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Evaluation of developmental toxicity of β-thujaplicin (hinokitiol) following oral administration during organogenesis in rats

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Abstract

The objective of this study was to evaluate the developmental toxicity of β-thujaplicin (TP) in rats. Pregnant rats were given TP by gastric intubation at 15, 45, or 135 mg/kg on days 6–15 of pregnancy. The maternal body weight gain during administration at 45 and 135 mg/kg and after administration at 136 mg/kg and adjusted weight gain at 45 and 135 mg/kg were significantly reduced. A significant decrease in food consumption during and after administration was found at 45 and 135 mg/kg. A significant increase in the incidence of postimplantation loss was found in pregnant rats given TP at 135 mg/kg. A significantly lower weight was found in female fetuses at 45 and 135 mg/kg and in male fetuses at 135 mg/kg. Although a significantly increased incidence of fetuses with skeletal variations and decreased degree of ossification were found at 135 mg/kg, no significant increase in external, skeletal and internal malformations was detected after administration of TP. The data demonstrated that TP had adverse effects on embryonic/fetal survival and growth only at maternal toxic doses. No adverse effects on morphological development were found in rats fetuses. Based on the significant decreases in maternal body weight gain and weight of female fetuses at 45 mg/kg and higher, it is concluded that the no-observed-adverse-effect levels (NOAELs) of TP for both dams and fetuses are considered to be 15 mg/kg in rats.

Keywords: β-Thujaplicin; Hinokitiol; Developmental toxicity; Teratogenicity; Rat

1. Introduction

β-Thujaplicin (TP; CAS No. 499-44-5; Hinokitiol; 4-isopropyltropolone) is a phenolic component of essential oils extracted from cypress trees. TP has been found to act as an antibacterial agent (Saeki et al., 1989; Osawa et al., 1990; Tonari, 1998) and an antitumor agent (Yamato et al., 1984; Inamori et al., 1993). In addition, it possesses phytogrowth-inhibitory effects (Inamori et al., 1991). TP is used as a natural food preservative in Japan.

Several reports on the toxicity of TP are available. In mutagenicity screening tests of TP, positive results were obtained in a Rec-assay with S9 mix at 1.0 mg/disk and chromosome aberration test in vitro at 0.002–0.003 mg/ml, but not in the Ames test or a micronucleus test in mice (Sofuni et al., 1993). The DNA damaging activity of TP was weak in a spore Rec-assay (Ueno and Ishizaki, 1992). The values of LD50 have been reported to be 504 mg/kg in male ddy mice and 469 mg/kg in female ddy mice after oral gavage of TP (Shimizu et al., 1993). Recently, Ogata et al. (1999) reported a significant increase in the incidence of fetuses with malformations after oral administration of TP at 560 mg/kg and higher on day 9 of pregnancy in ICR mice and that TP induced dysmorphogenicity in cultured mouse embryos at concentrations of 6.25 and 12.5 μg/ml. However, there is no information on the developmental toxicity of TP in rats. Therefore, the present study was conducted to evaluate the potential teratogenicity of TP after administration throughout organogenesis in rats.

Abbreviations: TP, β-thujaplicin; LOAEL, lowest-observed-adverse-effect level; NOAEL, no-observed-adverse-effect level.

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2. Materials and methods

2.1. Animals

Wistar rats (Jcl: Wistar, Clea Co., Ltd., Tokyo, Japan) were used throughout this study. Animals were reared on a basal diet (F-1; Funabashi Farm Co., Funabashi, Japan) and tap water ad libitum and maintained in an air-conditioned room at 24±1 °C, with a relative humidity of 55±5%, under a controlled 12-h light/dark cycle. Virgin female rats, weighing 216–244 g, 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 four groups of 16–17 rats each and housed individually.

2.2. Chemicals and dosing

The female rats were dosed once daily by gastric intubation with TP (purity >98%, SEIWA Technological Laboratories Ltd., Tokyo, Japan) at a dose of 0 (control), 15, 45, or 135 mg/kg from day 6 through day 15 of pregnancy. The dosage levels were determined based on the results of our range-finding study in which administration of TP by gastric intubation on days 6–15 of pregnancy caused maternal deaths and decreased maternal body weight gain and caused an increase in postimplantation loss and decrease in fetal weight at 125 mg/kg and higher in rats. TP was dissolved in olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The volume of each dose was adjusted to 5 ml/kg of body weight based on daily body weight. The control rats received olive oil only. The formulations were kept in a cool and dark place for no more than 7 days.

2.3. Observations

The maternal body weight and food consumption were recorded daily. The pregnant rats were euthanized by ether overdose on day 20 of pregnancy. The peritoneal cavity and uterus were opened, and the numbers of live and dead fetuses and of resorptions were counted. The gravid uterus was removed and the dams weighed again. The adjusted weight gain, i.e. maternal weight gain throughout pregnancy corrected for gravid uterine weight, was calculated. To confirm the dam's pregnancy status, the uteri were immersed in 2% sodium hydroxide solution for over 1 h. The uteri were cleared and the implantation traces were seen to be stained yellowish-brown (Yamada et al., 1985). The live fetuses removed from the uterus were sexed, weighed, and inspected for external malformations and malformations within the oral cavity. Approximately one-half 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 prior to dissection. To detect internal malformations, fetal heads were examined by the free-hand razor-blade sectioning method of Barrow and Taylor (1969) and the thoracic areas were examined by Nishimura's micro-dissecting method (1974), a modification of Barrow and Taylor's method.

2.4. Data analysis

The litter was considered the experimental unit. The initial body weight, body weight gain and food consumption of pregnant rats, numbers of implantations, postimplantation loss and live fetuses per litter and body weight of live fetuses were evaluated by analysis of variance, followed by Dunnett's multiple comparison test if differences were found. The incidences of post-implantation loss and fetal malformations per litter were analyzed by the Kruskal–Wallis test to assess the overall effects. Whenever a significant trend was noted, pairwise comparisons were made using the Mann–Whitney test. Fisher's exact test was used when the incidence in the control group was zero. The 0.05 level of probability was used as the criterion for significance.

3. Results

Table 1 shows the maternal findings in rats given TP during organogenesis. One pregnant rat was dead on day 20 of pregnancy at 135 mg/kg. The body weight gain on days 6–16 at 45 and 135 mg/kg and on days 16–20 at 135 mg/kg was reduced significantly. The adjusted weight gain, which indicates the net weight gain of pregnant rats, was significantly lower in the 45 and 135 mg/kg groups than in the control group. The food consumption on days 6–16 and days 16–20 was significantly lower in the 45 and 135 mg/kg groups than the control group. These findings indicate that the lowest-observed-adverse-effect level (LOAEL) and no-observed-adverse-effect level (NOAEL) of TP for pregnant rats are 45 and 15 mg/kg, respectively.

