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Developmental toxicity of dibutyltin dichloride in cynomolgus monkeys

Makoto Ema [a,*], Katsuhiro Fukunishi [b], Mariko Matsumoto [a], Akihiko Hirose [a], Eiichi Kamata [a], Toshio Ihara [b]

[a] *Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Shin Nippon Biomedical Laboratories Ltd., Kagoshima, Japan*

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Abstract

Dibutyltin dichloride (DBTCl) has been shown to be teratogenic in rats. The present study was conducted to determine the teratogenic potential of DBTCl given to pregnant monkeys during the entire period of organogenesis. Cynomolgus monkeys were dosed once daily by nasogastric intubation with DBTCl at 0, 2.5 or 3.8 mg/kg on days 20–50 of pregnancy, the whole period of organogenesis. The pregnancy outcome was determined on day 100 of pregnancy. In both DBTCl-treated groups, a significant increase in the incidence of pregnant females with soft stool and/or diarrhea, and with yellowish stool was observed. Maternal body weight gain at 3.8 mg/kg and food consumption at 2.5 and 3.8 mg/kg were decreased during the administration period. The survival rate of fetuses at terminal cesarean sectioning was decreased in the DBTCl-treated groups and significantly decreased at 2.5 mg/kg. There were no changes in the developmental parameters of surviving fetuses, including fetal body weight, crown-rump length, tail length, sex ratio, anogenital distance and placental weight, in the DBTCl-treated groups. No external, internal or skeletal malformations were found in the fetuses in any group. Although internal and skeletal variations were found, no difference in the incidence of fetal variation was noted between the control and DBTCl-treated groups. No effect on skeletal ossification was observed in fetuses in the DBTCl-treated groups. The data demonstrate that DBTCl is embryolethal but not teratogenic in cynomolgus monkeys.

Keywords: Dibutyltin; Organotin; Teratogenicity; Embryolethality; Monkey

1. Introduction

Organotin compounds are widely used in agriculture and industry. The most important non-pesticidal route of entry of organotin compounds into the environment is through the leaching of organotin-stabilized polyvinyl chloride (PVC) by water [1], and its use in antifouling agents, resulting in the entry of organotin into the aquatic environment [2]. Disubstituted organotin compounds are commercially the most important derivatives, being used as heat and light stabilizers for PVC plastics to prevent degradation of the polymer during melting and the forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers [3,4]. The identification of dibutyltin (DBT) and tributyltin (TBT) in aquatic marine organisms [5,6] and marine products [7] has been reported. TBT is degraded spontaneously and biochemically via a debutylation pathway to DBT in the environment [8,9]. Organotin compounds are introduced into foods by the use of pesticides and antifoulants and via the migration of tin from PVC materials [4].

We previously demonstrated that tributyltin chloride (TBTCl) during early pregnancy caused early embryonic loss [10–12], and TBTCl on days 10–12 and on days 13–15, but not on days 7–9 of pregnancy, produced fetal malformations in rats [13]. The predominant malformation induced by TBTCl was cleft palate [13,14]. It has been reported that TBT is metabolized to DBT and MBT, and DBT was metabolized to monobutyltin (MBT) [15–17]. DBT is also reported to have toxic effects on reproduction and development in rats [18]. The oral administration of dibutyltin dichloride (DBTCl) during early pregnancy caused early embryonic loss in rats [19–21]. The oral administration of DBTCl to rats throughout the period of organogenesis resulted in a significant increase in the incidence of fetuses with malformations [22], and rat embryos were highly susceptible to the

* Corresponding author. Tel.: +81 3 3700 9878; fax: +81 3 3700 1408.
E-mail address: ema@nihs.go.jp (M. Ema).

