Developmental Toxicity in Mice Following Paternal Exposure to Di-N-Butyl-Phthalate (DBP)*

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Abstract

Objective   The aim of the present study was to investigate the effects of paternal Di-N-butyl-phthalate (DBP) exposure pre- and postnatally on F1 generation offspring, and prenatally on F2 generation offspring.

Methods   Male mice were exposed to either 500 mg/kg or 2 000 mg/kg of DBP for 8 weeks, and mated with non-exposed females. Three-quarters of the females were sacrificed a day prior to parturition, and examined for the number of living and dead implantations, and incidence of gross malformations. Pups from the remaining females were assessed for developmental markers, growth parameters, as well as sperm quantity and quality.

Results   There were no changes in the fertility of parents and in intrauterine development of the offspring. Pups of DBP-exposed males demonstrated growth-retardation. Following paternal exposure to 500 mg/kg bw of DBP, there were almost twice the number of males than females born in the F1 generation. F1 generation females had a 2.5-day delay in vaginal opening. Paternal exposure to 2 000 mg/kg bw of DBP increased the incidence of sperm head malformations in F1 generation males; however, there were no changes in the fertility and viability of foetuses in the F2 generation.

Conclusion   Paternal DBP exposure may disturb the sex ratio of the offspring, delay female sexual maturation, and deteriorate the sperm quality of F1 generation males.

Key words: Di-n-butyl phthalate; Paternal exposure; Pre- and postnatal effects; Male-mediated developmental toxicity

INTRODUCTION

Phthalate esters are a large group of chemical agents used predominantly as plasticizers and solvents. Globally, more than 18 billion pounds of phthalates are used each year in the world[1]. They are used in consumer products such as shampoos, soaps, cosmetics, as well as in plastic goods, paint, toys, wood finishes, drug coatings, and particle formations[2-5]. Human exposure to phthalates can occur either orally, dermally or via inhalation[6]. Numerous reports suggest that most of population in the United States is exposed to phthalates[6-8]. Additionally, some reports suggest that phthalate exposure is also prevalent in European countries, such as Denmark

and Germany[9].

Di-n-butyl-phthalate (DBP) is one of the most common phthalates. Potential sources of DBP exposure are cellulose acetate plastics, personal care products (e.g. nail polish, perfumes, and lacquers), varnishes and coatings (e.g. pharmaceuticals), and food packing materials[9]. As Koo and Lee[10] reported, DBP was detected in 19 out of 21 nail polish and in 11 out of 42 perfumes tested, with an exposure level ranging from 444.6 to 1671.1 μg/mL. DBP leaches from many products and has become a common contaminant that is present in the environment, including air, water and soil[11]. Occupational exposure to DBP during phthalate manufacturing is estimated to be around 143 μg/kg bw/day, and at least 10 μg/kg bw/ day in the general population[12].

DBP, similar to other phthalates, belongs to a group of environmentally endocrine-active chemicals known to disrupt the development of the male reproductive tract in rodents[13-16]. Jobling et al.[17] suggest that DBP could reduce the binding of oestrogen to oestrogen receptors, and inhibit its transcriptional activity. DBP acts as a reproductive toxicant in male, but not in female laboratory animals[14-15, 18]. Colburn and Clement[19] suggested that the effects of DBP on the reproductive system would be greater in the second generation of animals. This suggestion was confirmed by Wine et al.[20], where oral DBP administration appeared to affect the second generation more than the first generation, and most F1 generation males were found to be infertile. Additionally, it was found that there is a larger risk of reproductive and developmental toxicity following gestational and lactational DBP exposure in comparison exposure in adulthood[21]. It was also reported that DBP at higher doses induces teratogenicity in rodents[22-23]. However, no studies to date have investigated the effects of paternal DBP exposure on the development of the reproductive system in offspring. Thus, the main aim of the present study was to investigate the effects of pre-conceptional, 8-week, paternal exposure to DBP during the full spermatogenic cycle on the development of F1 and F2 generation offspring. We studied pre- and postnatal effects of paternal DBP exposure on the F1 generation, including quantity and quality of sperm, and the intrauterine development of foetuses in the F2 generation

MATERIALS AND METHODS

Animals and Husbandry

Eight week old Pzh:Sfis outbred male mice (n=20) were housed in plastic cages in a room controlled for temperature (22 °C), humidity (55%) and light cycle (12 h light/12 h dark). Tap water and food were provided *ad libitum*. All of the procedures outlined in the present study followed the guidelines of the Ethical Commission for Animal Studies.

