ORIGINAL ARTICLE

Two generation reproductive and developmental toxicity following subchronic exposure of pubescent male mice to di(2-ethylhexyl)phthalate

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▌Abstract

Di-(2-ethylhexyl)phthalate (DEHP) is widely present in the human environment.
The study aimed at the investigation of potential genotoxic effects induced by subchronic exposure to DEHP in germ cells of male mice in the first period of puberty, and to check if the transmission of mutation to the next generation *via* the sperm is possible. 8-weeks exposure to 2,000 mg/kg and 8,000 mg/kg of DEHP diminished sperm count and quality, leading to a reduced percentage of pregnant females mated to exposed males. A slight increase in the frequency of prenatal deaths and dominant lethal mutations, as well as a significantly increased percentage of abnormal skeletons among the F1 offspring of males exposed to 8,000 mg/kg of DEHP, were observed. Exposure of the fathers did not cause a delay in the postnatal development of the offspring, except for fur development in the group of 8,000 mg/kg of DEHP. Gametes of male offspring of exposed fathers showed reduced motility. The results may suggest that diminished spermaozoa quality induced by DEHP may be coincidental with mutations leading to intrauterine deaths and skeletal abnormalities in the offspring.

▌Key words

DEHP, spermatozoa, male-mediated effects, progeny, mice

INTRODUCTION

Humans are increasingly exposed to various xenobiotic chemicals included in many products, such as pesticides and plastics [1]. Phthalate esters (esters of 1,2 benzenedicarboxylic acid) are a large group of chemical agents used as plasticizers to increase the flexibility and workability of high molecular–weight polymers. In some plastics, phthalates constitute up to 50% of the total weight. A quarter of the phthalates produced in the world is di(2-ethylhexyl)phthalate (DEHP) [2].

DEHP is a liquid of low volatility, widely used as a plasticizer in flexible polyvinyl chloride products (PVC). Plastics may contain from 1-40 % DEHP by weight and are used in consumer products such as artificial leather, waterproof clothing, footwear, upholstery, floor tiles, various types of furnishing, industrial tubing, wires and cables, tablecloths, shower curtains, food packing materials, children's toys, and a variety medical devices. DEHP is also used as a hydraulic fluid and dielectric fluids [3, 4]. Other uses are in rubbing alcohol, liquid detergents, decorative inks, industrial lubricating oils, and deforming agents during paper and paperboard manufacture [5].

DEHP is ubiquitous in the general environment as a result of its widespread manufacture, use and disposal, as well as their high concentration in plastics and ability to migrate from them [6]. DEHP leaks out from the plastics into the air, water and ground, and then enters foodstuffs [7, 8]. DEHP enters the human body also from blood storage bags, catheters and haemodialysis instruments, and DEHP residue concentration has been found in the blood and tissues of patients after numerous transfusions [9-12]. The important potential health effects of DEHP come from children's exposure to toys and other sources, and in the case of pregnant females, from dialysis treatment or blood transfusions [13].

Phthalic acid esters have been shown to reduce fertility and induce testicular atrophy in laboratory animals [7] due to its endocrine disrupting activity, which is capable of perturbing the reproductive process by mimicking or antagonizing steroid action [14]. It has been shown that the 'endocrine disrupting' potential of DEHP is directly associated with the synthesis of sex hormones. DEHP alters the expression of genes associated with testis development and steroid hormones synthesis [7, 15-16]. Both *in vivo* and *in vitro* experiments have demonstrated that the Sertoli cells are the primary site of phthalate-induced testicular toxicity. Sertoli cells alternations lead to progressive degeneration of spermatocytes and spermatids.

DEHP is a known reproductive and developmental toxicant in animals [17-18]. Effects on young and adult rodents include

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reduction of testosterone and sperm production, reduction in testis and epididymes weights, and pathological effects in the testis [18-22]. As Sjöberg et al. [12] reported, immature rats were much more sensitive to gonadal effects, induced following oral administration of DEHP, than adults; in some cases the occurrence of the testicular effects appeared earlier in young animals. Dominant lethal effects were reported to be induced in male mice [23].

The present study was designed to examine the potential genotoxic effects induced in male mice in the first period of puberty following subchronical exposure to DEHP, and to investigate the possibility of the transmission of mutations to following generations *via* the sperm.

MATERIALS AND METHODS

Animal husbandry. Outbreed Pzh: SFIS mice obtained from the Laboratory of Animal Breeeding 'Górzkowska' (Warsaw, Poland) were used in the study. Animals were housed in standard rodent cages in a room designed for the control of temperature and humidity, and the light cycle. Tap water and rodent diet were available *ad libidum*.

The authors obtained permission from Ethical Commission for Animal Experiments to conduct this study (Permission No. 3/03, 4th Local Commission, Warsaw).

30-35 days old Pzh:SFIS outbreed male mice were exposed by gavages to DEHP suspension in olive oil for 8 weeks, 3 days per week. The doses of DEHP were 2,000 mg/kg bw (1/16 LD_{50}) and 8,000 mg/kg bw (1/4 LD_{50}) daily. The doses were chosen on the basis of LD_{50} values for DEHP listed in IARC [24]. Control animals received olive oil only. The volume of administered suspension was 0.1 ml per mouse.

Study of effects in exposed generation. For this study, 5 males per group were weighed and sacrificed in the middle of exposure, at 24 h and at the 4th week after the last treatment. Both testes and epididymides were removed from each male and weighed.

One epididymis was macerated in 0.2 ml of 1% solution of trisodium citrate for 5-8 min. and minced. The solution was then 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 improved Neubauer haemocytometer [25-28].

