 and 8 weeks following the start of the treatments as well as 4 weeks after the last treatment (i.e. 12 weeks after the start of treatment) as is shown on the scheme 1.

Both epididymes (for sperm quantity and quality) and both testes (for comet assay and DBP concentrations) were removed and weighed from each male.

For sperm count, one epididymis was macerated in 0,2 ml of 1% solution of trisodium citrate for 5-8 min

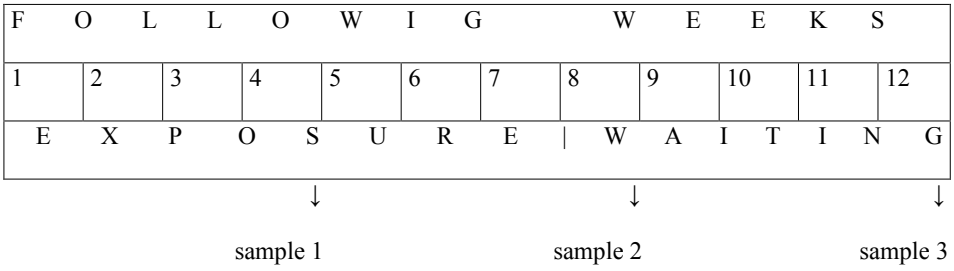

Figure 1. Scheme of DBP exposure and animal sampling
Schemat narażenia na DBP i pobieranie próbek

and minced. Then the solution was made up to 2 ml and mixed for about 1 min. The sperm suspension was diluted 1:1 in 10 % buffered formalin. The spermatozoa were counted using an improved Neubauer haemocytometer [20, 41]. The contents of the second epididymides were placed in prewarmed (37°C) physiological saline (0,2 ml). One drop of sperm suspension was placed on warm (37°C) microscope slide and covered with a cover slip. Two hundreds cells per animal were evaluated for motility (movement *vs*. no movement) within 5 min of their isolation from the epididymes, and data were expressed as percentages [53].

The study of frequency of morphologically abnormal spermatozoa was performed according to the procedure described by *Wyrobek and Bruce* [54]. Sperm remaining from the above experiment was dispersed evenly in saline. A drop of the sperm suspension placed on a slide was smeared with another slide, air-dried overnight, fixed and stained with eosin Y. Then 500 spermatozoa per mouse were analyzed using a light microscope, and abnormal sperm heads (e.g. lacking hook, amorphous, banana-shaped head) were recorded.

For comet assay analysis basic technique of *Singh* et al. [44] and further described by *Anderson* et al. [2] was used. One testis from each animal was decapsulated, placed in the RMPI 1640 medium and minced with scissors. Before using the cells, tubes were swirled so that the single cells remained in suspension. 5 µl of cell suspension was mixed in *Eppendorf* tube with 75µl 0,5 % low melting point agarose (LMA) for embedding on slides coated previously with 1 % normal melting point agarose (NMA). Slides were covered with cover slip and kept at 4°C for 5 min to allow the agarose to solidify. Then slides were covered with second layer of LMA and kept at 4°C for next 5 min. After removing the cover slips, the slides were immersed in freshly prepared cold lysing solution overnight at 4°C. Then they were drained and placed in gel electrophoresis tank, and left in electrophoresis solution for 20 min to allow unwinding of DNA. Electrophoresis was conducted at 4°C for 20 min using 19 V and 300 mA. After neutralization (0,4 M Tris, pH 7.5) slides were stained with ethidium bromide (EtBr) and examined using fluorescence microscope. Images of 100 randomly selected cells from each animal were recorded and analyzed using CASP image-analysis program [27]. The DNA tail moment was chosen as parameter for further analysis. Each experimental group was compared with appropriate control group using *Student t*-test.

Six weeks old male Pzh: SFIS mice were used in micronucleus study in spermatids. The method has been adapted from *Tates* et al. [45] and *Cliet* et al. [7]. The animals were treated with DBP (500 or 2000 mg/kg bw). Five mice were assigned randomly to each treatment or control group. Male mice were treated 6 times (3x2 times at a 24-h interval), begining on days 0 and 1 during the DNA synthesis phase of preleptotene spermatocytes I, followed by the treatment on days 7 and 8 during the residual DNA synthesis in pachytene spermatocytes I, (crossing-over and chromosome pairing), and finally on days 13 and 14 during the meiotic phase of spermatocytes I/II. On day 16 spermatidis which arose on Golgi spermatid stage were isolated by enzymatic dissociation of the testes tissue with trypsin and deoxyribonuclease I as described by *Meistrich* et al. [31]. For each dose of DBP, 5000 Golgi phase spermatids were scored (1000 per male mouse) for the presence of micronuclei. The statistical analysis between the negative control and treated group was performed using the *Kastenbaum-Bowman* [23] test. Testis weight (TW) was measured before spermatid isolation and testicular cytotoxicity was evaluated by comparing TW in the treated group with TW in the control group by *Student* t-test.