There were three groups: i) mice treated with DBP at a dose of 500 mg/kg bw (i.e. 1/16 LD_{50}), ii) mice treated with DBP at a dose of 2 000 mg/kg bw (i.e. 1/4 LD_{50}), and iii) control mice. DBP-treated mice were administered 0.1 mL of DBP solution in olive oil and control mice were given olive oil alone via gavage thrice a week for 8 weeks. The LD_{50} of orally administered DBP ranges in mice from approximately 5 to 16 g/kg bw. In a pilot study, we determined that the LD_{50} of DBP in our mouse line was 8 000 mg/kg bw. Immediately after 8-weeks of DBP exposure, which coincided with a full spermatogenesis cycle, male mice from the control and DBP-treated groups were caged with two unexposed, virgin females for one week. Mice were examined daily for the presence of a vaginal plug, which represented day 0 of pregnancy. Three-quarters (n=30) of the mated female mice were sacrificed by cervical dislocation a day prior to parturition. The other quarter of females (n=10) had delivered and reared their litters.

Dominant Lethality and Congenital Malformations: Prenatal Effects of DBP

The standard protocol for the dominant lethality assay[24] with additional modifications[25] was followed, as previously described. Females were sacrificed a day prior to expected parturition. Each male that mated with at least one female was defined as fertile. Each female with at least one living or dead implantation was defined as pregnant.

Females were examined for the number of implantations, the number of living foetuses, and the number of early and late post-implantation deaths. Post-implantation deaths were classified as early, if the embryo died and was resorbed, or late, if the dead embryo was at a stage beyond the onset of organogenesis.

Dominant lethal mutations (DLM) were calculated according to the following formula:

$$\%DLM = \left[1 - \frac{\text{living embryos/pregnant treated female}}{\text{living embryos/pregnant control female}}\right] \times 100\%$$

Living embryos were weighed and analysed for the presence and type of gross malformations (e.g. exencephaly). Mice were considered to be runts if

the living foetuses had body weights that were 75% lower than the mean of their littermates[26]. All of the malformed foetuses and half of the normal foetuses were randomly selected from each group and assessed for skeletal malformations following Alcian blue and Alizarin red staining.

Effects of DBP on Postnatal Development of the F1 Generation

Pups in each litter were counted and weighed at birth. Body weights were recorded weekly until pups were 8 weeks of age. Additionally, pups were assessed for developmental markers and growth parameters. Mortality was recorded from birth until pups were 8 weeks of age, and the percentage of mortality was calculated as follows:

$$\% \text{ Mortality} = \frac{\text{Total number of deaths}}{\text{Number of live births}} \times 100\%$$

The mean body weights (g) of each litter and treatment group were calculated weekly. Pups weighing less than 2 standard deviation of the mean body weight of the control group were considered growth-retarded[27]. The percentage of growth-retarded pups was calculated as follows:

$$\% \text{ Growth-retarded pups} = \frac{\text{Number of growth retarded pups}}{\text{Total number of live pups}} \times 100$$

The F1 generation mice were assessed for developmental markers, such as fur development, pinna detachment, eye opening, vaginal opening, and testes descent. Pinna detachment was recorded as the age (in days) at which the pinna of both ears unfolded into a fully erect position. Eye opening was defined as any visible break in the membrane covering the eye. Vaginal opening was defined as any visible break in the membrane when the vaginal lips were gently pulled laterally. Testes descent was recorded when the testes descended to lie in the scrotal sac[27].

Effects of DBP on Gonads and Gametes of F1 Males

Sperm Count   At 8 weeks of age, five F1 generation males from each group were weighed and sacrificed. Both the testes and epididymides were removed and weighed. An epididymis from each mouse was macerated in 0.2 mL of 1% of trisodium citrate for 5-8 min and minced. Then, the solution was brought up to 2 mL and mixed for about 1 min. The sperm suspension was diluted in 10% buffered formalin in a 1:1 ratio. The spermatozoa were counted using an improved Neubauer haemocytometer[28-29].