The contents of the second epididymis were 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. 200 cells per animal were evaluated for motility within 5 min. after the killing of the animal according to Working et al. [27].

The remaining sperm was distributed evenly in the saline. The study of frequency of morphologically abnormal spermatozoa was performed according to the procedure described by Wyrobek and Bruce [28]. Smears were prepared on microscope slides, air-dried overnight, and stained with eosin Y. 500 spermatozoa per mouse were analysed using a light microscope, and abnormal sperm heads (e.g. lacking hook, amorphous, banana-shaped head) were recorded.

For Comet assay, 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 single cells remained in the suspension. Basic technique of Singh et al. [29], further described by Anderson et al. [30] was used. 5 μl of cell suspension was mixed in an eppendorf tube with 75 μl low melting point agarose (LMPA) for embedding on slides covered previously with normal melting point agarose (NMPA). The slides were immersed in lysing solution overnight at 4°C. They were then drained, placed in a gel electrophoresis tank, and left in the solution for 20 min. The electrophoresis was conducted at 4°C for 20 min. using 24V and 300 mA. After neutralisation, the slides were stained with EtBr and examined using a fluorescence microscope. Images of 100 randomly selected cells from each animal were recorded and analyzed using CASP image-analysis software [31]. The DNA tail moment was chosen as the parameter for further analysis.

Effects in offspring of exposed males. Immediately after the end of 8-weeks exposure, 20 males from control as well as each of experimental group were caged for 1 week with 2 unexposed, virgin females. They were checked daily for the presence of vaginal plug, which determined day 0 of pregnancy. Three-quarters (30) of the mated females from each group were humanely killed 1 day before parturition. The other quarter (10) of the females from each group were allowed to deliver and rear their litters.

Study of dominant lethal and congenital malformation. The standard protocol for dominant lethal assay [32] with modifications proposed by Anderson et al. [33] was used.

Each male that mated with at least one female was defined as fertile. Each female with at least one live or dead implantation was defined as pregnant.

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

The dominant lethal mutations (DLM) were calculated according to the formula:

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

Live embryos were weighed and analysed for presence and type of gross malformations (e.g. exencephaly). Runts were defined as live foetuses having a body weight of less than 75 % of the mean of their litter mates [34]. After Alcian blue and Alizarin red staining, malformed foetuses and a randomly selected half of the number of normal foetuses from the exposed and control groups were processed for skeletal malformations.

Effect on postnatal development of F1 generation. Pups of females having parturition (5-7 litters from each group) were counted and weighed at birth, and then weighed weekly up to 8 weeks of age. They were observed for developmental markers and growth parameters.

Mortality was recorded from birth to the age of 8 weeks and percent of mortality was calculated as follows:

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

Mean body weight (g) of the individual litters and of each group was calculated weekly. Pups weighing less than

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2 standard deviations from the mean body weight of the control group were considered growth-retarded [35]. The percent of growth-retarded pups were calculated as follows:

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

Animals of the F1 generation were observed for developmental markers such as fur development, pinna detachment, eye opening, vaginal opening and testes descent. The appearance of pinna detachment unfolding was recorded as the age (in days) when pinnae of both ears unfolded to a fully erect position. Eye opening is 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 [35].

Five males from each group of the F1 generation were sacrificed at 8-9 weeks of age to check the weight of reproductive organs as well as the sperm count and quality. All parameters were estimated according to the methods described above for males of the F0 generation.

Statistical analysis. Statistical analysis was performed using ANOVA, Scheffe, Fisher and Chi-square tests. The results which showed statistical significance tested by ANOVA, were then tested by using Scheffe test.

RESULTS

The results of mean body, testes and epididymides weights, as well as the sperm quantity and quality of F0 male mice in the middle, following 8 weeks exposure and 4 weeks later, are shown in Table 1. The body weight of males exposed to 2,000 mg/kg DEHP was significantly reduced at 4 weeks after the end of exposure. There were no significant differences between testes as well as epididymides weight of exposed and unexposed males, except for males just after the end

of 8-weeks exposure to 8,000 mg/kg DEHP, when the epididymides weight was significantly decreased. There were no significant effects on the sperm count. Percentage of motile spermatozoa were significantly decreased at 24 h and 4 weeks after the termination of exposure to 8,000 mg/kg bw of DEHP. Eight-weeks exposure to DEHP significantly (approximately twice) increased the percentage of abnormal spermatozoa. Such results were not observed in the middle of exposure and 4 weeks following the end of exposure. Treatments with DEHP did not cause any statistically significant increase in DNA damage to germ cells; however, a slight enhancement in the tail moments after 8 weeks exposure were observed.

The results of male fertility and effects on the intrauterine survival of exposed males are shown in Table 2. There were no statistically significant effects of DEHP on male fertility nor on the frequency of pregnant females; however, the percentage of fertile males and pregnant females were reduced in the exposed groups. The mean number of implantations and the mean number of live foetuses were not significantly decreased in the groups exposed to DEHP. The mean number of dead foetuses in the group of 8,000 mg/kg bw of DEHP was approximately 2-fold higher than in the control group, although this was not statistically significant. The majority of dead foetuses constituted early deaths. The percentage of dominant lethal mutations induced by 2,000 m/kg and 8,000 mg/kg DEHP were calculated as 6 and 13, respectively.

The results of body weight, frequency and type of skeletal malformations in the F1 generation are shown in Table 3. There were no significant differences in body weight and incidence of gross malformations between unexposed and exposed male groups. The frequency of skeletal malformations was significantly increased (approx. 5-fold) in the group of 8,000 mg/kg bw DEHP.

