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Embryolethality Following Maternal Exposure to Dibutyl Phthalate During Early Pregnancy in Rats

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A wide spectrum of uses has been found for the various phthalic acid esters (PAEs) and the largest market for these esters is as plasticizing agents (Autian 1973). The plasticizers do not become part of the polymer matrix and, under certain use or disposal conditions, can migrate from the plastic to the external environment (Kluwe et al. 1982). PAEs have been widely distributed in the environment because of their widespread manufacture, use, disposal, high concentration in plastics, and ability to migrate from the plastics (CIRC 1992). The possibility of these compounds entering into biological systems caused concern about their toxic potential.

Dibutyl phthalate (DBP) is a PAE and is used as a plasticizer in explosives, elastomers, safety glass, printing inks, paper coatings, adhesives and cosmetics (CIRC 1985). DBP is reported to be developmentally toxic in mice (Shiota et al. 1980; Lamb et al, 1987) and rats (Singh et al. 1972; Nikonorow et al. 1973; Peters and Cook 1973; Gray et al. 1990; Ema et al. 1993, 1994a). We have previously reported that administration of DBP by gastric incubation to pregnant rats during the whole period of organogenesis caused high incidence of postimplantation loss at 630 mg/kg and above and of fetuses with malformations at 750 mg/kg (Ema et al. 1993). Teratogenicity was detected in pregnant rats given DBP on days 7-9 or days 13-15 of pregnancy (Ema et al. 1994a). Monobutyl phthalate (MBuP), which is formed as a major metabolize in rats given DBP (Albro and Moore 1974; Williams and Blanchfield 1975; Tanaka et al. 1978) and butyl benzyl phthalate (BBP) (Eigenberg et al. 1986; Mikuriya et al. 1988), induced similar teratogenicity to that observed with DBP and BBP (Ema et al. 1995, 1996). We have also shown that a markedly high postimplantation embryolethality was caused in pregnant rats given dietary BBP at 2.0% on days 0-11 of pregnancy (Ema et al. 1992) and suggested that the embryolethality due to BBP during early pregnancy was mediated via the reduction in plasma progesterone levels, an impairment of luteal function (Ema et all 1994b). The present study was conducted to determine the effects of DBP on pregnant dams and embryos during early pregnancy, in an attempt to compare it with BBP results.

MATERIALS AND METHODS

Wistar rats (Std: Wistar-KY, Japan SLC, Inc., Hamamatsu, Japan) were used throughout this study. Animals were maintained in an air-conditioned room at 24±1 °C, with a relative humidity of 55 ± 5%, under a controlled 12-hr light/dark cycle. The rats were reared on a basal diet (F-1; Funabashi Farm Co., Funabashi, Japan) and tap water ad Iibitum. Virgin female rats (10-14 wk old) were mated overnight with male rats. The day when sperm were detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats were distributed on a random basis into three groups and housed individually. The pregnant rats were fed a diet containing 2.0% DBP (98.2% pure, Wako

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Pure Chemical Industries, Ltd., Osaka, Japan) ad libitum on day 0 through day 11 and sacrificed on day 20 of pregnancy, or day 0 through the day of sacrifice, day 7, day 9, or day 11 of pregnancy. The diet containing DBP was prepared fresh weekly. A predetermined amount of DBP was weighed and added to a small aliquot of ground basal diet and hand-blended. This premix was then added to a preweighed ground basal diet and blended with a mill (Irie Shokai Co., Ltd., Tokyo, Japan) for 30 min. The pair-fed pregnant rats were given an amount of feed equal to the feed intake of rats fed the diet containing 2.0% DBP. Average daily intake of DBP was calculated by the method described by Tyl et al. (1988). The control rats were fed a basal diet only ad libitum.

Maternal body weight and food consumption were recorded daily. Ten pregnant rats in each group were sacrificed on day 20 of pregnancy. The peritoneal cavity was opened and the number of live and dead fetuses and resorption were recorded. The live fetuses removed from the uterus were sexed, weighed and inspected for external malformations and malformations within the oral cavity. Approximately two-thirds of the live fetuses in each litter were randomly selected and fixed in alcohol, stained with alizarin red S (Kawamura et al. 1990) and examined for skeletal malformations. The remaining live fetuses in each litter were fixed in Bouin's solution, sectioned with a razor blade and examined for internal malformations (Wilson 1965). To obtain more information on the dam's pregnancy status at earlier stages, six pregnant rats per time interval in each group were sacrificed on day 7, day 9 or day 11 of pregnancy. On the day of sacrifice, maternal blood samples were collected from the external jugular vein and serum was separated by centrifugation. Maternal serum progesterone was measured by Shionogi Biomedical Laboratories, Shionogi & Co., Ltd. (Osaka, Japan), using a radioimmunoassay kit (Diagnostic Products Corporation, Los Angels, USA). The detection limit of this assay was 0.05 ng/mL. The uterus and ovaries were removed and trimmed of fat, The uterus with embryonic contents and paired ovaries were weighed. Embryonic viability was determined by the presence of a heartbeat on day 11 of pregnancy. Statistical analysis of the offspring data was carried out using the litter as a sample unit. Analysis of variance and Dunnett's multiple comparison test, Kruskal-Wallis test and Mann-Whitney test or Fisher's exact test were used as appropriate. The 0.05 level of probability was used as the criterion for significance.