Sperm Motility   The second epididymis was placed into 0.2 mL of warm (37 °C), physiological saline. An aliquot was placed on a warm (37 °C) microscope slide and covered with a cover slip. Two-hundred cells per animal were evaluated for motility within 5 min after the sacrifice[30].

Sperm Morphology   The remaining sperm were mixed in saline. The frequency of morphologically abnormal spermatozoa was determined, as per the procedure described by Wyrobek and Bruce[31]. Smears were prepared on microscope slides, air-dried overnight and stained with eosin Y. Then, 500 spermatozoa per mouse were investigated for abnormal sperm heads (e.g. lacking hook, amorphous, banana-shaped head) using a light microscope.

Comet Assay   A testis from each animal was decapsulated, placed in RMPI 1 640 medium and minced with scissors. Prior to using the cells, tubes were shaken to ensure that cells remained in suspension. The comet assay procedure of Singh et al.[32] and Anderson et al.[33] was used. In an Eppendorf tube, 5 μL of the cell suspension was mixed with 75 μL of low melting point agarose (LMA) for sample embedding on slides previously coated with normal melting point agarose (NMA). Slides were immersed in lysing solution overnight at 4 °C. Then, slides were placed in a gel electrophoresis tank, and left in solution for 20 min. Gel electrophoresis was conducted at 4 °C for 20 min using the following settings: a voltage of 19V and a current of 300 mA. After neutralisation, slides were stained with ethidium bromide (EtBr) and examined under a fluorescence microscope. One-hundred images of randomly selected cells were analysed from each animal using the CASP image-analysis program[34]. DNA tail moment was assessed, as it functions as a marker of DNA damage.

Effects of DBP on Prenatal Development of the F2 Generation

At 8 weeks of age, F1 males from the control and DBP treatment groups were mated with two females from the same group, but from different litters, for one week. Females were sacrificed a day prior to expected parturition, and the uterus was removed and examined, as described above, with the exception of skeletal analyses.

Statistical Analysis

Statistical analysis was performed by using a Student's *t*-test, Fisher test and Chi-square test to

determine whether differences among groups were statistically significant. A *P* value of <0.05 was considered statistically significant.

RESULTS

Effects of DBP on Prenatal Development of the F1 Generation

The effects of preconceptional DBP exposure on the reproductive ability of males, as well as prenatal survival and mortality of their offspring are presented in Table 1. Eight weeks of DBP exposure (2 000 mg/kg bw) decreased male fertility, and as a result, reduced the percentage of successful pregnancies. However, these findings were not significantly different from the control group. The mean number of implantations and living foetuses per pregnant female were the lowest in the group treated with DBP at a dose of 2 000 mg/kg bw, however, there were no significant differences compared to control. In all groups, including control, the majority of foetuses that died, did so during the first 11 days of pregnancy (i.e. early deaths). In the group with paternal exposure to DBP at a dose of 2 000 mg/kg bw, all of the dead foetuses were classified as early deaths. The proportions of dominant lethal mutations in the groups with paternal exposure to DBP at a dose of 500 mg/kg bw and 2 000 mg/kg bw were -4% and 10%, respectively.

The effects of paternal DBP exposure on the induction of gross and skeletal malformations in foetuses are presented in Table 2. The incidence of gross congenital malformations was rare. Surprisingly, the percentage of malformed foetuses was the greatest, although not significantly different, in the control group. The most frequent malformation was growth retardation (i.e. runts), and one foetus had a bent tail in the control group. The percentage of abnormal skeletons was the lowest in the 1/16 LD_{50} DBP group, and the highest in the 1/4 LD_{50} DBP group. Although the percentage of abnormal skeleton was approximately twice as high as the control group, there was no significant difference among all groups. Additionally, there were no significant differences in the body weights of living foetuses of all groups.