The results regarding mean body weight and potential growth retardation of exposed and unexposed offspring of the males are shown in Table 4. The body weights of the offspring of unexposed and exposed to DEHP males were similar at birth and during the 8-weeks development.

Table 1. Mean reproductive organs weights and sperm quantity and quality after 8 weeks exposure of male mice to DEHP.

Dose	Time	Mean body weight (g) ±SD	Mean testes weight (mg) ±SD	Mean epididymides weight (mg) ±SD	Sperm count ×10⁶/ml ±SD	Percent of motile spermatozoa ±SD	Percent of abnormal spermatozoa ±SD	Comet tail moment
Control	4 weeks	34.63±0.74	214.6±20.5	42.8±6.7	2.75±0.60	79.6±3.29	3.76±0.93	1.33±1.03
2,000 mg/kg DEHP	4 weeks	32.85±2.10	201.4±22.9	44.4±6.1	2.03±0.59	82.2±4.71	3.92±1.35	1.80±1.10
8,000 mg/kg DEHP	4 weeks	34.22±2.25	198.8±19.2	45.6±6.0	2.08±0.33	81.0±12.92	4.44±0.99	1.51±0.82
Control	8 weeks	35.60±1.51	223.2±25.5	52.0±6.3	2.29±1.23	82.4±12.28	4.56±1.23	0.98±0.31
2,000 mg/kg DEHP	8 weeks	36.25±4.75	216±32.95	45.6±6.2	0.90±0.49	81.6±10.64	8.76±1.05[b]	1.90±2.41
8,000 mg/kg DEHP	8 weeks	33.53±2.31	196±19.10	41.6±1.3[a]	1.29±0.77	65.0±15.48[b]	9.44±2.89[b]	1.57±1.30
Control	8 + 4 weeks	39.62±2.19	234.2±14.9	53.2±7.3	2.91±0.53	73.0±15.28	5.64±1.33	3.96±3.35
2,000 mg/kg DEHP	8 + 4 weeks	35.82±1.77	208.0±41.0	54.2±6.9	2.79±0.67	71.6±11.93	5.92±1.95	4.40±3.33
8,000 mg/kg DEHP	8 + 4 weeks	38.67±1.38	213.4±42.3	55.4±4.3	2.67±0.51	63.8±14.32[b]	6.80±1.03	3.81±2.27

[a] p<0.05 compared to corresponding control by ANOVA and Scheffe tests.
[b] p<0.05 compared to corresponding control by Chi square test.

Table 2. Effects of DEHP on the reproductive outcome in mice after 8-weeks exposure of males.

Dose	% of fertile males	% of pregnant females	No. of implantations/ pregnant female± SD	No. of live foetuses per pregnant female± SD	No. of dead foetuses/ pregnant female's	% of early deaths	% of late deaths	% DLM
Control	94	72.0	10.25 ± 2.44	9.65 ± 2.33	0.59 ± 1.06	5.45	0.61	–
2,000 mg/kg DEHP	80	52.5	9.31 ± 4.13	9.08 ± 4.05	0.23 ± 0.44	0.83	1.65	6
8,000 mg/kg DEHP	80	55.0	9.43 ± 3.74	8.36 ± 3.97	1.07 ± 1.64	10.61	0.76	13

Table 3. Effects of sub-chronic paternal DEHP exposure on induction of gross and skeletal malformations of survival foetuses of mice.

Dose	Mean body weight of living foetuses (g)	% ab-normal fetuses	Type of gross malformations	% of abnormal skeletons	Type of skeletal malformations
Control	1.24	1.30	convex eyes - 2	2.59	missing rib – 1; retardation of development of dorsal arch of C1 vertebra (atlas) – 2.
2000 mg/kg DEHP	1.21	0	–	5.88	extra rib – 2; retardation of development of dorsal arch of C1 vertebra (atlas) – 2: concavity of skull bones (supraoccipitale and interparietal) – 1.
8000 mg/kg DEHP	1.17	0.97	abdominal hernia -1	13.92[c]	extra rib – 5; rudimentary rib – 2: retardation of development of dorsal arch of C1 vertebra (atlas) – 1: concavity of skull bones (supraoccipitale and interparietal) – 3.

[c] p<0.01 compared do corresponding control by Fisher test.

Table 4. The changes in postnatal body weight (g) and percentage of growth-retarded pups of males exposed to DEHP.

Time after birth	bw/ % g-r	Paternal dose		
		Control	2,000 mg/kg DEHP	8,000 mg/kg DEHP
at birth	bw	1.79 ± 0.34	1.66 ± 0.16	1.68 ± 0.26
	% g-r	0	0	0
1 week	bw	4.07 ± 0.59	4.31 ± 0.63	4.69 ± 0.93
	% g-r	0	1.64	0
2 weeks	bw	5.66 ± 0.89	5.82 ± 1.16	6.08 ± 1.31
	% g-r	0	0	0
3 weeks	bw	6.41 ± 0.87	6.72 ± 1.88	6.82 ± 1.63
	% g-r	0	1.64	0
4 weeks	bw	8.75 ± 2.14	9.49 ± 4.27	10.43 ± 4.73
	% g-r	6.52	0	0
5 weeks	bw	14.16 ± 3.57	14.83 ± 6.13	16.12 ± 6.64
	% g-r	2.56	3.77	10.71
6 weeks	bw	19.49 ± 3.91	19.7 ± 5.96	21.41 ± 5.8
	% g-r	2.56	9.43	8.00
7 weeks	bw	22.46 ± 3.57	23.58 ±4.76	24.12 ± 5.01
	% g-r	5.13	5.66	4.00
8 weeks	bw	24.9 ± 3.69	26.71 ± 4.28	25.86 ± 4.66
	% g-r	2.56	1.89	4.00

% g-r – percent of growth-retarded pups.

