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Teratogenicity of Di(2-ethylhexyl) Phthalate (DEHP) and Di-*n*-butyl Phthalate (DBP) in Mice

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Di(2-ethylhexyl) phthalate (DEHP) and di-*n*-butyl phthalate (DBP) were mixed with diet at graded levels of 0.05, 0.1, 0.2, 0.4 and 1.0 wt-% and given to pregnant ICR mice throughout gestation. Maternal weight gain was suppressed and fetal resorptions increased at 0.2, 0.4 and 1.0% levels of DEHP and 1.0% level of DBP. All the implanted ova died early in rats fed 0.4 and 1.0% levels of DEHP. External malformations increased significantly by 0.2% DEHP, and 1.0% DBP showed borderline significance. The major malformations in treated groups were neural tube defects (exencephaly and myeloschisis), suggesting that the phthalic acid esters (PAEs) affect neural tube closure in developing embryos. Treatment with the compounds caused intrauterine growth retardation and delayed ossification with an apparently dose-related response pattern. These results indicate that a high dose of DEHP and DBP might be embryotoxic and teratogenic in mice. The maximum nonembryotoxic doses of PAEs in mice were more than 2000 times the estimated level of human intake through the food chain. Thus it is assumed that the current "normal" exposure level of PAEs does not pose an imminent threat to human fetal development.

Introduction

Phthalic acid esters (PAEs) have been reported to leach into plasma from blood storage bags (*1*) and hemodialysis units (*2*). These compounds have also been found in a variety of materials in the environment, including foodstuffs (*3*). Thus, pregnant women might be at risk for unintentional exposure to minute amounts of PAEs derived from medical devices and diet.

In recent years, many studies have been carried out on the possible health hazards of PAEs, including the effects on reproduction and fetal development, and they have been extensively reviewed by several investigators (*4-6*). However, there is still a paucity or inconsistency of teratological information of PAEs. Singh et al. (*7*) injected several kinds of PAEs intraperitoneally into rats on days 5, 10 and 15 of gestation. Fetal resorptions and gross abnormalities increased on injection of 10 ml/kg di(2-ethylhexyl) phthalate (DEHP) and 1.02 ml/kg di-*n*-butyl phthalate (DBP). Skeletal abnormalities and hematomas were observed at 0.610 and 0.305 ml/kg levels of DBP. Nikonorow et al. (*8*) administered 0.34 and 1.70 g/kg/day DEHP and 0.12 and 0.60 g/kg/day DBP orally to rats during pregnancy and observed increased resorptions and decreased fetal weight, but no gross malformations. In a mouse study, consecutive oral administration of 10 mg/day DEHP and 10 and 50 mg/day DBP to pregnant females of ICR and ddY strains induced abnormalities in their offspring, including renal cysts and absence of tail (*9*). In this short report, however, the data were not compared with appropriate controls. Single oral administration of DEHP to pregnant mice of a random strain on day 7 of gestation resulted in decreased fetal weight at a dose level of 0.05 ml/kg and increased number of dead and malformed fetuses at 0.1 ml/kg (*10*). Teratogenicity in chicks has been reported by Haberman and his co-workers (*11-13*). Mutagenic and antifertility effects of DEHP have been shown by Singh et al. (*14*) by dominant lethal test in mice.

The present study was undertaken to ascertain the effects of orally administered DEHP and DBP

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on embryonic development and to determine the maximum nonembryotoxic dose in mice for extrapolation to the human. In the present experiment, the test compounds were given to the animals with diet throughout the whole gestation period, because they are ingested by people mainly through the food chain.

Materials and Methods

DEHP and DBP manufactured by Tokyo Kasei Industrial Co., Ltd. (Tokyo) were provided by the Ministry of Health and Welfare, Japan. They were individually mixed with the basal diet) OA-2 (CLEA Japan, Inc., Takatsuki) at graded levels of 0.05, 0.1, 0.2, 0.4 and 1.0 wt-%; the highest dose level was estimated to be near the maximum nontoxic dose for mature female ICR mice by a preparatory subchronic study for 3 weeks.