Pregnancy outcome in rats given TP during organogenesis are presented in Table 2. Litters totally resorbed were found in three of the 16 pregnant rats at 135 mg/kg. A significant increase in the number of resorptions per litter and incidence of postimplantation loss per litter and a significant decrease in the number of live fetuses per litter were also noted at 135 mg/kg. The weights of live fetuses were significantly decreased at 45 mg/kg and higher in females and at 135 mg/kg in males.

A summary of morphological findings in live fetuses of rats given TP during organogenesis is shown in Table 3. No fetus with external malformations was observed in any group. Skeletal examination revealed

Table 1
Maternal findings in rats given β-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

Dose (mg/kg)	0 (control)	15	45	135
No. pregnant rats	16	16	16	17
No. of dead rats	0	0	0	1
Initial body weight	227±8	227±7	227±6	227±6
Body weight gain during pregnancy (g)[a]				
Days 0–6	17±5	17±4	16±2	17±3
Days 6–16	45±4	39±6	32±7*	13±9*
Days 16–20	48±6	48±5	42±6	21±12*
Adjusted weight gain during pregnancy (g)[a,b]	39±7	36±8	28±10*	24±5*
Food consumption during pregnancy (g)[a]				
Days 0–6	105±7	101±6	98±5*	101±5
Days 6–16	157±12	147±13	129±12*	103±11*
Days 16–20	72±5	70±4	63±7*	66±6*

[a] Values are given as mean±S.D.
[b] Adjusted weight gain refers to maternal body weight gain excluding the gravid uterus.
* Significantly different from the control, $P<0.05$.

Table 2
Reproductive findings in rats givenβ-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

Dose (mg/kg)	0 (control)	15	45	135
No. litters	16	16	16	16
No. corpora lutea per litter[a]	16.3±1.3	16.3±1.3	15.7±1.4	16.3±0.9
No. implantations per litter[a]	15.4±1.4	15.5±1.2	14.8±1.7	15.6±1.3
No. of litters totally resorbed	0	0	0	3
No. resorptions per litter[a]	1.3±1.4	1.3±1.2	2.0±1.0	9.9±4.6*
No. dead fetuses per litter[a]	0.1±0.3	0	0	0
% Postimplantation loss per litter[b]	8.5	8.0	13.6	63.5*
No. live fetuses per litter[a]	14.1±1.4	14.3±1.5	12.8±1.8	5.7±4.6*
Sex ratio of live fetuses (male/female)	114/111	116/112	107/97	56/35
Body weight of live fetuses (g)[a]				
Male	3.39±0.19	3.26±0.19	3.25±0.18	2.71±0.21*
Female	3.19±0.18	3.13±0.18	3.02±0.19*	2.62±0.11*

[a] Values are given as mean±SD.
[b] (No. resorptions and dead fetuses/No. implantations)×100.
* Significantly different from the control, $P<0.05$.

one fetus with sternoschisis at 135 mg/kg. Skeletal variations in the vertebrae, ribs, and/or sternebrae were found in all groups. The incidences of fetuses with skeletal variations and fetuses with bipartite sternebrae and with rudimentary 14th ribs were significantly higher in the 135 mg/kg group than the control group. The numbers of ossification centers of the caudal vertebrae and of the sternebrae were significantly decreased at 135 mg/kg. Hypoplasia of the spleen occurred in two fetuses in one dam at 135 mg/kg. A few fetuses with thymic remnant in the nick and/or left umbilical artery were found in the control group and TP-treated groups. However, there was no significant difference in the incidence of fetuses with internal malformations and variations between the TP-treated groups and the control group. These findings indicate that the lowest-observed-adverse-effect level (LOAEL) and no-observed-adverse-effect level (NOAEL) of TP for fetal rats are 45 and 15 mg/kg, respectively.

4. Discussion

This study was designed to screen for general developmental toxicity in rats. Doses of TP expected to induce maternal and developmental toxicity, such as a decrease in maternal body weight gain and food consumption and in fetal weight and an increase in postimplantation loss, were given to pregnant rats to characterize the effects of TP on embryonic/fetal development. Maternal toxicity, as evidenced by a significant decrease in body weight gain and food consumption

Table 3
Morphological examinations in fetuses of rats given β-thujaplicin (TP) by gastric intubation on days 6–15 of pregnancy

Dose (mg/kg)	0(control)	15	45	135
External examination				
No. fetuses (litters) examined	225(16)	228(16)	204(16)	91(13)
No. fetuses (litters) with malformations	0	0	0	0
Skeletal examination				
No. fetuses (litters) examined	116(16)	117(16)	105(16)	49(13)
No. fetuses (litters) with malformations	0	0	0	1(1)
Sternoschisis	0	0	0	1(1)
No. of fetuses (litters) with variations	11(7)	8(4)	10(7)	21(11)**
Cervical rib	4(2)	3(1)	3(2)	1(1)
Splitting of thoracic vertebral bodies	0	1(1)	0	0
14th ribs				
Extra	0	0	0	4(3)
Rudimentary	2(1)	1(1)	2(2)	9(7)**
Bipartite sternebrae	1(1)	2(1)	1(1)	9(7)**
Asymmetry of sternebrae	5(5)	1(1)	4(3)	3(3)
Degree of ossification[a]				
No. of ossification centers of caudal vertebrae	3.3±0.4	3.1±0.4	3.2±0.4	2.8±0.3**
No. of sternebrae	4.9±0.4	4.9±0.6	4.8±0.5	3.9±0.7**
Internal examination				
No. fetuses (litters) examined	109(16)	111(16)	99(16)	42(12)
No. fetuses (litters) with malformations	0	0	0	2(1)
Hypoplasia of spleen	0	0	0	2(1)
No. of fetuses (litters) with variations	5(3)	3(3)	2(2)	2(2)
Thymic remnant in neck	4(3)	1(1)	2(2)	2(2)
Left umbilical artery	1(1)	2(2)	0	0

[a] Values are given as mean±SD.
* Significantly different from the control, $P<0.05$.

during the administration period was found at 45 mg/kg and higher. Although pregnant rats in the 45 mg/kg group recovered with respect to body weight after cessation of administration of TP, such recovery did not occur in the high dose group. This may be due to a lack of conceptuses at 135 mg/kg. However, a significantly low adjusted weight gain at 45 mg/kg and higher may suggest maternal toxicity. These findings indicate that TP exerts maternal toxicity at 45 mg/kg and higher when administered during organogenesis in rats.