The results of mean litter size, postnatal mortality, and appearance of developmental markers in pups of exposed to DEHP males are shown in Table 5. Mean litter size of the offspring of males exposed to DEHP was not significantly reduced in a dose-related manner. Mean female:male sex ratios in exposed and unexposed groups were similar. There were no significant differences in the mortality between groups, nor in the appearance of developmental markers in pups of exposed and control males, with the exception of fur development. In the group 8,000 mg/kg bw of DEHP the fur development was significantly delayed. Testes descent in the offspring of males exposed to DEHP appeared not significantly earlier than in the control group.

Table 6 shows the results of mean body, testes and epididymides weights, as well as of sperm quantity and quality of the offspring of DEHP exposed and control males. There were no significant differences in the body, testes and epididymides weights in male of the F1 generation. Similarly, there were no significant changes in the sperm count of the offspring of exposed to DEHP and control males. The motilities of spermatozoa were significantly reduced in the males paternally exposed to DEHP. The frequency of abnormal spermatozoa and the level of DNA damages in male germ cells were similar in the control and experimental groups.

DISCUSSION

Phthalates operate by decreasing androgen levels. There are some periods in life in which androgen levels are critical for normal development: during foetal gonad differentiation, neonatal testis development, and final maturation and differentiation of the testis in puberty [36].

The epidemiological study by Swan *et al.* [37] showed an increase in male reproductive disorder among male infants following prenatal phthalate exposure; this suggests that phthalate exposure might carry reproductive health risks for humans and animals.

According to EU criteria, DEHP is classified as a reproductive toxicant, labelled as: Repro cat 2: R61 – May cause harm to the unborn child, and R60 – May impair fertility [38].

A number of papers describe the effects on offspring of maternal exposure to DEHP during pregnancy and lactation [39-41], the majority of which concern exposure of rats. There is relatively little published about developmental toxicity following paternal exposure to this phthalate, especially

Table 5. Postnatal mortality and appearance of developmental markers in pups of males exposed to DEHP.

Paternal dose	Mean litter size	Percent of mortality	Mean female/male sex ratio	Time of appearance - days (mean ± SD)				
				Pinna detachment	Fur development	Eye opening	Vagina opening	Testes descent
Control	9.67±1.97	4.10±4.96	56:44	4.50± 0.61	4.75 ± 0.52	15.22 ± 1.68	22.58 ± 2.3	29.4 ± 3.49
2,000 mg/kg DEHP	8.71±2.43	1.98±3.37	48:52	4.81 ± 0.37	5.07 ± 0.79	15.87 ± 1.56	23.36 ± 2.3	28.36 ±1.46
8,000 mg/kg DEHP	7.20±2.59	3.82±4.18	61:39	4.60 ± 1.14	5.90 ± 0.22[a]	14.90 ± 1.95	21.56 ± 1.2	27.9 ± 1.02

[a] p<0.05 compared to corresponding control by ANOVA and Scheffe tests.

Table 6. Mean testes and epidydymides weight in offspring of males exposed to DEHP Sperm count and quality in males of F1 generation.

Dose	Mean body weight (g) ±SD	Mean testes weight (mg) ±SD	Mean epididymides weight (g) ±SD	Sperm count ×10⁶/ml ±SD	Percent of motile spermatozoa±SD	Percent of abnormal spermatozoa ±SD	Comet score of DNA damage
Control	27.28±4.05	183.8±27.4	35.2±5.3	1.78±0.70	80.6±11.78	6.24±1.27	0.86±0.19
2,000 mg/kg DEHP	29.50±4.09	196.4±21.6	38.8±7.5	1.68±0.33	70.4±5.94[b]	6.68±1.37	1.32±0.77
8,000 mg/kg DEHP	28.94±4.34	195.4±23.3	37.6±5.7	1.76±0.57	73.4±18.94[b]	6.44±1.18	1.32±0.48

[b] p<0.05 compared to corresponding control by Chi square test.

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before conception. The production of morphologically and genetically healthy sperm is very important for the reproduction and development of offspring since sperm delivers the paternal genome to the oocyte.

In the present study, male mice in the first period of puberty were exposed to DEHP throughout whole spermatogenesis cycle, and were then mated with unexposed females. The effects induced in the F0 and F1 generations were investigated.

The main source of human exposure is through food contamination. The average daily exposure from food in the United States has been estimated to be about 0.3 mg/day per individual with a maximum exposure of 2 mg/day [42]. The other frequent route of human exposure is wide use of DEHP in the medical devices. DEHP was detected in whole blood at levels ranging from 16.8-46.1 mg/l, and in packed cells at levels ranging from 32.6-55.5 mg/l in PVC blood bags [42]. The doses used in animal studies are usually much higher compared to human exposure, and varied depending on the way of administration and type of exposure. The doses used in this study were similar to those described by other authors [24]. For example, Yagi *et al.* [43] exposed pregnant females to several doses between 1/30-1/1. Shiota and Mima [44] used doses of 500-8,000 mg/kg DEHP by i.p. injection and 250-2000 mg/kg by gavage. Dostal *et al.* [45] evaluated the effect of DEHP in male rats using oral doses of 10-2,000 mg/kg.