Table 1. Effects of Paternal DBP Exposure on Prenatal Development of the F1 Generation

Dose	% of Fertile Males	% of Pregnant Females	No of Implantations/ Pregnant Female± SD	No of Live Foetuses /Pregnant Female ± SD	No of Dead Foetuses/ Pregnant Female±SD	% of Early Deaths	% of Late Deaths	% DLM
Control	100	87	10.18 ± 1.33	9.76 ± 1.35	0.42 ± 0.52	2.88	1.44	-
500 mg/kg bw DBP	90[NS]	85[NS]	10.43 ± 3.03[ns]	9.82 ± 2.24[ns]	0.61 ± 1.06[ns]	5.42	1.25	-4
2 000 mg/kg bw DBP	86[NS]	69[NS]	9.42 ± 3.13[ns]	9.00 ± 3.35[ns]	0.42 ± 0.69[ns]	4.57	0.00	10

Note. [ns]-not significantly different from control, as determined by the Student's *t*-test. [NS] -not significantly different from control, as determined by the chi-square-test (numbers of pregnant and non-pregnant females in control vs. DBP-treated groups).

Table 2. Paternal DBP Exposure, and the Induction of Gross and Skeletal Malformations in Surviving Foetuses

Dose	Mean Body Weight of Living Foetuses (g)	% Abnormal Foetuses	Type of Gross Malformations	% of Abnormal Skeletons	Type of Skeletal Malformations
Control	1.28	1.5	2 runts: 69.6 %, 71.8 %; 1 bent tail	3.60	missing rib – 3; rudimentary rib - 1
500 mg/kg bw DBP	1.27	0.9[ns]	2 runts: 64.2%, 67.3 %;	2.65[ns]	extra rib – 3;
2 000 mg/kg bw DBP	1.22	0.6[ns]	1 runt: 65.5%	7.45[ns]	extra rib – 7;

Note. [ns]-not significantly different from control, as determined by the Fisher test.

Effects of DBP on Postnatal Development of the F1 Generation

The effects of paternal DBP exposure on postnatal body weights and growth retardation of pups are presented in Table 3. At birth and 1 week of age, there were no significant changes in offspring body weights of DBP exposed and not exposed males. Growth-retardation of pups was also rare. At 2 and 3 weeks of age, the mean offspring body weights of males exposed to DBP were significantly lower. At 4 and 5 weeks of age,

the mean offspring body weights of DBP exposed and not exposed males were not significantly different compared with the control. At 3, 4, and 5 weeks of age, the highest percentage of growth-retarded pups in both paternally DBP exposed groups (i.e. 10.11% to 22.34%) were observed. Between 6 and 8 weeks of age, there were no significant differences in the offspring body weights of DBP exposed and not exposed males, and only a few pups were growth-retarded.

The effects of paternal DBP exposure on litter size, postnatal mortality, and appearance of developmental markers in the offspring are presented in Table 4. Mean litter size was the largest, although not significantly different, in the group treated with DBP with a dose of 500 mg/kg bw. However, the percentage of mortality was also greater, but not significantly different, in this group than in any other group. Furthermore, there were almost twice as many males than females born in

Table 3. Changes in Postnatal Body Weights (g) and Percent of Growth-retarded Pups Resulting from Paternal Exposure to DBP

Time after Birth	bw/ % g-r	Paternal Dose		
		Control	500 mg/kg bw DBP	2 000 mg/kg bw DBP
at birth	bw	1.68 ± 0.17	1.71 ± 0.21^{ns}	1.75 ± 1.22^{ns}
	% g-r	0	3.13^{ns}	1.10^{ns}
1 week	bw	4.38 ± 0.71	4.35 ± 0.87^{ns}	4.42 ± 1.04^{ns}
	% g-r	0	3.19^{ns}	1.10^{ns}
2 weeks	bw	6.67 ± 0.91	$6.28 \pm 0.92^{*}$	6.36 ± 1.55^{ns}
	% g-r	0	6.38^{ns}	7.69^{*}
3 weeks	bw	9.95 ± 1.48	$9.29 \pm 1.98^{*}$	$8.92 \pm 2.57^{**}$
	% g-r	0	19.15^{***}	17.78^{***}
4 weeks	bw	16.57 ± 2.27	15.56 ± 3.82^{ns}	15.73 ± 3.69^{ns}
	% g-r	2.90	22.34^{***}	15.73^{**}
5 weeks	bw	22.47 ± 2.36	21.10 ± 3.80^{ns}	21.74 ± 3.53^{ns}
	% g-r	1.45	20.43^{***}	10.11^{ns}
6 weeks	bw	25.36 ± 3.03	24.88 ± 3.74^{ns}	25.22 ± 3.54^{ns}
	% g-r	0	6.45^{ns}	0
7 weeks	bw	27.61 ± 3.39	27.35 ± 3.87^{ns}	27.57 ± 3.84^{ns}
	% g-r	0	3.22^{ns}	0
8 weeks	bw	28.89 ± 3.32	28.89 ± 3.76^{ns}	28.92 ± 3.83^{ns}
	% g-r	1.45	3.23^{ns}	1.75^{ns}