Four-week-old male and female mice of ICR-JCL strain were purchased from CLEA Japan, Inc. and reared in our laboratory with laboratory chow (OA-2) and tap water *ad libitum*. The room temperature was kept at 22 ± 2°C and the relative humidity at 55 ± 5%. The daily light cycle was 12 hr light and 12 hr dark. Each female mouse at 8-16 weeks of age was placed overnight with a male of proven fertility. The day on which a vaginal plug was found was taken as day zero of pregnancy. The females with vaginal plugs were randomly distributed into ten test groups and one control group. The females in the test groups were fed pellets containing DEHP or DBP at the graded levels mentioned above from day zero until the day of sacrifice. The control animals were fed the basal diet only. During the gestation period, the females were weighed daily, and food and water consumptions recorded daily.

On day 18 of pregnancy, the females were sacrificed by cervical dislocation. The uteri were removed, and implantations, resorptions and dead fetuses were recorded. The live fetuses were weighed, sexed and inspected for gross external abnormalities. About half of the fetuses from each litter, randomly selected, were eviscerated, fixed in 95% ethanol, cleared in 1% KOH, and stained with alizarin red S for detection of skeletal abnormalities. The remaining fetuses were fixed in Bouin solution for several days and examined for internal soft tissue anomalies by microdissection under a dissecting microscope.

The data concerning maternal weight and food and water consumption were analyzed by Student's *t* test for comparing means between groups. The data relating fetuses were analyzed in the following way in order to minimize biases resulting from the litter effect (*15*). The percentages or means for a group were computed by first obtaining the percentage or mean for each litter and then calculating the average of these percentages or means. The distribution of a percentage or a mean for treated litters was compared with the control distribution by use of the Wilcoxon rank sum test (*16*).

Results

Maternal Food, Water and PAE Intake

The average food intake by the treated females ranged from 6.1 to 7.4 g/day, and these did not differ significantly from the amount taken by the control mice (Table 1). No dose-related trends were noted in food consumption. The average daily doses of DEHP calculated from food intake and body weight were 2200, 830, 410, 190 and 70 mg/kg for dose levels of 1.0, 0.4, 0.2, 0.1 and 0.05%, respectively. The corresponding doses of DBP were 2100, 660, 370, 180 and 80 mg/kg for the respective dose levels. The daily water intake was not significantly different in treated and control groups.

Table 1. Effects of DEHP and DBP on pregnant mice.

Phthalate	Phthalate dose, %	Number of dams	Average food intake, g/day	PAE dosage, mg/kg/day	Average maternal weight, g (mean ± SE)	
					Day 0	Day 18
DEHP	1.0	12	7.2	2,200	30.2 ± 0.4	37.4 ± 1.2[a]
DEHP	0.4	7	7.2	830	31.4 ± 0.9	38.1 ± 1.1[a]
DEHP	0.2	24	6.9	410	29.0 ± 0.8	42.6 ± 2.2[b]
DEHP	0.1	9	7.4	190	29.2 ± 0.6	49.9 ± 1.3
DEHP	0.05	8	6.1	70	31.6 ± 0.8	56.6 ± 1.1
DBP	1.0	15	7.1	2,100	30.4 ± 0.4	39.7 ± 1.6[a]
DBP	0.4	9	6.9	660	29.9 ± 0.3	53.5 ± 2.9
DBP	0.2	21	7.1	370	30.1 ± 0.7	52.6 ± 2.5
DBP	0.1	8	7.3	180	29.5 ± 0.4	52.2 ± 1.8
DBP	0.05	7	6.9	80	29.3 ± 0.3	56.3 ± 1.5
Control	—	8	6.2	–	29.2 ± 0.4	52.6 ± 1.1

[a]Significantly different from control mice ($p < 0.01$).
[b]Significantly different from control mice ($0.01 < p < 0.05$).