RESULTS AND DISCUSSION

No deaths of pregnant females were observed in any group (Table 1). The majority of the pregnant rats in the pair-fed and 2.0% DBP groups showed slight piloerection during the treatment period. Body weight gain on days 0-11 in the pair-fed and 2.0% DBP groups was similar and significantly lower than the control group. Body weight gains on days 11-20 and days 0-20 in the 2.0% DBP group were significantly lower than the pair-fed and control groups. The adjusted weight gain in the 2.0% DBP group was significantly lower than the control group. Food consumption in the pair-fed group was comparable to the 2.0% DBP group. Food consumption on days 0-11 and days 0-20 in the pair-fed and 2.0% DBP groups were significantly lower than the control group. Food consumption on days 11-20 in the pair-fed and 2.0% DBP groups was significantly higher than the control group.

No significant difference among the groups was found in the number of corpora lutea per female, implantations per female, the incidence of preimplantation loss per female, and the sex ratio of live fetuses (Table 2). Complete resorption of all implanted embryos was found in nine of the ten litters in the 2.0% DBP group. The incidence of postimplantation loss per female in the 2.0% DBP group was significantly higher than the control and pair-fed groups; the pair-fed group was similar to the control group. No significant difference in the incidence of fetuses with external, skeletal, and internal malformations was found between the control and pair-fed groups (Table 3). Thus, the adverse effect on embryo-fetal survival was detected in the 2.0% DBP group but not in the pair-fed group. These findings suggest that the markedly high embryolethality observed in pregnant rats given 2.0% DBP is attributable to the effect of dietary DBP but not to maternal malnutrition from reduced food consumption.

Table 1. The effects of DBP on pregnant rats when administered on days 0-11 of pregnancy and assessed on day 20 of pregnancy

Group	Control	Pair-fed	2.0% DBP
No. of mated rats	10	10	10
No. of dead rats	0	0	0
No. of pregnant rats	10	10	10
Initial body weight (g)"	231±8	232±7	229±7
Body weight gain during pregnancy (g)[a]			
Days 0-11	45±9	-4±4**	-7±7**
Days 11-20	69±9	103±12**	39±9**††
Days 0-20	114±11	100±12*	32±9**††
Adjusted weight gain during pregnancy (g)[b]	46±11	37±11	28±8**
Food consumption during pregnancy (g)"			
Days 0-11	201±15	109±3**	113±18**
Days 11-20	191±13	226±13**	228±16**
Days 0-20	392±25	335±5**	341±19**
Daily intake of DBP (mg/kg)[a,c]	0	0	895±156**††

[a]Values are given as mean+ SD.

[b]Adjusted weight gain refers to maternal body weight gain excluding the gravid uterus.

[c][(Food consumption on days 0-11 / 11) X %DBP] / initial body weight.

* $P < 0.05$, ** $P < 0.01$, significantly different from the control group.

†† $P < 0.01$, significantly different from the pair-fed group.

Table 2, The effects of DBP on reproduction when administered on days 0-11 of pregnancy and assessed on day 20 of pregnancy

Group	Control	Pair-fed	2.0% DBP
No. of litters	10	10	10
No. of corpora lutea per female[a]	15.1 ± 1.2	14.7 ± 1.6	15.4 ± 1.2
No. of implantations per female[a]	14.0 ± 1.8	13.7 ± 2.3	14.5 ± 1.4
Preimplantation loss per female (%)[b]	7.4	7.1	5.9
No. of litters totally resorbed	0	0	9**††
No. of resorption and dead fetuses per female[a]	2.2 ± 1.5	2.4 ± 2.2	14.5 ± 1.3**††
Postimplantation loss per female (%)[c]	15.8	16.8	98.7**††
No. of live fetuses per female[a]	11.8 ± 2.3	11.3 ± 2.5	0.2 ± 0.6**††
Sex ratio of live fetuses (male/female)	59 / 59	52 / 61	0 / 2
Body weight of live fetuses (g)[a]			
Male	4.10 ± 0.19	4.12 ± 0.18	
Female	3.85 ± 0.19	3.88 ± 0.16	2.74

[a]Values are given as mean±SD.

[b][(No. of corpora lutea - no. of implantations) / no. of corpora lutea] X 100.