Developmental endpoints should include the number and percent of pre-and postimplantation loss, morphological alterations in fetuses, and decreased fetal weight (Kimmel and Price, 1990; Schardein, 2000; OECD, 2001). Schardein (2000) stated that fetal size is an important in the assessment of potential teratogen as an indicator of developmental toxicity, and reduction in size or growth retardation commonly occurs among fetuses of dams given dosages that are toxic to the dam, to the offspring, or both. In the present study, a significant increase in the incidence of postimplantation loss was found at 135 mg/kg and a significantly decreased weight of female fetuses was found at 45 mg/kg and higher. These findings indicated that TP is embryolethal at 135 mg/kg and toxic to fetal growth at 45 mg/kg and higher when administered during the period of organogenesis.

As for morphological examinations in the fetuses of exposed mother, a few fetuses with skeletal or internal malformations were found in the 135 mg/kg group. The malformations observed in the present study are not thought to be due to the administration of TP, because they occurred at a very low incidence and are of types that occur sporadically among control rat fetuses (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997). Several types of skeletal and internal variations were also found in both the control group and TP-treated groups. These variations are frequently observed in fetuses of rats at term (Kimmel and Wilson, 1973; Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997). In the 135 mg/kg group, a significant increase in the incidence of fetuses with skeletal variations and fetuses with bipartite sternebrae and with rudimentary 14th ribs, but no extra ribs, and a significant decrease in the degree of ossification were accompanied by a significant decrease in the fetal weight. These findings show a correlation between these morphological alterations and growth retardation in fetuses. Although a skeletal variation, i.e. super-

numerary extra 14th ribs, is a warning sign of possible teratogenicity, the rudimentary 14th ribs, sternebral variations, and bilobed centra of the vertebral column are a normal variation (Kimmel and Wilson, 1973). Chahoud et al. (1999) noted that variations are unlikely to adversely affect survival or health and this might result from a delay in growth or morphologenesis that has otherwise followed a normal pattern of development. Consideration of these findings together suggests that the morphological changes observed in the present study do not indicate a teratogenic response and that TP possesses no teratogenic potential in rats.

In a developmental toxicity study in mice in which a single administration of TP was given at 420, 560, 750, or 1000 mg/kg by gastric intubation on day 9 of pregnancy, maternal deaths, dams with litter totally resorbed, and a significant increase in embryolethality were found at 750 mg/kg and higher (Ogata et al., 1999). A significant increase in the incidence of fetuses with malformations was accompanied by a significant decrease in fetal weight at 560 mg/kg and higher. Two highest doses, 750 and 1000 mg/kg, were maternally lethal, and the dose level of 560 mg/kg was very close to the maternally lethal dose. Thus, fetal malformations occurred after a single administration of TP at high doses in a single species. In other words, TP may be capable to produce fetal malformations under extreme experimental conditions in mice. Studies in additional species would be of great value in evaluating developmental toxicity of TP in conventional experimental conditions. We demonstrated here that TP possesses no adverse effects on morphological development in rat fetuses when administered during the whole period of organogenesis at doses which caused a decreased fetal weight, increased incidence of postimplantation loss, and maternal toxicity.

In conclusion, the administration of TP to pregnant rats throughout organogenesis had adverse effects on maternal rats and embryonic/fetal survival and growth but had no adverse effects on morphological development of fetuses even at maternally toxic and embryolethal doses. The data indicate that TP adversely affected the embryonic/fetal survival and growth only at maternally toxic doses in rats. Based on the significant decreases in maternal body weight gain and weight of female fetuses at 45 mg/kg and higher, it is concluded that the no-observed-adverse-effect levels (NOAELs) of TP for both dams and fetuses are considered to be 15 mg/kg in rats.

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Decreased anogenital distance and increased incidence of undescended testes in fetuses of rats given monobenzyl phthalate, a major metabolite of butyl benzyl phthalate

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Abstract

The objective of this study was to determine the adverse effects of monobenzyl phthalate (MBeP), a major metabolite of butyl benzyl phthalate (BBP), on the development of the reproductive system, and to assess the role of MBeP in the antiandrogenic effects of BBP. Pregnant rats were given MBeP by gavage at 167, 250, or 375 mg/kg on days 15–17 of pregnancy. Fetuses were examined on day 21 of pregnancy. Maternal body weight gain and food consumption were significantly decreased at 167 mg/kg and higher. Fetal weight was significantly decreased at 375 mg/kg. A significant increase in the incidence of undescended testes and decrease in the anogenital distance (AGD) and ratio of AGD to the cube root of body weight was found in male fetuses at 250 mg/kg and higher. The AGD and ratio of AGD to the cube root of body weight of female fetuses in the MBeP-treated groups were comparable to those in the control group. The present data indicate that MBeP produces adverse effects on the development of the reproductive system in male offspring and suggest that MBeP may be responsible for the antiandrogenic effects of BBP.

Keywords: Monobenzyl phthalate; Butyl benzyl phthalate; Developmental toxicity; Male reproductive system; Anogenital distance; Undescended testes; Antiandrogenic effect; Rat

1. Introduction

A wide range of uses has been found for the various phthalic acid esters (PAEs) and the largest market for these esters is as plasticizing agents for polyvinyl chloride products [1]. The plasticizers are not irreversibly bound in the polymer matrix and, under certain conditions, can migrate from the plastic to the external environment. PAEs have been ubiquitous environmental pollutants because of their widespread manufacture, use, and disposal as well as their high concentration in and ability to migrate from plastics [2,3]. Butyl benzyl phthalate (BBP) is used as a plasticizer in polyvinyl chloride (PVC) for vinyl tiles, food conveyor belts, carpet tiles, artificial leather, traffic cones, and to a limited extent, vinyl gloves, and is also used in some adhesives [4,5]. BBP may be released into the environment during its production and also during incorporation into plastics or adhesives, and PAEs released into the environment can be deposited on or taken up by crops that are intended for human or livestock consumption, and thereby enter the food supply [4,5]. The most important route of human exposure to BBP is via food. The estimated intake for adults is 2 μg/kg per day; intake values for infants and children are up to three-fold higher [4].