The reduction in sperm count, however not statistically significant, is in line with the results of earlier papers [46-48]. We also observed diminished sperm motility after sub-chronic exposure to 8,000 mg/kg bw of DEHP daily, and an increased percentage of malformed spermatozoa at 24 h following 8-weeks exposure to both doses of DEHP. Similarly, a reduction in epidydymal sperm density, as well as increased frequency of abnormal spermatozoa, were observed previously in rats [49] and in adult mature male mice [50]. The diminished sperm motility associated with increase in the level of malformed spermatozoa observed here, may lead to reduced fertility because it is known that malformed spermatozoa are usually less motile and have less ability to fertilize eggs [51]. The decreased percentage of fertile males and pregnant females, as well as reduction in the mean of total and live foetuses – however not statistically significant – most probably resulted from diminished sperm count and quality observed in this experiment.

More than 45 % of non-pregnant females may reflect an excess of unfertilized eggs or pre-implantation losses as a result of fertilization of oocytes by spermatozoa carrying irregular genetic material. Such defects in male germ cells might be induced during any stages of spermatogenesis. In this study, a marked, but not statistically significant decrease in the percentage of pregnant females was noted, in contrast to an earlier study on the adult males exposed to the same doses of DEHP [50].

The frequency of early deaths increased 2-fold with 14 % of DLM induced in the group of male exposed to 8,000 mg/kg bw DEHP. This finding may reflect tendencies towards a genetic effect. The foetuses losses observed in the dominant lethal test are usually caused by numerical and structural chromosome damage or lethal gene mutations delivered from the fertilizing spermatozoa [52]. Such mutations lead to death shortly after implantation [53], as occurred in this study. The frequency of abnormal skeleton increased in a dose-related manner, leading to a statistically significant result in the group of males exposed to higher

dose of DEHP. The highly increased percentage of gross and skeletal malformations were observed previously after maternal exposure to 1,000 mg/kg bw of DEHP daily [21, 39]. In an earlier study on the 8-weeks paternal exposure to DEHP of mature male mice, a reduction in the mean number of total and live implantations was noted after exposure to 8,000 mg/kg bw DEHP, but there were no effects on the induction of intrauterine deaths or on the frequency of gross and skeletal malformations in the surviving foetuses [50]. The enhanced frequency of abnormal skeletons observed in the offspring of males exposed to 8,000 mg/kg bw of DEHP might be the effects of changes induced in the genetic material of the male gametes before fertilization. Five days exposure of male rats to 10-2,000 mg/kg bw DEHP did not affect the number of total and live foetuses [54]. Intrauterine foetal deaths and increased level of gross and skeletal malformations were sometimes observed after exposure of pregnant females to DEHP [21, 39-40].

In this study, we observed a slight, not statistically significant reduction in the litter size of exposed males, and there were no changes in the percentage of mortality of the offspring of exposed and control males. In earlier studies, a differences in litter size after exposure of both sexes of mice to DEHP [55] and after exposure of female rats during pregnancy [22] were observed. The average litter size was reduced in rats paternally exposed to 20,000 ppm DEHP administered for 60 days [49]. In previous studies, no effects were noted on the number of pups born alive, and in their postnatal mortality in the offspring of exposed adult male mice [50], as well as in the infants of male and female rats exposed to DEHP [56].

Pups of control and exposed foetuses developed similarly, while those coming from males exposed to DEHP grew faster. The reason of this finding may be the bigger size of the litter of control males, but rather not reflecting the benefit of DEHP exposure.

The only difference in developmental markers was delayed appearance of fur development in the offspring of males exposed to higher dose of DEHP. This finding is unusual and difficult to interpret. Testes descent was slightly, but not statistically significantly accelerated, contrary to earlier results [50, 57]. The study by Noriega *et al.* [58] showed that pubertal administration of DEHP delayed the onset of puberty, and reduced androgen-dependent tissue weights in both Long-Evans and Sprague-Dawley male rats.

In this study, the male offspring of exposed males showed significantly decreased sperm motility. Previously, anogenital distance, sperm counts, and reproductive organ weights were reduced in F1 male offspring of female rats exposed from gestational day 8 to day 17 of lactation with 300 mg/kg/day DEHP [21]. DEHP reduced testis weight in the offspring of female KM mice exposed from 12-19 days of gestation with 500 mg/kg bw per day [59]. *In utero* and lactational exposure reduces sperm concentration and quality in the male offspring of rats [60-62].

The results of this paper show that exposure of male mice to DEHP in the first period of puberty causes similar effects in the F0 generation, but has rather less effect in the offspring than exposure of mature adult male rats. The only considerable effect seems to be enhanced frequency of skeletal anomalies in the offspring of younger males, which might to be the effect of defects in spermatozoa of their fathers. Nevertheless, the exposure of young adult male

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to DEHP may have harmful effects on their reproductive health, as well as on the development and health of their future offspring. Mutations induced by DEHP in male gametes might be transmitted to the next generations via the sperm. It is necessary to pay attention to the higher frequency of non-pregnant females mated to males exposed to DEHP, which may result from diminished sperm count and quality. In human populations, such an effect of DEHP on male gametes may be manifested as infertility of the couples, which nowadays is the growing problem in highly industrialized communities. As numerous authors have reported, the fertilization potential of males in Europe is highly diminished in recent years [63-65].

Contrary to our study, the results of earlier studies on rats have suggested that immature males were more sensitive for induction of testicular damages than older individuals [66-67]. The reason for the different response between younger and older animals, as well as between species, may also be caused by different metabolism of the phthalates.