[c](No. of resorption and dead fetuses/no. of implantations) X 100.

** P <0.01, significantly different from the control group.

†† P <0.01, significantly different from the pair-fed group.

Table 3. The effects of DBP on incidence of fetal malformations when administered on days 0-11 and assessed on day 20 of pregnancy

Group	Control	Pair-fed	2.0% DBP
External malformations			
No. of fetuses (litters) examined	118 (10)	113 (10)	2 (1)
No. of fetuses (litters) with malformations	1 (1)	0	0
No. of fetuses (litters) with encepholocele	1 (1)	0	0
Skeletal malformations			
No. of fetuses (litters) examined	80 (10)	76 (10)	1 (1)
No. of fetuses (litters) with malformations	1 (1)	0	0
No. of fetuses (litters) with fusion of cervical vertebral arches	1 (1)	0	0
Internal malformations			
No. of fetuses (litters) examined	38 (10)	37 (10)	1 (1)
No. of fetuses (litters) with malformations	0	0	1 (1)
No. of fetuses (litters) with dilatation of renal pelvis	0	0	1 (1)

Table 4. Reproductive parameters in pregnant rats (n = 6 per time interval) on day 7, 9 or 11 of gestation

Group	Control	Pair-fed	2.0% DBP
Uterine weight (mg)[a]			
Day 7	583 ± 68	568 ± 71	551 ± 39
Day 9	1493 ± 96	1506 ± 144	1344 ± 135
Day 11	3331 ± 346	3331 ± 389	2470 ± 233**††
Postimplantation loss per female (%)[a,b]			
Day 11	9.5	5.0	84.6**††
Ovarian weight (mg)'			
Day 7	72 ± 5	69 ± 11	59 ± 4*
Day 9	74 ± 3	72 ± 4	60 ± 2**††
Day 11	81 ± 5	75 ± 3	64 ± 3**††
Serum progesterone level (ng/ml)[a]			
Day 7	79 ± 15	74 ± 3	66 ± 8
Day 9	80 ± 15	77 ± 7	72 ± 6
Day 11	87 ± 7	85 ± 10	72 ± 13*

[a]values are given as mean ± SD.

[b](No. of dead embryos / no. implantations) X 100.

* $P < 0.05$, ** $P < 0.01$, significantly different from the control group,

†† $P < 0.01$, significantly different from the pair-fed group.

To determine the effects of DBP on reproductive parameters during early pregnancy, rats were sacrificed on day 7, day 9, or day 11 of pregnancy. Although uterine weights on day 7 and day 9 of pregnancy were similar among groups, uterine weight on day 11 in the 2.0% DBP group was significantly lower than the control and pair-fed groups (Table 4). This suggested that DBP adversely affected development of conceptus after day 9 and prior to day 11 of pregnancy. The incidence of postimplantation loss per female on day 11 of pregnancy in the 2.0% DBP group was significantly higher than the control and pair-fed groups. Thus, it appeared that most of the postimplantation loss induced by DBP occurred before day 11 of pregnancy. A significantly lower ovarian weight on day 7 was found in the 2.0% group when compared to the control group, and the ovarian weights on day 9 and day 11 in the 2.0% DBP groups were significantly lower than the control and pair-fed groups. These findings suggest that DBP affects development of the corpora lutea. It is expected that poor development of the corpora lutea should cause impairment of luteal function. Corpora lutea are the primary sources of progesterone (Niswender and Nett 1988). Progesterone plays a dominant role in the maintenance of pregnancy and the well-being of the conceptus (Niswender and Nett 1988). A trend toward decreased serum progesterone levels was observed after treatment with 2.0% DBP. A significantly lower level of serum progesterone on day 11 was found in the 2.0% DBP when compared to the control group. Gray et al. (1990) showed that DBP administered to female rats from weaning reduced the pregnancy rate, the number of implantations, embryo viability, and progesterone secretion. Their results suggest that DBP can impair ovarian function and induce reproductive failure. Therefore, the possibility that embryolethality could result from effects of DBP on luteal function still remains.

The present data show that DBP administered during the first half of pregnancy is developmentally toxic in a manner similar to BBP (Ema et al. 1992, 1994b). Consideration of these findings together suggests that MBuP and/or its further metabolizes may participate, at least in part, in the induction of the postimplantation embryolethality of DBP and BBP. However, decrease in maternal progesterone levels was more striking during administration of BBP (Ema et al. 1994b) than during administration of DBP. One possible explanation for this difference may be MBeP, which is one of the primary metabolizes of BBP in rats (Eigenberg et al. 1986; Mikuriya et al. 1988), and/or its further metabolizes. Further studies are necessary to assess the embryotoxicity of metabolizes of DBP and BBP, in an attempt to get a better understanding of the embryolethality of these PAEs.

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