Recently, in vitro screening tests revealed that PAEs such as BBP and dibutyl phthalate (DBP) are estrogenic in estrogen-responsive human breast cancer cells [6–9] and in a recombinant yeast screen [9,10]. The possibility of these compounds entering into biologic systems has caused great concern among the public about their reproductive and developmental toxicity. BBP was shown to be developmentally toxic in mice [11] and rats [12–17]. BBP was noted to produce an impairment of development of the male reproductive system in offspring after maternal exposure [18,19]. We showed that maternal exposure to BBP on days 15–17 of pregnancy at 500 mg/kg and higher caused decreased anogenital distance (AGD) and an increased incidence of male fetuses with undescended testes in rats [19]. BBP was metabolized and converted to monobutyl phthalate (MBuP)

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as one of the major metabolites [20–22]. Maternal administration of MBuP also caused a decrease in male AGD and increased incidence of undescended testes when administered at 250 mg/kg and higher during the susceptible period for the adverse effects on development of the male reproductive system [23]. We hypothesized that MBuP is responsible for the induction of the adverse effects of BBP on development of the male reproductive system. Following administration of BBP in rats, monobenzyl phthalate (MBeP) is formed as another one of the major metabolites of BBP [20–22].

This study was conducted to determine the adverse effects of MBeP on development of the reproductive system in offspring following maternal administration during late pregnancy, and to assess the role of MBeP in the adverse effects of BBP on reproductive development.

2. Materials and methods

2.1. Animals

Wistar rats (Jcl: Wistar, CLEA Japan, Tokyo, Japan) were used throughout this study. Animals were maintained in an air-conditioned room at 23–25 °C with a relative humidity of 50–60% under a controlled 12 h:12 h light/dark cycle. The rats were reared on a basal diet (F-1; Funabashi Farm, Funabashi, Japan) and tap water ad libitum. Virgin female rats, about 14 weeks of age, were mated overnight with male rats of the same strain from the same supplier. 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 four groups of 16 each and housed individually.

2.2. Chemicals and administration

The pregnant rats were given MBeP (100% pure by neutralized titration, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) by gastric intubation at a dose of 167, 250, or 375 mg/kg on day 15 through day 17 of pregnancy. The dosage levels were determined based on the results of our previous study in which MBeP caused a significant increase in the incidences of postimplantation embryonic loss after administration by gavage on days 13–15 of pregnancy at 500 mg/kg and higher, but not at 375 mg/kg [24]. The days of administration were determined based on the results of our previous study in which BBP given by gastric intubation on days 15–17 of pregnancy caused a significant decrease in the AGD and increase in the incidence of undescended testes in male rat offspring [19]. MBeP was given to rats in aqueous solution as the ammonium salt. The solution of each dose was adjusted to pH 6.8–7.2. The volume of each dose was adjusted to 5 ml/kg of body weight based on daily body weight. The control rats received an equivalent amount of ammonium chloride on days 15 through 17 of pregnancy.

2.3. Observations

Pregnant rats were examined daily for obvious signs of toxicity and their weights and food consumption were recorded daily. The pregnant rats were sacrificed by an ether overdose on day 21 of pregnancy. The peritoneal cavity and the uterus were opened and the numbers of live fetuses and of resorptions and dead fetuses were counted. The live fetuses removed from the uterus were weighed and the AGD (the distance between the anus and the genital tubercle) was measured using calipers. The ratio of AGD to the cube root of body weight was calculated [25]. All live fetuses were fixed in Bouin's solution and sectioned through both kidneys. The intestines were removed from the caudal end of the trunk, and fetuses were sexed by examination of the gonads. Male fetuses were examined for undescended testes. Undescended testes were defined by the criterion that the distance between the bladder neck and the lower pole of the testis was greater than one-third of the distance between the bladder neck and the lower pole of the kidney. The degree of transabdominal testicular ascent was determined by measuring the distance from the bladder neck to the lower pole of the testes using calipers, and the measurements were standardized by defining the distance between the bladder neck and the lower pole of the kidney as 100 U [26].

2.4. Data analysis

The litter was used as the basis for analysis of fetal variables. Analysis of variance and Dunnett's multiple comparison test, the 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.

3. Results

The maternal findings in rats given MBeP on days 15 to 17 of pregnancy are shown in Table 1. No deaths were found in any groups. A reddish-staining of facial fur was observed in one pregnant rat at 375 mg/kg. Significant decreases in the maternal body weight gains on days 15–18 at 167 mg/kg and higher and on days 18–21 at 250 mg/kg and higher were found. Adjusted weight gain, which indicates the net weight gain of maternal rats during pregnancy, was significantly reduced at 250 mg and higher. Food consumption on days 15–18 at 167 mg/kg and higher and on days 18–21 at 250 mg/kg and higher was significantly decreased.

The reproductive and fetal findings are presented in Table 2. No significant difference between the MBeP-treated groups and the control group was found in the number of corpora lutea, implantations, resorptions, and dead fetuses, the incidence of postimplantation loss per litter, or the sex ratio of live fetuses. The weights of male and female fetuses in the 375 mg/kg group were significantly less than those in the control group. The incidence of fetuses with

Table 1
Maternal findings in rats given monobenzyl phthalate (MBeP) on days 15–17 of pregnancy

MBeP (mg/kg)	0 (control)	167	250	375
Number of pregnant rats	16	16	16	16
Number of dead pregnant rats	0	0	0	0
Initial body weight (g)[a]	236 ± 7	237 ± 7	235 ± 10	236 ± 10
Body weight gain during pregnancy (g)[a]				
Days 0–15	42 ± 7	42 ± 5	44 ± 5	43 ± 7
Days 15–18	31 ± 4	24 ± 4*	23 ± 7*	15 ± 12*
Days 18–21	40 ± 4	38 ± 4	34 ± 7*	31 ± 9*
Adjusted weight gain[b]	27 ± 8	21 ± 6	15 ± 11*	9 ± 18*
Food consumption during pregnancy (g)[a]				
Days 0–15	245 ± 19	249 ± 19	246 ± 15	246 ± 21
Days 15–18	54 ± 2	46 ± 4*	40 ± 12*	33 ± 10*
Days 18–21	52 ± 3	48 ± 4	44 ± 6*	39 ± 12*

[a] Values are given as the mean ± S.D.
[b] Adjusted weight gain refers to maternal weight gain excluding the gravid uterus.
* Significantly different from the control, $P < 0.05$.