For the determination of DBP residues, germ cells were extracted as described by *Ljungvall* et al. [29]. Prior the extraction samples were homogenized and mixed with cyclohexane and MTBE. The mixture was placed in to ultrasonic bath and the extract was filtered using the paper filter.

HPLC-UV analysis was performed on the Waters LC module I plus coupled with UV/VIS detector. The system was controlled by Millennium software. The Lichrospher 60 RP – selected B (5µm particle size) column (Merck) was used. Separation was performed at 35°C. The mobile phase consisted of methanol and water (93:7, v/v) and delivered at the flow rate of 1 ml/min. The column elute was monitored at 225 nm.

The limit of quantification (LOQ) for DBP in blood samples was 0,12 µg/ml and the linear range was up to 7 µg/ml. The extraction procedure has been validated for mice germ cells samples by spiking with DBP and showed the average recovery 65 %.

The gas chromatography with mass spectrometry as detector was used to confirm identity of DBP in the samples. GC-MS analysis was performed on the Varian, Saturn 4D. The DB5-MS column was used (0,25 mm i.d. x 30 m and 0,25 µm film thickness). The splitless injection mode was used with setting the injector temperature - 260°C (injection volume 2 µl). The initial oven temperature was 60 °C. 1 min after the temperature was increased at the rate of 10 °C/min to 260 °C and was maintained for 14 min. Helium was used as a carrier gas (the column flow 1.25 ml/min). The interface temperature was 280°C and the electron impact were used as a ionization mode. The detector temperature was 170°C. Identification was carried out by selected ions (149 m/z, 223 m/z) and by comparing the retention time for samples and for pure standard.

Table 1. Mean testes and epididymes weights at different time periods after the start of exposure of male mice to DBP
Średnie masy jąder i najądrzy samców myszy w różnych odstępach czasu od rozpoczęcia narażania na DBP

Dose	Time after the start of treatment	Mean body weight (g) ±SD	Mean testes weight (mg) ±SD	Relative testes weight (%)	Mean epididymes weight (mg) ±SD	Relative epididymes weight (%)
Control	4 weeks	36.71±2.17	191.3±60.7	0.52	42.0±8.3	0.11
500 mg/kg bw DBP	4 weeks	37.34±1.09	232.0±18.1	0.62	50.2±4.8	0.13
2000 mg/kg bw DBP	4 weeks	35.90±2.43	208.8±36.4	0.58	47.8±1.9	0.11
Control	8 weeks	41.38±2.34	186.8±47.6	0.45	51.0±14.7	0.12
500 mg/kg bw DBP	8 weeks	38.87±2.19	214.0±47.7	0.55	49.2±6.1	0.13
2000 mg/kg bw DBP	8 weeks	36.06±2.28	186.4±64.6	0.52	43.4±13.6	0.12
Control	12 weeks [a]	41.93±2.16	192.6±30.1	0.46	46.4±10.1	0.11
500 mg/kg bw DBP	12 weeks [a]	40.51±1.46	225.4±22.3	0.56	52.6±9.8	0.13
2000 mg/kg bw DBP	12 weeks [a]	37.76±1.74	182.8±41.0	0.49	48.8±7.9	0.13

Student t-test: ns - not significant $p<0.05$
[a] 4 weeks after the end of exposure

$$\text{Relative organ weight} = \frac{\text{Mean weight of examined organ}}{\text{Mean body weight}} \text{ x } 100$$

These experiments obtained clarification No 3/03 for conducting studies on experimental animals from the Fourth Local Ethical Commission acting as a part of the National Ethical Commission.

RESULTS

Body weight, epididymes and testes weights, and relative weights of both organs are shown in Table 1.

Male mice exposed to the highest concentration of DBP – 2000 mg/kg b.w. after 8 and 12 weeks of the experiment, showed mean body weights lover than the other animals, although the results were not statistically significant. Mean and relative testes and epididymes weights were not significantly different compared to control animals.

Results of sperm quantity and quality are shown in Table 2. In the middle of exposure sperm count was not tested.