CONCLUSIONS

The results of this paper support the idea that endocrine disruptors present in the human environment, such as DEHP, may contribute to reduced sperm quantity and quality, leading to diminished birth-rate in numerous populations. Particular attention should be paid by people medically exposed to high doses of DEHP, for instance, during haemodialysis or blood transfusions.

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REFERENCES

1. Iovdijova A, Bencko V. Potential risk of exposure to selected xenobiotic residues and their fate in the food chain. Part 1: Classification of xenobiotics. Ann Agric Environ Med. 2010; 17: 183-192.
2. Van Wezel AP, van Vlaardingen P, Posthumus R, Crommentuijn GH, Sijm DTHM. Environmental risk limits for two phthalates with special emphasis on endocrine disruptive properties. Ecotoxicol Environ Saf. 2000; 46: 305-321.
3. ATSDR. Toxicological profile for di(2-ethylhexyl)phthalate. Agency for Toxic Substances and Disease Registry, United States Public Health Service, Atlanta (GA) 1989.
4. Tanaka T. Reproductive and neurobehavioral toxicity study of bis(2-ethylhexyl) phthalate (DEHP) administered to mice in the diet. Food Chem Toxicol. 2002; 40: 1499-1506.
5. Environmental Protection Agency. Technical Factsheet on: Di(2-ethyl)Phthalate (DEHP). Office of Ground Water and Drinking Water, Washington DC 1998.
6. CIRC (Cosmetic Ingredient Review Committee): Final report on the safety assessment of butyl benzyl phthalate. J Am Coll Toxicol. 1992; 11: 1-23.
7. Albro PW. The biochemical toxicology of di(2-ethylhexyl) and related phthalates: Testicular atrophy and hepatocarcinogenesis. Rev Biochem Toxicol. 1987; 8: 73-119.
8. Thomas JA, Darby TD, Wallin RF, Gavin PJ, Martis L. A review of the biological effects of di(2-ethylhexyl)phthalate. Toxicol Appl Pharmacol. 1978; 45: 1-27.
9. Faouzi A, Dine T, Gressier B, Kambia K, Lucky M, Pagniez D, Brunet C, Cazin M, Belabed A, Cazin JC. Exposure of hemodialysis patients to di(2-ethylhexyl)phthalate. Int J Pharmacol. 1999; 180: 113-121.
10. Hillman LS, Goodwin LS, Sherman WR. Identification and measurement of plasticizer in neonatal tissues after umbilical carters and blood products. New England J Med. 1975; 292: 381-85.
11. Manojkumar V, Deepadevi KV, Arun P, Nair KG, Lakshmi LR, Kurup PAS. Changes in the concentration of erythrocyte membrane during storage of blood in di(2-ethylhexyl)phthalate (DEHP) plasticized poly vinyl chloride (PVC) blood storage bags. Ind J Med Res. 1999; 109: 157-163.
12. Sjöberg P, Lindquist NG, Montin G, Ploen L. Effects of repeated intravenous infusions of the plasticizer di(2-ethylhexyl)phthalate in young male rats. Arch Toxicol. 1985; 58: 78-83.
13. Parks LG, Ostby JS, Lambright CR, Abbott BD, Klinefelter GR, Barlow NJ, Gray LE Jr. The plasticizer diethylhexyl phthalate induced malformations be decreasing fetal testosterone synthesis during sexual differentiation in the male rat. Toxicol Sci. 2000; 58: 339-349.
14. Sharpe RM. Hormones and testis development and possible adverse effects of environmental chemicals. Toxicol Lett. 2001; 120: 221-232.
15. Shultz VD, Philips S, Sar M, Foster PM, Gaido KW. Altered gene profiles and fetal rats testes after in utero exposure to di(n-buthyl)phthalate. Toxicol Sci. 2001; 64: 235-242.
16. Wong JS, Gill SS. Gene expression changes induced in mouse liver by di(2- ethylhexyl)phthalate. Toxicol Appl Pharmacol. 2002; 185: 180-96.
17. DFG, Deutsche Forschungsgemeinschaft. Di(2-ethylhexyl)phthalate (DEHP). In: Greim H (Ed.): Gesundheitsschadliche Arbeitsstoffe-Toxikologischarbeitsmedizinische Begrundungen von MAK-Werten. 35. Erganzungslieferung, Wiley-VCH 2002.
18. Kavlock R, Boekelheide K, Chapin R, Cunningham M, Faustman E, Foster P, Golub M, Henderson R, Hinberg I, Little R, Seed J, Shea K, Tabacova S, Tyl R, Wiliams P, Zacharewski T. NTP Center for Evaluation of Risks to Human Reproduction: phthalates expert panel report on the reproductive and developmental toxicity of di(2-ethylhexyl) phthalate. Reprod Toxicol. 2002; 16: 529-653.
19. Arcadi FA, Costa C, Imperatore C, Marchese A, Rapisardi A, Salemi M, Trimarchi GR, Costa. G. Oral toxicity of bis(2-ethylhexyl)phthalate during pregnancy and sucking in the Long-Evans rat. Food Chem Toxicol. 1998; 35: 963-970.
20. ATSDR. Toxicological profile for di(2-ethylhexyl)phthalate (DEHP). Agency for Toxic Substances and Disease Registry, United States Public Health Service, Atlanta (GA) 2002.
21. Gray LE Jr, Ostby J, Furr J, Price M, Veeramachaneni DN, Parks L. Prenatal exposure to the phthalates DEHP, BBP, and DINP, but not DEP, DMP, or DOTP, alters sexual differentiation of the male rat. Toxicol Sci. 2000; 58: 350-65.
22. Moore RW, Rudy TA, Lin TM, Ko K, Peterson RE. Abnormalities of sexual development in male rats with in utero and lactational exposure to the antiandrogenic plasticizer di(2-ethylhexyl)phthalate. Environ Health Perspect. 2001; 109: 229-237.
23. Ashby J, Clapp MJL. The rodent dominant lethal assay: a proposed format for data presentation that alerts to pseudodominant lethal effects. Mutat Res. 1995; 330: 209- 218
24. IARC (1982) Monographs on the Evaluation of Carcinogenic Risk to Humans. Vol. 29: Some Industrial Chemicals and Dyestuffs. Summary of Data Reported and Evaluation, 269-293, IARC, Lyon 1982.
25. Harrison A, Moore PC. Reduction in sperm count and increase in abnormal sperm in the mouse following X-radiation or injection of ^{22}Na. Health Phys. 1980; 39: 219-224.
26. Searle AG, Beechey CV. Sperm count, egg-fertilization and dominant lethality after X-irradiation of mice. Mutat Res. 1974; 22: 69-74.
27. Working PK, Bus JS, Hamm TE Jr. Reproductive effects of inhaled methyl chloride in the male Fisher 344 rat. II. Spermatogonial toxicity and sperm quality. Toxicol Appl Pharmacol. 1985; 77: 144-57.
28. Wyrobek AJ, Bruce WR. Chemical induction of sperm abnormalities in mice. Proceed Nat Acad Sci USA. 1975; 72: 4425-4429.
29. Singh NP, Mc Coy M, Tice RR, Schneider EL. A simple technique for quantization of low level of DNA damage in individual cells. Exp Cell Res. 1988; 175: 184-191.
30. Anderson D, Yu TW, Phillips BJ, Schmezer P. The effects of various antioxidants and other modifying agents on oxygen-radical-generated damage in human lymphocytes in Comet assay. Mutat Res. 1994; 307: 261-71.
31. Końca K, Lankoff A, Banasik A, Lisowska H, Kuszewski T, Góźdź S, Koza Z, Wójcik A. A cross-platform public domain PC image-analysis program for comet assay. Mutat Res. 2003; 534: 15-20.
32. Knudsen I, Hansen EV, Meyer OA, Poulsen E. A proposed method for the simultaneous detection of germ cell mutations leading to foetal death (dominant lethality) and of malformations (male teratogenicity). Mutat Res. 1977; 48: 267-270.
33. Anderson D, Edwards AJ, Brinkworth, MH. Male-mediated F1 effects in mice exposed to 1,3-butadiene. In: Sorsa M, Peltonen K, Vainio H, Hemminki K (Eds.). Butadiene and Styrene: assessment of Health Hazards, 171-181. IARC Scientific Publications No. 127, Lyon 1993.