Table 2
Reproductive and fetal findings in rats given monobenzyl phthalate (MBeP) on days 15–17 of pregnancy

MBeP (mg/kg)	0 (control)	167	250	375
Number of litters	16	16	16	16
Number of corpora lutea per litter[a]	15.7 ± 1.1	15.1 ±1.3	15.9 ± 1.2	16.1 ± 1.1
Number of implantations per litter[a]	14.3 ± 2.0	13.5 ± 1.5	15.1 ± 1.2	14.8 ± 1.2
Number of litters totally resorbed	0	0	0	0
Number of resorptions and dead fetuses per litter[a]	1.4 ± 1.1	0.7 ± 0.9	1.1 ± 0.8	1.3 ± 1.9
Percent postimplantation loss per litter[b]	9.7	5.3	8.1	10.9
Number of live fetuses per litter[a]	14.1 ± 1.8	12.8 ± 1.9	13.8 ± 0.8	13.2 ± 1.9
Sex ratio of live fetuses (male/female)	105/101	109/96	107/114	117/94
Body weight of live fetuses (g)[a]				
Male	4.95 ± 0.25	4.95 ± 0.24	4.70± 0.30	3.82 ± 0.65*
Female	4.63 ± 0.20	4.58 ± 0.20	4.39 ± 0.24	3.67 ± 0.56*
Number of male fetuses (litters) with undescended testes	2 (2)	1 (1)	21 (12)*	79 (16)*
Degree of transabdominal testicular ascent[a,c]	18.9 ± 0.3	18.4 ± 2.3	23.8 ± 7.1*	40.1 ± 8.2*

[a] Values are given as the mean ± S.D.
[b] (No. of resorptions + no. of dead fetuses)/(no. of implantations) × 100.
[c] (Distance between the bladder neck and the lower pole of the testes)/(distance between the lower pole of the kidney and the bladder neck) × 100.
* Significantly different from the control, $P < 0.05$.

undescended testes was significantly increased at 250 mg/kg and higher. The degree of transabdominal testicular ascent in relation to the bladder was also significantly increased at 250 mg/kg and higher.

Fig. 1 shows the AGD and AGD per cube root of body weight ratio in male and female fetuses of rats given MBeP on days 15–17 of pregnancy. AGD was significantly reduced at 250 and 375 mg/kg in male offspring. Male AGD at the highest dose was female-like. The AGD of female fetuses in the MBeP-treated groups was comparable to that in the control group. The ratio of AGD to the cube root of body weight of male fetuses in the 250 and 375 mg/kg groups was significantly lower than that in the control group. No significant difference in the AGD per cube root of body weight ratio of female fetuses was detected between the control group and the MBeP-treated groups.

4. Discussion

We previously showed that MBuP, one of the major metabolites of BBP, adversely affected the development of the reproductive system in male offspring when administered on days 15–17 of pregnancy [23], the most susceptible period for the adverse effects on development of the male reproductive system [27]. The present study demonstrated that MBeP, another of the major metabolites of BBP, administered during this period caused a significant decrease in the male AGD and increase in the incidence of undescended testes in a dose-dependent manner.

Adverse effects of MBeP on maternal rats, as evidenced by a significant decrease in the maternal body weight gain and food consumption, were found at all doses. Although no embryolethality was found after treatment with MBeP,

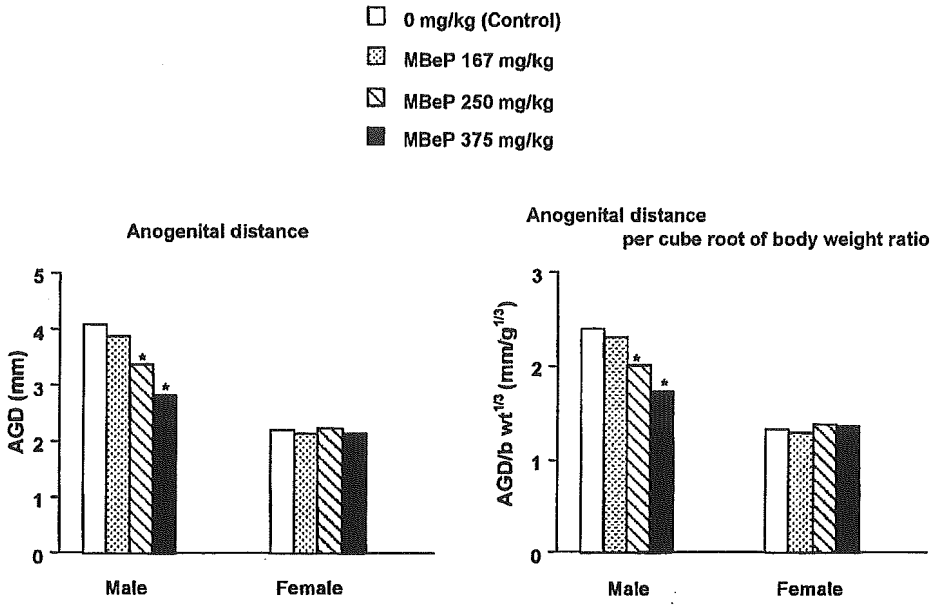

Fig. 1. Anogenital distance (AGD) and AGD per cube root of body weight ratio in male and female fetuses of rats given monobenzyl phthalate (MBeP) on days 15–17 of pregnancy. Values are given as the mean. *Significantly different from the control group, $P < 0.05$.

adverse effects on the growth of offspring, as evidenced by a significantly lower fetal weights, were detected at 375 mg/kg, but not at 250 mg/kg. These findings indicate that MBeP is maternal toxic at 167 mg/kg and is toxic to growth of the embryo/fetus at 375 mg/kg when administered on days 15–17 of pregnancy in rats.

The AGD and ratio of AGD to the cube root of body weight in male fetuses, but not in female fetuses, were significantly decreased at 250 mg/kg and higher. A significantly higher incidence of fetuses with undescended testes was also found at 250 mg/kg and higher following administration of MBeP on days 15–17 of pregnancy. We previously reported that a significant increase in the incidence of malformed fetuses was detected following administration of MBeP on days 7–15 of pregnancy at 313 mg/kg and higher, but not at 250 mg/kg [28]. Thus, the doses that produced impairment of development of the male reproductive system were lower than those that produced malformations in major organs. These findings suggest that the male reproductive system may be more susceptible than other organ systems to MBeP toxicity after maternal exposure and changes in development of the male reproductive system may be a sensitive parameter for toxic effects. This phenomenon was noted after maternal administration of DBP [27,29], BBP [19], and MBuP [23].