After 8 weeks following the start of treatment the results in control as well as in experimental groups were similar. Four weeks after the end of exposure sperm count were slightly enhanced following exposure to 1/16 LD_{50} and slightly decreased after treatment with 1/4 LD_{50}, but results were not statistically significant. However, sperm motility was significantly decreased after 4 weeks of exposure to lower dose, after 8 weeks of exposure to both 1/4LD_{50} and 1/16 LD_{50} DBP doses, and 4 weeks after the end of exposure to 2000 mg/kg b.w. of DBP. Four weeks after the start of treatments the percent of abnormal spermatozoa slightly, but not statistically significant increased. Eight weeks after the start and 4 weeks after the termination of exposure to higher DBP dose, the percent of malformed sperm heads was enhanced over 2 times as compared to the results obtained in the control group ($p<0.001$ by Chi-square test). DNA tail moment in male mice germ cells, generally remained unchanged following both doses at all time points. The highest response was observed just after the end of 8-weeks exposure to 2000 mg/kg b.w. DBP, but results were not statistically significant.

Induction of micronuclei in spermatids is shown in Table 3. Testes weight was the lowest and frequency of

Table 2. Sperm quantity and quality at different time periods after the start of 8 weeks exposure of male mice to DBP
Liczebność plemników i jakość w różnych odstępach czasu od rozpoczęcia 8-tygodniowego narażania samców myszy na DBP

Dose	Time	Sperm count x10^6 ±SD	Percent of motile spermatozoa ± SD	Percent of abnormal spermatozoa ± SD	Germ cells tail moment ± SD
Control	4 weeks	Not tested	67.50±9.27	2.52±0.66	2.00±0.59
500 mg/kg bw DBP	4 weeks	Not tested	57.20±8.30 ##	3.12±0.61 NS	2.08±0.96 ns
2000 mg/kg bw DBP	4 weeks	Not tested	66.70±12.74 NS	3.32±1.32 NS	2.39±1.55 ns
Control	8 weeks	1.89±0.65	71.10±5.25	4.36±1.05	2.46±2.24
500 mg/kg bw DBP	8 weeks	1.89±0.37 ns	61.60±5.63 ###	5.00±1.62 NS	2.76±0.58 ns
2000 mg/kg bw DBP	8 weeks	1.84±1.28 ns	61.70±7.25 ###	9.65±5.69 ###	3.91±1.30 ns
Control	12 weeks [a]	2.39±0.99	76.80±15.99	5.64±1.40	1.95±0.88
500 mg/kg bw DBP	12 weeks [a]	2.92±0.95 ns	75.50±8.12 NS	6.08±1.04 NS	2.38±1.45 ns
2000 mg/kg bw DBP	12 weeks [a]	1.79±1.43 ns	53.20±17.48 ###	12.68±7.65 ###	2.73±0.42 ns

Chi-square test: NS - not significant , ##$p<0.01$, ###$p<0.001$
Student t-test: ns - not significant
[a] 4 weeks after the end of exposure

micronuclei was the highest after treatments of males with 2000 mg/kg bw of DBP, but results were not statistically significant.

The DBP concentration were not found in none of the tested germ cells samples over LOQ of the method (results not shown).

Table 3. Frequency of micronuclei in spermatids of male mice exposed to DBP
Częstość występowania mikrojąder w spermatydach samców myszy narażanych na DBP

Dose	Testes weight mg ±SD	Spermatocytes with micronuclei per 1000 cells ±SD
Control	213±9	1.4±1.34
500 mg/kg bw DBP	219±11	1.8±1.64
2000 mg/kg bw DBP	213±11	2.5±2.29

DISCUSSION

People are chronically exposed to usually low doses of natural and synthetic chemicals coming from natural and occupational environment. These dose levels after single exposure do not cause measurable adverse health effects. However, chronic exposure can induce changes in somatic as well as in germ cells. Exposure to phthalates may affect especially the developing organisms. For this reason all uses of di(2-ethylheksyl) phthalate (DEHP), butyl benzyl phthalate (BBP) and DBP in toys and other children-specific items was prohibited by EU in 2005 [15].