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teratogenic effects of DBTCl when it was administered on day 7 and 8 of pregnancy [23]. Dibutyltin diacetate (DBTA) [24–28], dibutyltin maleate, dibutyltin oxide, and dibutyltin dilaurate [26] were teratogenic in rats when administered orally. Developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from that of tetrabutyltin (TeBT), TBT and MBT in its mode of action because the period of susceptibility to teratogenicity and the types of malformations induced by DBT are different from those induced by TeBT, TBT and MBT [29,30]. DBTCl had dysmorphogenic effects in rat embryos in a whole embryo culture system [31,32]. DBT was detected in rat maternal blood at 100 ng/g and embryos at 720 ng/g at 24 h after gavage of DBTA at 22 mg/kg on day 8 of pregnancy [27]. The dysmorphogenic concentrations of DBTCl in cultured embryos were within the range of levels detected in maternal blood after the administration of a teratogenic dose of DBT. These findings suggest that DBT itself is a causative agent in DBT teratogenesis, which may be due to direct interference with embryos.

As described above, the teratogenic effects of organotin compounds, including DBT, were extensively investigated in rodents [18]. No reports on the assessment of the teratogenicity of DBT in any other species are available. It appears that conclusive evidence in support of the teratogenicity of DBT is still lacking, because the teratogenicity of DBT only has been reported in a single animal species. Studies in non-rodents would be of great value in estimating the teratogenicity of DBT in humans. The present study was conducted to determine the teratogenic potential of DBTCl given to pregnant cynomolgus monkeys during the entire period of organogenesis.

2. Materials and methods

2.1. Animals

Cynomolgus monkeys (*Macaca fascicularis*) were used in this study. The monkeys were obtained from Guangxi Primate Center of China (Guangxi, China) through Guangdong Scientific Instruments and Materials Import/Export Co. (Guangzhou, China). The monkeys were quarantined for 4 weeks, and confirmed to be free from tuberculosis, *Salmonella* and *Shigera*. The animals were maintained in an air-conditioned room at 23.0–29.0 °C, with a relative humidity of 45–58%, under a controlled 12/12 light/dark cycle, with a ventilation rate of 15 air changes/hour, and were housed individually, except during the mating period. The monkeys were fed 108 g/day of diet (Teklad global 25% protein primate diet; Harlan Sprague-Dawley Inc., Madison, USA) and tap water ad libitum from automatic lixit devices. Healthy male and female monkeys were selected for use. Only females showing 25–32 days menstrual cycles were used in the experiment. Each female monkey was paired with a male of proven fertility for three consecutive days between days 11–15 of the menstrual cycle. The visual confirmation of copulation and/or the presence of sperm in the vagina were considered evidence of successful mating. When copulation was confirmed, the

Table 1
Maternal findings in monkeys given DBTCl on days 20–50 of pregnancy

	Dose (mg/kg)		
	0 (control)	2.5	3.8
Number of pregnant females	12	12	10
Number of females showing toxicological signs			
Death	0	0	0
Soft stool/diarrhea	1	12*	10*
Yellowish stool	0	8*	8*
Vomiting	0	3	3
Initial body weight	3.53 ± 0.59	3.49 ± 0.43	3.79 ± 0.36
Body weight gain during pregnancy (g)[a]			
Days 0–20	76 ± 114	42 ± 160	73 ± 142
Days 20–51	57 ± 237	−242 ± 423	−556 ± 526*
Days 51–100	710 ± 162	755 ± 174	848 ± 263
Food consumption during pregnancy (g/day)[a]			
Days 20–21	99 ± 18	93 ± 23	76 ± 33
Days 23–24	91 ± 27	71 ± 31	55 ± 31*
Days 27–28	77 ± 28	47 ± 19*	37 ± 34*
Days 30–31	63 ± 32	33 ± 15*	22 ± 10*
Days 34–35	88 ± 25	53 ± 42	23 ± 17*
Days 37–38	86 ± 28	53 ± 42*	25 ± 24*
Days 41–42	87 ± 27	59 ± 59	36 ± 29*
Days 44–45	95 ± 22	62 ± 40	41 ± 31*
Days 48–49	98 ± 18	70 ± 48	59 ± 44
Days 51–52	94 ± 20	97 ± 24	71 ± 39
Days 55–56	102 ± 12	107 ± 2	100 ± 20
Days 58–59	106 ± 7	108 ± 0	104 ± 10
Days 62–63	106 ± 7	108 ± 0	106 ± 5
Days 80–81	108 ± 0	108 ± 0	108 ± 0
Days 90–91	106 ± 7	108 ± 0	108 ± 0
Days 99–100	108 ± 0	108 ± 0	108 ± 0