BBP administered orally was rapidly metabolized to MBuP and MBeP by pancreatic lipase and esterases in the small intestine [20–22]. These monoesters were absorbed from the gut and excreted in the urine. Although MBuP and MBeP were detected in rat urine, BBP, the parent compound, was never recovered in urine [22]. These phenomena were observed in the biotransformation of other PAEs and Lake et al. [30] described that any toxic effects of orally ingested PAEs would be governed essentially by the properties of the corresponding monoesters ad/or alcohols rather than by those of the intact diesters.

Following administration of DBP, MBuP is also formed as a major metabolite [31–34]. We previously observed that the phase specificity of teratogenicity and the most frequent types of fetal malformations after administration during major organogenesis induced by MBuP [35] were consistent with those induced by DBP [36] and BBP [14] and that those induced by MBeP [24] were consistent with those induced by BBP [14]. These findings suggested that the teratogenicity of DBP is mediated via MBuP and the teratogenicity of BBP is mediated via MBuP and MBeP. We also previously reported that BBP and MBuP produced a decrease in the male AGD and increase in the incidence of undescended testes in offspring of rats treated during late pregnancy [19,23] and showed here that MBeP had adverse effects on the development of the male reproductive system in a dose-dependent manner. It appears that MBuP and MBeP may participate in the induction of the antiandrogenic effects of BBP. Therefore, both of the major metabolites, MBuP and MBeP, are considered to be responsible for the induction of the developmental toxicity, including the teratogenic and antiandrogenic effects, of BBP.

A decrease in testosterone levels was found in the fetal testis of rats given DBP at 500 mg/kg on days 12–21 of pregnancy; testicular testosterone was 34 and 26% of control on days 18 and 21 of pregnancy, respectively [37]. DBP and MBuP were negative in the competitive binding and transcriptional activation assay with androgen receptor [38]. These finding suggest that the antiandrogenic effects of DBP

are induced by a reduction in fetal T levels and mediated via MBuP, but not mediated directly at the level of the androgen receptor. MBeP and MBuP were reported to have no estrogenic activity in a yeast screen [9], but the effects of MBeP on androgenic receptors and rat fetal T levels are unknown. Further studies are needed to determine the effects of MBeP on the androgenic receptor and fetal rat T levels.

The results of the present study suggest that MBuP may be responsible for the induction of the antiandrogenic effects of BBP and MBeP may also participate, at least in part, in the induction of the antiandrogenic effects of BBP.

In this study, a no observed adverse effect level (NOAEL) for offspring was 250 mg/kg but a NOAEL for dams was not established. The lowest NOAEL for BBP is reported to be 20 mg/kg based on a decrease in body weight of F1 offspring in a two-generation reproductive study in rats [17]. This value is at least 3000-fold higher than the human exposure (adults 2 μg/kg per day, infants and children 6 μg/kg per day [4]). Thus, the risk to the human fetuses and neonates appears to be extremely low. However, the combined risk associated with exposure to DBP should be considered because BBP and DBP have a common active metabolite, MBuP.

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Protective effects of progesterone on implantation failure induced by dibutyltin dichloride in rats

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Abstract

We previously showed that dibutyltin dichloride (DBTCl) at 7.6 mg/kg and higher on days 0–3 of pregnancy caused implantation failure and a decline in serum progesterone levels in rats and hypothesized that the decline is responsible for the implantation failure. This study was conducted to determine the protective effects of progesterone on the DBTCl-induced implantation failure in rats. Rats were given oral DBTCl at 0, 7.6, or 15.2 mg/kg on days 0–3 of pregnancy and/or subcutaneous progesterone at 2 mg/rat on days 0–8 of pregnancy. The reproductive outcome was determined on day 9 of pregnancy. No effects of administration of progesterone alone on the pregnancy rate and number of implantations were found. The pregnancy rate and number of implantations were significantly decreased after administration of DBTCl alone. The pregnancy rate and number of implantations were higher in the groups given DBTCl and progesterone than the groups given DBTCl alone. The present data indicate that progesterone protects, at least in part, against the DBTCl-induced implantation failure and support our hypothesis that the decline in progesterone levels is a primary mechanism for the implantation failure due to DBTCl.

Keywords: Dibutyltin chloride; Organotin; Implantation failure; Early embryonic loss; Progesterone

1. Introduction

Organotin compounds are chemicals widely used in agriculture and industry. Disubstituted organotin compounds are commercially the most important derivatives, being used as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during melting and forming of the resin into its final products as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers (Piver, 1973; WHO, 1980). The amounts of organotin released into the environment have increased with its widespread use. The most important non-pesticidal route of entry of organotin compounds into the environment is through leaching of organotin-stabilized PVC by water (Quevauviller et al., 1991), and use in antifouling agents resulting in the entry of organotin into the aquatic environment (Maguire, 1991).

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Data are available regarding the identification of dibutyltin (DBT) and tributyltin (TBT) in aquatic marine organisms (Sasaki et al., 1988; Lau, 1991) and marine products (Suzuki et al., 1992). In the environment, TBT is degraded spontaneously and biochemically via a debutylation pathway to DBT (Seligman et al., 1988; Stewart and de Mora, 1990). Organotin compounds are introduced into foods by the use of pesticides and antifoulants and via migration of tin from PVC materials (WHO, 1980). The dietary exposure of Japanese consumers to organotin compounds was estimated and reported that daily intake was 1.7 μg/person for TBT, 0.45 μg/person for DBT, 0.09 μg/person for triphenyltin, and 0 μg/person for diphenyltin (Toyoda et al., 2000).

Although the toxicity of organotins has been extensively reviewed, the developmental and reproductive toxicity of these compounds is much less well understood (Boyer, 1980; WHO, 1980; Snoeij et al., 1987). We previously reported that oral administration of dibutyltin dichloride (DBTCl) at 5 mg/kg throughout the period of organogenesis resulted in a significant increase in the incidence of fetuses with malformations (Ema et al., 1991). Rat embryos were highly susceptible to the teratogenic effects of DBTCl when administered on days 7 and 8 of pregnancy (Ema et al., 1992). We also reported that DBTCl had dysmorphogenic effects in rat embryos in a whole embryo culture system (Ema et al., 1995, 1996).