Effects on Dams

No mortality or behavioral abnormalities were observed in the pregnant mice throughout the experimental period. However, the maternal weight was significantly depressed during the later gestation period in the groups given 1.0, 0.4 and 0.2% DEHP and 1.0% DBP (Table 1). Such weight suppression of the mothers could be accounted for by the increased early resorptions in these groups. At autopsy, no gross pathological changes were noted in maternal major organs.

Effects on Fetal Viability and Weight

There was no significant difference in the mean numbers of corpora lutea and implantations between the treated and control groups (Table 2). Fetal mortality increased in a dose-related manner, and it was significantly higher in 1.0, 0.4 and 0.2% DEHP and 1.0% DBP groups. No viable term fetuses were obtained at the two highest doses of DEHP. Most of the fetal deaths were very early resorptions.

The average weight of viable fetuses showed a dose-related decrease in the treated groups, and that in 0.2% DEHP group and 0.4% DBP group (male only) was significantly lower than in controls (Table 2). Out of the 15 pregnant females which were given 1.0% DBP, only three fetuses from two dams survived to term. Their weights were far lower than those of controls.

External Anomalies

Several kinds of malformations were observed in the treated groups (Table 3). The incidence of malformed fetuses in 0.2% DEHP group was significantly higher than in the control group ($p < 0.05$). The difference between 1.0% DBP and control groups was at the borderline level of significance ($p \approx 0.05$). The most common malformations were neural tube defects (exencephaly and myeloschisis), and

Table 2. Effects of DEHP and DBP on fetal viability and development.

Phthalate	Phthalate dose, %	Number of litters	Number of implants (means ± SE)	Resorptions and dead fetuses, %	Fetal weight, g (mean ± SE)	
					Male	Female
DEHP	1.0	12	12.6 ± 0.5	100.0[a]	–	–
DEHP	0.4	7	15.0 ± 1.2	100.0[a]	–	–
DEHP	0.2	24	11.1 ± 0.9	67.8[a]	1.12 ± 0.07[a]	1.16 ± 0.04[b]
DEHP	0.1	9	12.6 ± 2.1	31.0[c]	1.28 ± 0.08	1.21 ± 0.07
DEHP	0.05	8	13.3 ± 1.7	7.5	1.32 ± 0.07	1.29 ± 0.06
DBP	1.0	15	12.1 ± 0.5	98.4[a]	1.03, 1.24	0.80
DBP	0.4	9	12.0 ± 0.8	11.4	1.10 ± 0.18[b]	1.06 ± 0.20
DBP	0.2	21	12.4 ± 0.8	22.3	1.31 ± 0.07	1.24 ± 0.06
DBP	0.1	8	12.0 ± 2.9	11.2	1.29 ± 0.10	1.22 ± 0.08
DBP	0.05	7	12.7 ± 3.1	3.7	1.31 ± 0.04	1.29 ± 0.03
Control	—	8	10.8 ± 1.9	5.0	1.41 ± 0.07	1.35 ± 0.07

[a]Significantly different from control mice ($p < 0.01$).
[b]Significantly different from control mice ($0.01 < p < 0.05$).
[c]Difference from controls is at the borderline level of significance ($p \approx 0.05$).

Table 3. Malformations in mouse fetuses from dams treated with DEHP or DBP.

Phthalate	Phthalate dose, %	Number (%) of malformed fetuses	Type of malformation and (no. of cases)[c]
DEHP	1.0	—	—
DEHP	0.4	—	—
DEHP	0.2	14 (25.8)[a]	Ex (3), Ex+OE (2), Ex+TA (1), My (1), My+GS+CF (1), My+OE+GE (1), GS+TA (1), TA (4)
DEHP	0.1	3 (5.3)	Ex (2), OE (1)
DEHP	0.05	0 (0.0)	—
DBP	1.0	2 (75.0)[b]	Ex (2)
DBP	0.4	0 (0.0)	—
DBP	0.2	1 (0.5)	OE (1)
DBP	0.1	0 (0.0)	—
DBP	0.05	0 (0.0)	—
Control	—	0 (0.0)	—

[a]Significantly different from control mice ($p < 0.05$).
[b]Difference from controls is at the borderline level of significance ($p \approx 0.05$).
[c]Type of malformation: CF club foots; Ex, exencephaly; GE, generalized edema; GS, gastroschisis; My, myeloschisis; OE, open eyelids; TA, tail anomaly.