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

median day of the mating period was regarded as day 0 of pregnancy. Pregnancy was confirmed on day 18 or 19 of pregnancy by ultrasound (SSD-4000, Aloka Co., Mitaka, Japan) under anesthesia induced by intramuscular injection of 5% ketamine hydrochloride (Sigma Chemical Co., St. Louis, USA). Pregnant females, weighing 2.51–4.50 kg on day 0 of pregnancy, were allocated randomly to three groups, each of 10–12 monkeys, and housed individually. Animal experiments were performed at Shin Nippon Biomedical Laboratories, Ltd. (SNBL; Kagoshima, Japan) during 2004–2005 in compliance with the Guideline for Animal Experimentation (1987) [33], and in accordance with the Law Concerning the Protection and Control of Animals (1973) [34] and the Standards Relating to the Care and Management of Experimental Animals (1980) [35]. This study has been approved by the Institutional Animal Care and Use Committee of SNBL and performed in accordance with the ethics criteria contained in the bylaws of the committee of SNBL.

2.2. Dosing

The monkeys were dosed once daily with DBTCl (lot no. GG01, 98% pure, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) at 0, 2.5 or 3.8 mg/kg by nasogastric intubation on days 20–50 of pregnancy, i.e., the entire period of organogenesis [36]. Dosing was terminated in the dams in which embryonic/fetal loss occurred. The dosage levels were determined from the results of previous studies in rats, in which DBTCl administered by gavage at 7.6 or 15.2 mg/kg on days 0–3 and days 4–7 of pregnancy caused significant increases in pre- and/or post-implantation embryonic loss in rats [19–21], and in which DBTCl by gavage at 5, 7.5 or 10.0 mg/kg throughout the period of organogenesis resulted in a significant increase in the incidence of fetuses with malformations [22]. DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The dose volume was adjusted to 0.5 ml/kg of the most recent body weight. The control monkeys received olive oil only.

2.3. Observations

The pregnant monkeys were observed for clinical signs of toxicity twice a day during the administration period and once a day during the non-administration period. The body weight was recorded on days 0, 20, 27, 34, 41, 51, 60, 70, 80, 90 and 100 of pregnancy. The food consumption was recorded on days 20, 23, 27, 30, 34, 37, 41, 44, 48, 51, 55, 58, 62, 80 and 90 of pregnancy. Embryonic/fetal heartbeat and growth were monitored using ultrasound under anesthesia induced by intramuscular injection of 5% ketamine hydrochloride on days 25, 30, 35, 40, 50, 60, 70, 80, 90 and 99 of pregnancy. In the dams in which embryonic/fetal cardiac arrest was confirmed by ultrasound, necropsy was performed under anesthesia induced by intraperitoneal injection of pentobarbital Na (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan). The uterus, including the embryo/fetus and placenta and ovaries, was removed from the maternal body and stored in 10% neutral buffered formalin. Dead or aborted embryos/fetuses were morphologically examined.

Terminal cesarean sectioning was performed on day 100 of pregnancy, under anesthesia induced by intramuscular injection of 5% ketamine hydrochloride (0.1–0.2 ml/kg) and inhalation of isoflurane (0.5–2.0%, Dainippon Pharmaceutical Co. Ltd., Osaka, Japan), and contraction was induced with atropine (0.01 mg/kg, Tanabe Seiyaku Co. Ltd., Osaka, Japan). The fetus and placenta were removed from the dams. The placenta was weighed and stored in 10% neutral buffered formalin. Dams that underwent cesarean sectioning were not necropsied.