Note. % g-r-percentage of growth-retarded pups. ^{ns}-not significant, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control, as determined by either a Student's t-test (body weight) or a Chi-square test (growth retardation).

Table 4. Effects of Paternal Exposure to DBP on Postnatal Mortality and Appearance of Developmental Markers in Pups

Paternal Dose	Mean Litter Size	Percent of Mortality	Mean Female: Male sex Ratio	Time of Appearance, Days (mean $\pm$ SD)				
				Pinna Detachment	Fur Development	Eye Opening	Vagina Opening	Testes Descent
Control	8.9±1.05	1.43	52:48	4.67 ± 1.12	5.11 ± 0.60	13.63 ± 1.93	23.58 ± 2.01	22.52 ± 1.23
500 mg/kg bw DBP	9.6 ± 2.01^{ns}	4.17^{NS}	36:64	4.70 ± 0.48^{ns}	5.10 ± 0.74^{ns}	$14.43 \pm 1.06^{**}$	$26.14 \pm 3.31^{**}$	22.93 ± 2.22^{ns}
2000 mg/kg bwDBP	9.1 ± 2.33^{ns}	3.30^{NS}	49:51	4.20 ± 1.03^{ns}	5.10 ± 0.74^{ns}	$14.64 \pm 2.36^{**}$	23.93 ± 2.98^{ns}	23.02 ± 1.86^{ns}

Note. Student's t-test: ^{ns}-not significant, $^{**}P<0.01$ vs. control. Chi-square test: ^{NS}-not significant.

the group treated with DBP at a dose of 500 mg/kg bw. The female:male ratio was approximately 50:50 in the control and 2 000 mg/kg bw of DBP-treated groups. There were no significant differences in the time of pinna detachment, fur development and testes descent between experimental and control groups. Eye opening was delayed by one day in both DBP-treated groups ($P<0.01$). There was roughly a 2.5-day delay in vaginal opening of pups in the 1/16 LD_{50} DBP-treated group ($P<0.01$).

Effects of DBP on Gonads and Gametes of F1 Males

Mean and relative testes and epididymal weights of 8 weeks old, F1 generation males are presented in Table 5. Mean and relative testes weights were greater in offspring of males exposed to 500 mg/kg bw of DBP. Conversely, mean epididymal weights were the lowest in this group. However, the relative epididymal weights were similar among all groups.

Table 5. Mean Testes and Epididymidal Weights of F1 Males Following 8 weeks of Paternal Exposure to DBP

Dose	Mean Body Weight (g)	Mean Testes Weight (mg)±SD	RTW [%]	Mean Epididymides Weight (mg)±SD	REW [%]
Control	31.99	198.8±22.8	0.62	42.8±7.3	0.13
500 mg/kg bw DBP	30.22	215.8±16.9[ns]	0.71	40.2±6.8[ns]	0.13
2000 mg/kg bw DBP	30.97	186.0±27.9[ns]	0.60	41.2±5.7[ns]	0.13

Note. Relative testes weight (RTW) $= \dfrac{\text{Testes weight}}{\text{Body weight}}$ x100. Relative epididymes weight (REW) $=$ $\dfrac{\text{Epididymides weight}}{\text{Body weight}}$ x100. *Student's t - test :* [ns]-not significantly different from control.