Table 4. Effects of DEHP and DBP on skeletal development of mouse fetuses.

Phthalate	Phthalate dose, %	Number of fetuses examined	Skeletal malformations	Variations (%)		Number of ossified coccygia (mean ± SE)
				Lumbar rib	Deficient ossification of sternebrae	
DEHP	1.0	0	–	–	–	–
DEHP	0.4	0	–	–	–	–
DEHP	0.2	40	0	18.7	8.3	6.5 ± 0.6[a]
DEHP	0.1	35	0	20.7	14.7	9.2 ± 0.3
DEHP	0.05	31	0	7.6	0.0	7.3 ± 0.4[a]
DBP	1.0	0	0	–	–	–
DBP	0.4	42	0	36.9	0.0	2.6 ± 0.4[a]
DBP	0.2	65	0	26.3	0.0	6.0 ± 0.4[a]
DBP	0.1	38	0	17.2	0.0	4.5 ± 0.3[a]
DBP	0.05	41	0	23.8	6.3	5.1 ± 0.3[a]
Control	—	0	0	13.3	0.0	9.4 ± 0.2

[a]Significantly different from control mice ($p < 0.05$).

Table 5. Reported teratogenicity/embryotoxicity of DEHP in rodents.

Species (strain)	Route of administration	Period of administration	Dose	Principal findings	Authors
Mouse (ICR)	PO	Throughout gestation	190-2200 mg/kg/day	Neural tube defects, tail anomalies, decreased fetal weight, delayed ossification, resorptions	Present study
Mouse (ICR, ddY)	PO	Throughout gestation	10 mg/day	Renal cysts	Onda et al. (*9*)
Mouse (ddY-Slc × CBA)	PO	Day 7	0.05 ml/kg 0.1 ml/kg	Decreased fetal weight Fetal deaths, skeletal abnormalities	Nakamura et al. (*10*)
Rat (Wistar)	PO	Throughout gestation	0.34, 1.70 g/kg/day	Resorptions, decreased fetal weight, decreased placental weight	Nikonorow et al. (*8*)
Rat (Sprague-Dawley)	PO	Days 3, 6, 9	2.4 ml/kg/day	Decreased implantations	Peters and Cook (*31*)
Rat (Sprague-Dawley)	IP	Days 5, 10, 15	10 ml/kg/day	Malformations, resorptions, decreased fetal weight	Singh et al. (*7*)

these anomalies were occasionally encountered also in dead fetuses. Other anomalies consisted of gastroschisis, absence or shortness of tail, club foot, open eyelids and generalized edema.

Skeletal and Visceral Anomalies

As shown in Table 4, extra lumbar ribs and deficient ossification of sternebrae increased in treated groups, although the difference from the controls was not statistically significant. Ossification was significantly retarded in treated groups except 0.1% DEHP group. Delayed ossification in those groups was probably related to the general underdevelopment of the fetuses. No internal anomalies were observed by microdissection.

Discussion

The results of the present experiment indicate that DEHP and DBP can affect fetal development when they are taken orally during pregnancy. Although there appears to be no adverse effect on implantation, a dose-related response was observed for fetal mortality and fetal body weight. It was also shown that DEHP and DBP provoke their teratogenicity in mice at high dosages.