Recently we reported that a significant increase in implantation failure, preimplantation embryonic loss, was caused following oral administration of DBTCl on days 0–3 of pregnancy at 7.6 mg/kg and higher in rats (Ema and Harazono, 2000a,b). We also showed that DBTCl caused the suppression of uterine decidualization and a decrease in serum progesterone levels in pseudopregnant rats at doses which induced implantation failure (Harazono and Ema, 2001). These findings suggest that a decline in progesterone levels causes the suppression of uterine decidualization and impairment of uterine function, and these effects are responsible for the DBTCl-induced implantation failure. This study was designed to determine whether the administration of progesterone protects against the DBTCl-induced implantation failure in rats.

2. Materials and methods

2.1. Animals

Wistar rats (Jcl: Wistar, CLEA Japan, Tokyo, Japan) were used in this study. The animals were maintained in an air-conditioned room at 23–25 °C, with a relative humidity of 50–60%, under a controlled 12/12 light/dark cycle. The rats were reared on a basal diet (F-1; Funabashi Farm Co., Funabashi, Japan) and tap water ad libitum. Daily vaginal smears were monitored from virgin female rats, about 13 weeks of age. On the evening of proestrus, female rats were caged overnight for 15 h with untreated, proven-fertile male rats and checked the following morning for signs of successful mating by examining vaginal smears. The day when sperm were detected in the vaginal smear was considered to be day 0 of pregnancy.

2.2. Administration of dibutyltin dichloride (DBTCl) and/or progesterone

The rats were dosed once daily by gastric intubation with DBTCl (98% pure, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) at 0, 7.6, or 15.2 mg/kg on days 0–3 after mating. The dosage levels were determined based on the results of our previous study in which DBTCl at 7.6 and 15.2 mg/kg caused significant increases in implantation failure and preimplantation embryonic loss in rats (Ema and Harazono, 2000a,b). The DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Successfully mated females were distributed on a random basis into six groups of 14–15 rats each and housed individually. Three groups were subcutaneously injected with progesterone at 2 mg/rat on days 0–8 after mating. The remaining three groups received no progesterone. The volume of each dose of DBTCl was adjusted to 5 ml/kg of body weight based on the daily body weight. The control rats received olive oil only.

2.3. Observations

The female rats were sacrificed by ether overdose on day 9 after mating and the reproductive

outcome was determined. The numbers of corpora lutea and implantations were counted under a dissecting microscope. The uteri were placed in 2% sodium hydroxide for confirmation of the pregnancy status.

2.4. Data analysis

The initial body weight, body weight gain and food consumption of the female rats, and number of implantations were evaluated by analysis of variance, followed by a Dunnett's multiple comparison test if differences were found. Statistical comparisons of the pregnant females and non-pregnant females were made using Fisher's exact test. The 0.05 level of probability was used as the criterion for significance.

3. Results

Table 1 shows the body weight gain and food consumption in female rats given DBTCl and/or progesterone. The body weight gain and food consumption on days 0–4 and 4–9 of pregnancy in the groups given DBTCl with or without progesterone were significantly lower than those in the control group and the group given progesterone alone. No significant differences in body weight gain and food consumption were found between the control group and the group given progesterone alone, or between the groups given DBTCl alone and the groups given DBTCl and progesterone.

Reproductive findings in female rats given DBTCl and/or progesterone are presented in Table 2. There were no significant differences in the reproductive parameters between the control group and the group given progesterone alone. The pregnancy rate and number of implantations per female in the groups given DBTCl alone at 7.6 or 15.2 mg/kg were significantly lower than those in the control group and in the group given progesterone alone. The pregnancy rate and number of implantations per female were higher in the groups given DBTCl and progesterone than in the groups given DBTCl alone, and significantly higher values were found in the group given DBTCl at 7.6 mg/kg and progesterone. The incidence of preimplantation embryonic loss was significantly higher in the groups given DBTCl with or without progesterone than in the control group and in the group given progesterone alone. The incidence of preimplantation loss was lower in the groups given DBTCl and progesterone than in the groups given DBTCl alone, and significantly lower values were found in the group given DBTCl at 7.6 mg/kg and progesterone.

4. Discussion

We previously showed that DBTCl caused implantation failure (Ema and Harazono,

Table 1
Body weight gain and food consumption in female rats given DBTCl with or without progesterone

DBTCl (mg/kg)	0 (control)	0	7.6	7.6	15.2	15.2
Progesterone (mg/rat)	0	2	0	2	0	2
Number of females successfully mated	14	14	15	14	15	14
Initial body weight (g)[a]	236±12	237±9	232±14	237±12	234±14	235±14
Body weight gain (g)[a]						
Days 0–4	8±4	7±6	−24±12*†	−24±11*†	−31±4*†	−28±5*†
Days 4–9	12±4	14±4	−11±22*†	−22±17*†	−35±5*†	−31±9*†
Food consumption (g)[a]						
Days 0–4	48±8	46±9	10±11*†	9±10*†	4±1*†	3±2*†
Days 4–9	80±8	78±8	25±30*†	15±27*†	2±1*†	4±4*†

*, Significantly different from the control group; $P < 0.05$. †, Significantly different from the group given progesterone alone, $P < 0.05$.

[a] Values are given as the mean±S.D.

Table 2
Reproductive findings in female rats given DBTCl with or without progesterone

DBTCl (mg/kg)	0 (control)	0	7.6	7.6	15.2	15.2
Progesterone (mg/rat)	0	2	0	2	0	2
Number of females successfully mated	14	14	15	14	15	14
Number of pregnant females (%)	14 (100)	14 (100)	7 (46.7)*†	13 (92.9)#	5 (33.3)*†	9 (64.3)*†
Number of non-pregnant females (%)	0 (0)	0 (0)	8 (53.3)*†	1 (7.1)#	10 (66.7)*†	5 (35.7)*†
Number of corpora lutea[a]	16.3±1.3	17.0±1.9	15.1±1.3†	15.6±1.7	15.3±1.5†	15.3±1.1†
Number of implantations[a]	14.9±2.1	15.1±1.3	5.6±6.6*†	11.6±5.2†#	2.9±5.1*†	6.1±6.3*†
Preimplantation loss (%)	8.6	10.5	62.8*†	25.9*†#	81.3*†	60.0*†

*, Significantly different from the control group, $P < 0.05$. †, Significantly different from the group given progesterone alone, $P < 0.05$. #, Significantly different from the group given DBTCl alone, $P < 0.05$.
[a] Values are given as the mean±S.D.