Sperm count and quality of F1 males are presented in Table 6. Sperm counts were similar among all groups. The percentage of mobile spermatozoa was surprisingly increased in a dose-dependent manner of DBP, although not significantly different from the other groups. The percentage of morphologically abnormal sperm heads in the offspring of males exposed to 500 mg/kg bw of DBP was similar to the control group, whereas offspring of males exposed to 2 000 mg/kg bw of DBP had an increase of about 5% (i.e. 1.6 times) compared with control group ($P<0.001$ by Chi-square test). DNA damage, determined via DNA tail moment, was significantly decreased in offspring of males exposed to 500 mg/kg bw of DBP.

Table 6. Sperm Quantity and Quality of F1 Male Mice after 8 Weeks of Paternal Exposure to DBP

Dose	Sperm Count x10^6±SD	Percent of Motile Spermatozoa±SD	Percent of Abnormal Spermatozoa ±SD	Tail Moment
Control	2.04±0.52	75.00±8.08	7.90±2.67	4.53±2.08
500 mg/kg bw DBP	2.05±0.54[ns]	79.16±10.69[NS]	7.72±3.10[NS]	1.66±1.16[*]
2000 mg/kg bw DBP	2.16±1.01[ns]	81.00±13.70[NS]	12.76±3.25[###]	4.42±3.02[ns]

Note. Chi-square test: [NS]-not significant, [###]$P<0.001$ vs. control. Student's *t*-test: [ns]-not significant, [*]$P<0.05$ vs. control.

Effect of DBP on Prenatal Development of the F2 Generation

The effects of paternal DBP exposure of on the reproductive outcome of the F1 generation and intrauterine viability of the F2 generation are presented in Table 7. All males in the F1 generation were fertile. The lowest percentage of pregnant females were noted in the paternal DBP exposed group treated with 500 mg/kg bw of DBP, however this finding was not significantly different from the other groups. The lowest mean number of total and live implantations was in the group exposed to DBP at a dose of 2 000 mg/kg bw, but this finding was not statistically significant. There were no significant differences in the incidence of dead foetuses.

Table 7. Effects of Paternal DBP Exposure on Prenatal Development of the F2 Generation.

Dose of F0 Males	% of Fertile Males	% of Pregnant Females	No of Implantations/ Pregnant Female±SD	No of Live Foetuses± SD	No of Dead Foetuses/Pregnant Female±SD	% of Early Deaths	% of Late Deaths	% DLM
Control	100	92	11.55 ± 1.35	10.97 ± 1.35	0.58 ± 0.52	4.16	0.78	-
500 mg/kg bw DBP	100	82[NS]	11.43 ± 3.03[ns]	11.04 ± 2.24[ns]	0.39 ± 1.06[ns]	2.19	1.25	-1
2 000 mg/kg bw DBP	100	93[NS]	11.03 ± 3.13[ns]	10.47 ± 3.35[ns]	0.59 ± 0.69[ns]	4.90	0.49	5

Note. [ns]-not significantly different from control, as determined by the Student's *t*-test. [NS]-not significantly different from control, as determined by the Chi-square-test (number of pregnant and non-pregnant in control vs. DBP-treated mice).

DISCUSSION

During the past decade, numerous studies have examined the developmental and reproductive effects of a wide range of synthetic chemicals on wildlife populations. Several research groups have expressed concern regarding to potential adverse effects of environmental contaminants, such as di-n-butyl phthalate (DBP), di-2-ethylhexyl phthalate (DEHP), and benzyl butyl phthalate (BBP), on human health and reproduction. Animal studies on phthalates found that they have anti-androgenic properties, which disrupt Leydig cell function and interfere with normal reproductive development[14-15, 35-38]. Until now, research on absorption and metabolism of phthalates is equivocal. Harris et al.[39] noted that phthalates are metabolized into their corresponding monoesters, which are less active in *in vivo* conditions. Kavlock et al.[40] indicated that there is no bioaccumulation of DBP or its metabolites.