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34. Kirk KM, Lyon MF. Induction of congenital malformations in the offspring of male mice treated with X-rays at pre-meiotic and post-meiotic stages. Mutat Res. 1984; 125: 75-85.

35. Hossain M, Devi PU, Bisht KS. Effect of prenatal gamma irradiation during the late fetal period on the postnatal development on the mouse. Teratology. 1999; 59: 133-38.

36. Lottrup G, Andersson A-M, Leffers H, Martensen GK, Toppari J, Skakkebaek NE, Main, KM. Possible impact of phthalates on infant reproductive health. Int J Androl. 2006; 29: 172-180.

37. Swan SH, Main KM, Liu F, Stewart SL, Kruse RL, Calafat AM, Mao CS, Redman JB, Termand CL, Sullivan S, Tregue JL. Study of future families. Research Team. Decrease in anogenital distance among male infants with prenatal phthalate exposure. Environ Health Perspect. 2005; 113: 1056-61.

38. ECB. Search Appendix to Directive 67/48/EEC on classification and labeling of dangerous substances, 2004. Last updated 30/04/2004b. http//ecb.jrc.it/classification-labeling (accessed: 11.2004).

39. Hellwig J, Freudenberger J, Jackh R. Differential prenatal toxicity of branched phthalate esters in rats. Food Chem Toxicol. 1997; 35: 501-12.

40. Shirota M, Saito Y, Imai K, Horihucki S, Yoshimura S, Sato M, Nagao T, Ono H, Katoh M. Influence of di(2-ethylhexyl)phthalate on fetal testicular development by oral administration to pregnant rats. J Toxicol Sci. 2005; 30: 175-94.

41. Gray LE, Barlow NJ, Howdeshell KL, Ostby JS, Furr JR, Gray CL. Transgenerational effects of di(2-ethylhexyl)phthalate in the male CRL:CD(SD) rat: added value of assessing multiple offspring per litter. Toxicol Sci. 2009; 110: 411-25.

42. IARC (2000) Monographs on the Evaluation of Carcinogenic Risk to Humans. Vol. 77: *Some Industrial Chemicals Summary of Data Reported and Evaluation,* 41-148, IARC, Lyon, 2000.

43. Yagi Y, Nakamura Y, Tomita I, Tsuchikawa K, Shimoi N. Teratogenic potential of di- and mono-(2-ethyl)phthalate in mice. J Environ Pathol Toxicol. 1980; 4: 533-544.

44. Shiota K, Mima S. Assessment of the teratogenicity of di(2-ethylhexyl) phthalate and mono(2-ethylhexyl)phthalate in mice. Arch Toxicol. 1985; 56: 263-266.