Fetal viability was recorded, and the fetuses were anesthetized by intraperitoneal injection of pentobarbital Na and euthanized by submersion in saline for 30–40 min at room temperature. Fetuses were sexed and examined for external anomalies after confirmation of the arrested heart-beat. Fetal and placental weights were recorded. The head width, tail length, crown-rump length, chest circumference, paw and foot length, distance between the eyes, umbilical cord length, volume of amniotic fluid and diameters of the primary and secondary placentae were measured. After the completion of external examinations, fetuses were examined for internal anomalies. The peritoneal cavity was opened and the organs were grossly examined. The brain, thymus, heart, lung, spleen, liver, kidneys, adrenal glands and testes/uterus and ovaries were weighed and stored in 10% neutral buffered formalin. The eyeballs, stomach, small and large intestine, head skin and auricles were stored in 10% neutral buffered formalin. Fetal carcasses were fixed in alcohol, stained with alizarin red S [37] and examined for skeletal anomalies. The number of ossification centers of the vertebral column, and lengths of the ossified parts of the humerus, radius, ulna, femur, tibia and fibula were recorded. Histopathological evaluations were performed on single

Table 2
Reproductive and developmental findings in monkeys given DBTCl on days 20–50 of pregnancy

	Dose (mg/kg)		
	0 (control)	2.5	3.8
Number of pregnant females	12	12	10
Number of females with embryonic/fetal loss	1	8*	4
Number of females with live fetuses until terminal cesarean section	11	4*	6
Number of live fetuses at terminal cesarean section	11	4*	6
Sex ratio of live fetuses (male/female)	6/5	1/3	3/3
Body weight of live fetuses (g)			
Male	133 ± 13	125	112 ± 24
Female	118 ± 12	108 ± 20	118 ± 13
Anogenital distance (cm)[a]			
Male	2.0 ± 0.2	1.9	1.7 ± 0.4
Female	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1
Crown-rump length (cm)[a]			
Males	12.8 ± 0.6	12.4	12.4 ± 0.7
Female	12.6 ± 0.4	12.3 ± 0.5	12.6 ± 0.1
Tail length (cm)[a]			
Male	11.8 ± 1.2	11.8	11.4 ± 0.7
Female	11.9 ± 0.8	11.7 ± 1.7	12.4 ± 0.6
Placental weight (g)[a]	42.4 ± 7.2	38.9 ± 6.2	37.5 ± 9.1
Number of a single placenta	1	1	3

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

placentas and accessory spleens after fixation, paraffin embedding, sectioning and staining with hematoxylin and eosin.

2.4. Analysis of plasma steroids hormone levels

Blood samples were collected from the femoral vein on day 51 of pregnancy, 24 h after the last administration of DBTCl. The plasma was separated and stored at −80 °C for the later assay of steroid hormones. Plasma progesterone and 17β-estradiol were measured by Teizo Medical Co. Ltd. (Kawasaki, Japan) using liquid chromatography-electrospray ionization Tandem Mass Spectrometry (LC-MS/MS, Applied Biosystems/MDS SCIEX). The detection limits of plasma progesterone and 17β-estradiol were 10.0 pg/ml and 0.25 pg/ml, respectively. The intra- and inter-assay coefficients of variation for 17β-estradiol were below 6.4 and 8.9%, respectively. The intra- and inter-assay coefficients of variation for progesterone were below 9.0 and 7.9%, respectively.