DBP markedly affects the development of the male reproductive tract. Earlier studies on adult rats have indicated that Sertoli cells are potential targets of phthalate esters[17,41-43]. Sertoli cells do not proliferate following puberty, therefore damage to their cellular function may affect spermatogenesis[44-48]. Previous studies on DBP found that it affects testosterone-mediated reproductive development[20, 49].

Currently, the effects of paternal DBP exposure prior to mating on offspring and developmental toxicity remain unknown. Numerous studies have assessed the effects of DBP exposure on the development of offspring, however these studies focused on the effects of maternal DBP exposure during pregnancy or lactation. Furthermore, studies have found that DBP has anti-androgenic effects on male rat offspring exposed to DBP *in utero*[50-52]. *In utero* DBP exposure induces cryptorchidism, hypospadias, decreases the sperm count in adulthood,

and decreases testosterone production[14, 37, 53]. Ema et al. noted[23, 51, 54-56] that DBP exposure of pregnant female rats results in an increased incidence of pre-implantation or post-implantation loss and teratogenicity.

In the present study, the effects of paternal DBP exposure on pre- and postnatal development of the offspring were investigated. This is the first study to assess the effects of paternal DBP exposure during the full spermatogenesis cycle on the offspring. Eight weeks of DBP exposure did not affect the total and living implantation. This finding corroborated that our other finding, that is that there were no effects of paternal DBP exposure on the mean sperm quantity and quality[57].

The pups of males exposed to DBP had poor development. Paternal DBP exposure resulted in a reduction of the mean body weights at 2 and 3 weeks of postnatal life, and growth retardation between 2 and 5 weeks of age. The time course of body weight differences suggests that there may be differences during the switch from maternal milk to solid food. However, defects in sperm DNA or chromosomes may also explain the decreased viability of foetuses, and increased risk of health problems[58].

There were approximately twice the number male to female offspring in the group where paternal DBP exposure was induced at a dose of 500 mg/kg bw. This finding is unusual because the typical sex ratio of males to females is 50:50, which was found in litters of males not exposed to DBP or exposed to the highest dose of DBP. Unusual sex ratios at birth indicate that there was something unusual in the hormonal levels of fathers at the time of conception. Interestingly, James[59] suggested that high levels of oestrogen and testosterone are associated with increases in birth of males. In the present study, the offspring of DBP-exposed males demonstrated a delay in the appearance of developmental markers (i.e. eye opening, vaginal

opening), suggesting that there may be an abnormality in the germ cells of males exposed to DBP. In another study, it was found that eye opening of rat pups was not affected by female DBP exposure during mating, pregnancy, and lactation[60]. In the present study, paternal exposure to DBP seems to be responsible for the delayed sexual maturation of females. Lee et al.[61] reported that after DBP exposure from late gestation to lactation, female rat offspring have a slight delay in the onset of puberty. Conversely, Mylchaest et al.[14] reported that maternal DBP exposure from gestation to lactation did not affect vaginal opening of the offspring. Similarly, in another study, administration of DBP to female rats from weaning did not affect the onset of vaginal opening[62]. There was an approximate 2.5-day delay in the vaginal opening of offspring of males treated with DBP at a dose of 500 mg/kg bw. Taken together, the lower number of female born and delayed sexual maturation of females may suggest that the effects of paternal DBP exposure at a lower dose is reflected in the F1 generation. Thus, eight weeks of DBP exposure may affect the X chromosomes of males, which in turn may reduce the pre-implantation of eggs fertilized with spermatozoa carrying the X chromosome.

Sperm count and motility of F1 generation males were not affected; however, the percentage of abnormal spermatozoa of offspring of males treated with a dose of 2 000 mg/kg bw of DBP was increased 1.5-fold in the comparison to control males. Thus, F1 generation males were fertile and the increased number of abnormal spermatozoa did not affect the *in utero* viability of the F2 generation offspring.

Thus, the findings of the present study suggest that paternal exposure to DBP during the full spermatogenesis cycle may cause genetic defects in male gametes, which in turn may affect the reproductive system of the offspring. Paternal exposure may also result in delayed sexual maturation of female offspring, and slightly deteriorate sperm quality of male offspring. These findings should be taken into consideration, especially in populations at risk of high phthalate exposure (i.e. DBP), as these agents may affect future generations.

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