45. Dostal LA, Jenkins WL, Schwetz BA. Hepatic peroxisome proliferation and hypolipidemic effects of di(2-ethylhexyl)phthalate in neonatal and adult rats. Toxicol Appl Pharmacol. 1987; 87: 81-90.

46. Kwack SJ, Kim KB, Kim HS, Lee BM. Comparative toxicological evaluation of phthalate diesters and metabolites in Sprague-Dawley male rats for risk assessment. J Toxicol Environ Health A. 2009; 72: 1446-54.

47. Parmar D, Srivastava SP, Seth PK. Effect of di(2-ethylhexyl)phthalate (DEHP) on spermatogenesis in adult rats. Toxicology. 1986; 42: 47-55.

48. Siddiqui A, Srivastava SP. Effect of di(2-ethylhexyl)phthalate administration on rat sperm count and on sperm metabolic enzymes. Bull Environ Contam Toxicol. 1992; 48: 115-19.

49. Agarwal DK, Eustis S, Lamb JC, Reel JR, Kluwe WM. Effects of di(2-ethylhexyl) phthalate on the gonadal pathophysiology, sperm morphology, and reproductive performance of male rats. Environ Health Perspect. 1986; 65: 343-50.

50. Dobrzyńska MM, Czajka U, Tyrkiel EJ. Male-mediated F1 effects in mice exposed to di(2-ethylhexyl)phthalate (DEHP). In: Anderson D, Brinkworth MH (Eds.). *Male-mediated developmental toxicity.* RSC Publishing 2007.

51. Katz DF, Diel L, Overstreet JW. Differences in the movement of morphologically normal and abnormal human seminal spermatozoa. Biol Reprod. 1982; 26: 566-70.

52. Hales BF, Cyr DG. Study designs for the assessment of male-mediated developmental toxicity. In: Robaire B, Hales BF (Eds.). *Advances in male mediated developmental toxicity.* 271-77. Kluwer Academic/Plenum Publishers, New York 2003.

53. Bateman AJ. The dominant lethal asssay in the mouse. In: Kilbey B, Legator M, Nicholl W, Ramel C (Eds.). *Handbook of Mutagenicity test procedures*, 325-35. Elsevier, Amsterdam 1977.

54. Dostal LA, Chapin RE, Stefanski SA, Harris MW, Schwetz BA. Testicular toxicity and reduced Sertoli cell numbers in neonatal rats by di(2-ethylhexyl)phthalate and the recovery of fertility as adults. Toxicol Appl Pharmacol. 1988; 95: 104-21.

55. Lamb JC 4th, Chapin RE, Teaque J, Lawton AD, Reel JR. Reproductive effects of four phthalic acid esters in the mouse. Toxicol Appl Pharmacol. 1987; 88: 255-69.

56. Faber WD, Deyo JA, Stump DG, Ruble K. Two-generation reproduction study of di-2-ethylhexyl terephthalate in Crl:CD rats. Birth Defects Res Part B Dev Reprod Toxicol. 2007; 80: 69-81.

57. Ema M, Miyawaki NE, Hirose A, Kamata E. Decreased anogenital distance and increased incidence of undescended testes in fetuses of rats given monobenzyl phthalate, a major metabolite of butyl benzyl phthalate. Reprod Toxicol. 2003; 17: 407-12.

58. Noriega NC, Howdeshell KL, Furr J, Lambright CR, Wilson VS, Gray LE Jr. Pubertal administration of DEHP delays puberty, suppresses testosterone production, and inhibits reproductive tract development in male Sprague-Dawley and Long-Evants rats. Toxicol Sci. 2009; 111: 163-78.

59. Song XF, Deng YJ, Zhang DY, Liu X, Wu SD, Wei GH. Effects of di(2-ethylhexyl)phthalate on the testis and testicular gubernaculums of fetal KM mice (Abstr). Zhonghua Nan Ke Xue. 2009; 15: 195-99.

60. Andrade AJ, Grande SW, Talsness CE, Gericke C, Grote K, Golembiewski A, Sterner-Kock A, Chahoud I. A dose response study following *in utero* and lactational exposure to di(2-ethylhexyl)phthalate (DEHP): reproductive effects on adult male offspring rats. Toxicology. 2006; 228: 85-97.

61. Dalsenter PR, Santana GM, Grande SW, Andrado AJ, Araujo SL. Phthalate affects the reproductive function and sexual behavior of male Wistar rats. Hum Exp Toxicol. 2006; 25: 297-303.

62. Vo TTB, Jung EM, Dang VH, Jung R, Baek J, Choi KC Jeung EB. Differential effects of flutamide and di(2-ethylhexyl) phthalate on male reproductive organs in rat model. J Reprod Develop. 2009; 55: 400-11.

63. Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during the past 50 years. BMJ. 1992; 305: 609-612.

64. Horak S, Kamińska J, Olejek A. The fertilization of donor semen between 1982 and 2004 in the industrial area of Upper Silesia (Poland). Ann Agric Environ Med. 2008; 15: 113-118.

65. Swan SH, Elkin EP, Fenster L. The question of declining sperm density revisited: An analysis of 101 studies published 1934-1996. Environ Health Perspect. 2000; 108: 961-966.

66. Gray TJ, Butterworth KR. Testicular atrophy produced by phthalate esters. Arch Toxicol. 1980; Suppl 4: 432-455.

67. Gray TJ, Beamand JA. Effect of some phthalates esters and other testicular toxins on primary cultures of testicular cells. Food Chem Toxicol. 1984; 22: 123-31.