In the two previous experiments, in which DEHP and/or DBP was given orally to pregnant mice (*9*, *10*), cysts of the kidney, absence of tail (*9*) and skeletal abnormalities (*10*) were noted, but no neural tube defects were observed. On the contrary, renal cysts were not found in the present study by careful visceral examination. It is also interesting to note that a certain difference in embryotoxicity has been shown between mice and rats, and between oral (PO) and parenteral (IP) administration (Tables 5 and 6). At present, there is no clear explanation for such inconsistency between experiments. Although recent metabolic studies have shown that PAEs are metabolized in rats after oral administration (*17-19*) and IV injection (*20*), comparative data are still insufficient in the mouse and human. It seems possible that the metabolism of PAEs is not the same between species and between strains. Thus comparative aspects of PAE metabolism and potential embry-

Table 6. Reported teratogenicity/embryotoxicity of DBP in rodents.

Species (strain)	Route of administration	Period of administration	Dose	Principal findings	Authors
Mouse (ICR)	PO	Throughout gestation	660, 2100 mg/kg/day	Neural tube defects, resorptions, decreased fetal weight, delayed ossification	Present study
Mouse (ICR, ddY)	PO	Throughout gestation	10, 50 mg/day	Renal cysts, absence of tail	Onda et al. (*9*)
Rat (Wistar)	PO	Throughout gestation	0.12, 0.60 g/kg/day	Resorptions, decreased fetal weight	Nikonorow et al. (*8*)
Rat (Sprague-Dawley)	IP	Days 5, 10, 15	0.61, 0.305 ml/kg/day	Decreased fetal weight, skeletal abnormalities	Singh et al. (*7*)
			1.02 ml/kg/day	Decreased fetal weight, resorptions	

otoxicity of the metabolites should be ascertained. Pertinent animal models in terms of the pharmacokinetic similarity to humans are needed.

From the findings of our experiment, it seems reasonable to assume that the maximum nonembryotoxic doses of orally administered PAEs in mice would be at least 70 mg/kg/day for DEHP and 370 mg/kg/day for DBP. On the other hand, the current human intake of PAEs from the diet has been estimated to be 0.03 mg/kg/day at maximum in the case of the Japanese (*10*, *21*). Thus the maximum nonembryotoxic doses in mice are more than 2000 times the current human exposure level reported so far. For this reason, it seems unlikely that ingestion of PAEs under the current environmental level would pose an imminent threat to human early development. The following facts also favor this assumption. First, pharmacokinetic studies show that DEHP is metabolized and eliminated rapidly in mice (*22*, *23*), rats (*18*, *24*) and humans (*1*, *25*). Second, Singh et al. (*26*) found that in rats the levels of phthalate diesters in a whole fetus did not exceed the level in the maternal blood. In the human, DEHP and DBP in cord blood were at about the same levels as in the maternal blood (*27*). Thus, no fetal accumulation of PAEs is likely to occur. Third, Garvin et al. (*28*) and Lewandowski et al. (*29*) injected IV the plasma-soluble extracts of poly(vinyl chloride) (PVC) to pregnant rats and found no evidence of teratogenic or embryotoxic effects. In one of the experiments (*29*), the highest dose of the extract was equivalent to 5.3 mg DEHP/kg/day, which is similar to the amount predicted to be received by a 60-kg human undergoing an exchange transfusion of 21-day-old blood.

Although placental transfer of PAEs has been studied in rats (*26*), there is no information concerning the accumulation of PAEs in human conceptuses. Since there is little expectation of human *in vivo* study, actual determination of the PAE levels in human embryonic and fetal tissues and comparison of those with the levels in experimental animals with recognizable embryotoxicity would enable us to assess how safe the current environmental level is to human conceptuses. Such determination of fetal tissue levels could be done by utilizing the tissues from induced human abortuses (*30*).

From the discussion above, it can be concluded that the current "normal" dietary exposure level of PAEs may be harmless to human development. However, the problem of high level exposure in the human has not yet been solved. Epidemiological studies are needed regarding the reproductive outcome in populations under high level exposure of PAEs, e.g., among woman workers in the PVC and car industries.

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