2000a,b) and the suppression of uterine decidualization correlated with the reduction in serum progesterone levels in rats, and hypothesized that this decline in progesterone levels may be responsible for the DBTCl-induced reproductive failure (Harazono and Ema, 2001). In this study, we determined the effects of progesterone on reproductive parameters in pregnant rats, and showed that progesterone protects against the DBTCl-induced implantation failure.

Normal reproductive function in females involves the interaction of the central nervous system, ovary, and uterus, and toxic effects at these sites can affect embryonic survival. The function of the uterine endometrium is one of the principle factors for the initiation and maintenance of pregnancy. Adequate levels of progesterone are required for normal uterine decidualization and a normal decidualization is required for normal implantation of the embryos (Yochim and De Feo, 1962; Hashimoto and Wiest, 1969). We showed here that lowered reproductive parameters in the groups given DBTCl were recovered by the administration of progesterone, and the values in the groups given DBTCl at 7.6 mg/kg in combination with progesterone were comparable to those in the control group and group given progesterone alone. These findings indicate that progesterone protects against the DBTCl-induced reproductive failure, and support our previous hypothesis that the decline in progesterone levels is the primary factor responsible for the DBTCl-induced implantation failure. However, the number and percent of implantations were less in the groups given DBTCl and progesterone compared with the control group and group given progesterone alone, and these values in the group given DBTCl at 15.2 mg/kg in combination with progesterone were different from the control values. Thus, incompletely protective effects of progesterone against the DBTCl-induced implantation failure were noted, especially at higher dose of DBTCl. It is likely that other mechanisms act in the induction of implantation failure.

In this study, no significant difference in maternal body weight gain or food consumption was found between the females given DBTCl and progesterone and females given progesterone alone. These results indicate that the progesterone did not protect against the maternal toxicity induced by DBTCl, and that the progesterone protects, at least in part, against the implantation failure without the recovery of overt maternal damage. In other words, the implantation failure after the administration of DBTCl during early pregnancy may be due to the direct effect of DBTCl, not to any secondary effects of maternal toxicity. However, DBTCl at high dose was severely maternal toxic and progesterone incompletely protected the DBTCl-induced implantation failure.

In our previous study, significant increases in the incidences of preimplantation embryonic loss were observed after administration of TBTCl, the

parent compound of TBTCl, at 16.3 mg/kg and higher on days 0–3 of pregnancy (Harazono et al., 1998a,b). TBTCl at 16.3 mg/kg is equivalent to 50 μmol/kg. We also showed that DBTCl on days 0–3 of pregnancy induced a significant increase in the incidence of preimplantation embryonic loss at 7.6 mg/kg (Ema and Harazono, 2000a,b). DBTCl at 7.6 mg/kg is equivalent to 25 μmol/kg. More precisely, the doses of DBTCl that caused early embryonic loss were lower than those of TBTCl. The DBT compound was identified as the main metabolite of the tributyltin compound in rats (Iwai et al., 1981). If on a mole equivalent basis a metabolite is as effective or more effective than the parent compound, this is consistent with the view that the metabolite is the proximate toxicant or at least an intermediate to the proximate toxicant. It is apparent that DBTCl participates in the induction of early embryonic loss due to TBTCl. Furthermore, TBTCl (Harazono and Ema, 2000; Ema and Harazono, 2000b) and DBTCl (Harazono and Ema, 2001) also caused the suppression of uterine decidualization correlated with decreased levels of serum progesterone in pseudopregnant rats at doses that induced implantation failure. These findings suggest the same mechanisms act in the induction of implantation failure due to TBTCl and DBTCl. Consideration of these findings together suggests that the TBTCl-induced implantation failure is mediated by the decline in the maternal serum progesterone levels due to DBT.

In summary, the administration of progesterone protects, at least in part, against the DBTCl-induced implantation failure. The present data support our hypothesis that the decline in progesterone levels is a primary mechanism for the implantation failure due to DBTCl.

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HIGHER SUSCEPTIBILITY OF NEWBORN THAN YOUNG RATS TO 3-METHYLPHENOL

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ABSTRACT — To determine susceptibility of infants to 3-methylphenol, a repeated dose toxicity study was conducted with oral administration to newborn and young rats. In an 18-day newborn study from postnatal days 4 to 21 at doses of 30, 100 and 300 mg/kg/day, various clinical signs including deep respiration, hypersensitivity on handling and tremors under contact stimulus, and depressed body weight gain were observed at 300 mg/kg. At 100 mg/kg, hypersensitivity and tremors were also noted in a small number of males only on single days during the dosing period. No adverse effects were observed in the 30 mg/kg group. There were no abnormalities of physical development, sexual maturation and reflex ontogeny. The no observed adverse effect level (NOAEL) for newborn rats was considered to be 30 mg/kg/day and the unequivocally toxic level 300 mg/kg/day. In a 28-day study starting at 5 weeks of age, clinical signs and depression of body weight gain, as observed in the newborn rats, appeared in both sexes at 1000 mg/kg but not 300 mg/kg. The NOAEL and the unequivocally toxic level were 300 mg/kg/day and 1,000 mg/kg/day, respectively. From these results, newborn rats were concluded to be 3 to 10 times more susceptible to 3-methylphenol than young rats. However, the realistic no adverse effect dose for the newborn must be slightly lower than 100 mg/kg/day, at which the toxicity incidence was very low, rather than 30 mg/kg/day. Based on this speculation and the equal toxicity at unequivocally toxic levels, the differences in the susceptibility to 3-methylphenol could be concluded to be 3 to 4 times. This is consistent with the results of our previous comparative studies on 4-nitrophenol, 2,4-dinitrophenol and 3-aminophenol, which showed 2 to 4 times differences in the susceptibility between newborn and young rats.

KEY WORDS: Toxicity in newborn rats, 3-Methylphenol

INTRODUCTION

It is known that neonates have specific physiological characteristics with regard to water volume per body, weight of liver and brain relative to body size, cardiac output, respiratory rate, and blood flow to brain and kidney, for example. In fact, the toxicokinetic ability of infants seems to differ from that of adults with respect to their metabolism, clearance, protein binding and volume of distribution, based on data obtained with therapeutic drugs (Besunder *et al.*, 1988; Kearns and Reed, 1989; Morselli, 1989), although there is very little information regarding environmental chemicals. Furthermore, the sensitivity of rapidly developing tissues/systems in neonates may also differ from that in adults (Vesselinovitch *et al.*, 1979; Pope *et al.*, 1991; Faustman *et al.*, 2000). Since infants are always exposed to various chemicals by putting fingers, toys and other objects into their mouths as well as via mother's milk, there is growing concern about effects

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