DBP is metabolizing by nonspecific esterases in the gastrointestinal tract to mono-n-butyl phthalate (MBP) [37, 48]. *White* et al. [48] have shown that DBP crossed the intestine much less rapidly than MBP. High oral doses of DBP might exceed the capacity of esterases present in the gut to convert DBP to MBP thereby affecting the rate of absorption. Possibility of accumulation in the adipose tissue in rats was reported by *Williams and Blanchfied* [50]. In our experiments, presence of DBP has not been ascertained in germ cells probably because phthalate have short biological half life and are rapidly metabolized to their monoesters and excreted through the urine and feces [1, 21, 40]. In the present study MBP as possible DBP metabolite was not analyzed. Our results partly confirmed outcome of *Kavlock* et al. [24] showing that there is no bioaccumulation of DBP or its metabolities in rodent tissues (including testes and prostate tissue).

It is not without of importance that many of chemicals present in the environment, including phthalates, due to chemical similarity to endogenous hormones can disrupt hormone metabolism, block the effects of endogenous hormones or disrupt reproduction and development [32, 39]. The presence of endocrine-disruptors in the environment is believed to be one of the reason of impaired sperm count and quality in general population in recent years.

Reproductive and developmental toxicity of DBP is described in *Kavlock* et al. [24] report. DBP is known to be reproductive toxicant in males, but not in females, because the development of tissues, which are the most sensitive to DBP, depend on androgenic activity [18, 35]. Previously obtained data showed that DBP is developmental and reproductive toxicant in the rat [12, 14, 16]. Results of other animal studies suggest that phthalates exposure including DBP is associated with damage to the testis and decreased sperm production [17, 42]. Exposure to phthalates including DBP of young and adult rodents causes pathological and biochemical changes in the testis [5]. The main target for a toxic action of phthalates seems to be Sertoli cells. Phthalate monoesters affect the normal nurse function of Sertoli cells [51]. They cause gross morphological changes in testes and may result in abnormal maturation and death of germ cells [5, 38]. In adult male rats received DBP, testis and epididymal weights were less and hypospermia of epididymis were observed [30]. In our experiment there were no toxic effects in gonads of exposed to DBP males (reduction of mass). We did not observe in these studies any significant decrease in sperm count, although slight biological effect was found 4 weeks after the end of exposure to 1/4 LD_{50}. This is in good agreement with results obtained for testes weights. Similarly, in rats *Zhang* et al. [55] did not observe significant difference between semen concentrations and sperm density following the exposure to phthalates. We have found that the exposure to DBP resulted in diminished quality of spermatozoa. The motility was decreased and the percent of abnormal sperm heads was significantly increased, especially at the end of 8-weeks exposure and 4 weeks after the end of exposure to higher dose. Effects on germ cells quality 4 weeks after the termination of exposure may be explained by the fact that in this case gametes were exposed to DBP during 4-weeks period including development of their younger stages. Above results showed that stem cells, spermatogonia and early spermatids are the most sensitive to DBP.

In the study of men who were partners in infertile couples *Duty* et al. [10] observed an inverse dose-response relationship between monobutyl phthalate (MBP), a metabolite of DBP and sperm motility and concentrations. Sperm DNA damage was associated with enhanced urinary concentrations of phthalate metabolites [22]. Higher urinary metabolite levels were observed in men with lower semen quality [10].

In the present experiment we observed slight biological effect on the DNA damage induced by DBP in germ cells by alkaline comet assay. This effect was however not significant. *Duty* et al. [11] found increased

sperm DNA damage or no linear association between sperm DNA migration measured by neutral comet assay following environmental exposure to phthalates.

Our results showed that the exposure to DBP might cause harmful effect in germ cells. Effects induced in germ cells are of considerable importance, because they can affect next generation. Results of other studies showed that *in utero* or neonatal exposure to DBP, at the dose levels above estimated environmental and occupational human exposure, leads to abnormal reproductive tract development and significantly increase the number of abnormal germ cells [25, 26, 55]. This clear experimentally provided biological activity of DBP does not confirm however that the much lower environmental levels are safe. There is well known that the larger risk of DBP is associated with reproductive and developmental toxicity following gestation and lactation exposures as compared to adult exposures [36]. *Colburn and Clement* [8] showed that DBP which act as an estrogen, can induce greater reproductive effect in second generation of animals. The lack of DBP in male gonads may suggest an indirect influence of this chemical or perhaps the changes we observed in these experiments were caused by its metabolite (MBP) which however was not measured in this study. The above question needs to be elucidated in the future study.

CONCLUSIONS

1. Eight-weeks DBP exposure, especially at high dose leads to decrease of sperm quality. Effects are rather not depending on DNA damage.
2. Earlier stages of spermatogenesis i.e. stem cells, spermatogonia seem to be more sensitive to DBP
3. There were no bioacumulation of DBP in male mice gonads.

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