2.5. Data analysis

The data was analyzed by MUSCOT statistical analysis software (Yukums Co. Ltd., Tokyo, Japan) using the dam or fetus as the experimental unit [38]. Data were analyzed using Bartlett's test [39] for the homogeneity of variance. When the variance was homogeneous, Dunnett's test [40] was performed to compare the mean value in the control group with that in each DBTCl group. When the variance was heterogeneous, the data were rank-converted and a Dunnett-type test [41] was performed to compare the mean value in the control group with that in each DBTCl group. The incidences of maternal and embryonic/fetal deaths and anomalous fetuses were analyzed by Fisher's exact test. The 0.05 level of probability was used as the criterion for significance.

3. Results

Table 1 presents maternal findings in monkeys given DBTCl on days 20–50 of pregnancy. No maternal death occurred in any group. In both DBTCl-treated groups, a significant increase in the incidence of females with soft stool and/or diarrhea, and with yellowish stool was observed. Soft stool and/or diarrhea were observed in one of the 12 females in the control group and in all females of the DBTCl-treated groups. In both groups treated with DBTCl, yellowish stool was noted in eight females and vomiting was observed in three females. Body weight gain on days 0–20, during the pre-administration period, did not significantly differ among the groups. Body weight gain on days 20–50, during the administration period, was lower in the DBTCl-treated groups, and significantly decreased at 3.8 mg/kg. No significant decrease in body weight gain on days 51–100, during the post-administration period, was found in the DBTCl-treated groups. Food consumption during the administration period was significantly reduced at 2.5 mg/kg and higher. Relatively marked decreases in the body weight gain and food consumption were observed in dams showing abortion or embryonic/fetal death.

The reproductive and developmental findings in monkeys given DBTCl on days 20–50 of pregnancy are shown in Table 2. The incidence of females with embryonic/fetal loss was increased in the DBTCl-treated groups, and a significant difference was noted at 2.5 mg/kg. Embryonic/fetal loss was observed in one of the 12 females in the control group, eight of the 12 females in the 2.5 mg/kg group and four of the 10 females in the 3.8 mg/kg group. Abortion occurred on day 30 of pregnancy in the control group, and on day 35, 44, 46, 49 or 60 of pregnancy at 2.5 mg/kg. Embryonic/fetal death was found on day 35, 40 or 64 of pregnancy at 2.5 mg/kg, and on days 38, 40 or 50 (two embryos) of pregnancy at 3.8 mg/kg. External examinations was performed in five of the eight embryonic/fetal losses at 2.5 mg/kg and four of the four embryonic/fetal losses at 3.8 mg/kg, and no anomalies were detected. Eleven, four and six females in the control, 2.5 and 3.8 mg/kg groups, respectively,

Table 3
Morphological findings in fetuses of monkeys given DBTCl on days 20–50 of pregnancy

	Dose (mg/kg)		
	0 (control)	2.5	3.8
Number of fetuses examined	11	4	6
External examination			
Number of fetuses with malformations	0	0	0
Internal examination			
Number of fetuses with malformations	0	0	0
Number of fetuses with variations	0	0	1
Accessory spleen	0	0	1
Skeletal examination			
Number of fetuses with malformations	0	0	0
Number of fetuses with variations	0	1	1
Short supernumerary rib	0	1	1
Degree of ossification[a]			
Number of ossified centers of vertebral column	53.6 ± 0.8	53.0 ± 1.2	54.2 ± 1.0
Skeletal length (mm)[a]			
Humerus	23.6 ± 0.8	23.3 ± 1.3	23.6 ± 1.2
Radius	23.0 ± 1.0	22.3 ± 1.6	23.1 ± 1.7
Ulna	24.6 ± 1.0	23.9 ± 1.5	24.3 ± 2.2
Femur	22.3 ± 1.2	21.8 ± 1.3	22.7 ± 1.6
Tibia	21.5 ± 1.3	20.5 ± 1.7	21.7 ± 1.4
Fibula	19.8 ± 1.0	19.0 ± 1.8	19.9 ± 1.6

[a] Values are given as the mean ± S.D.

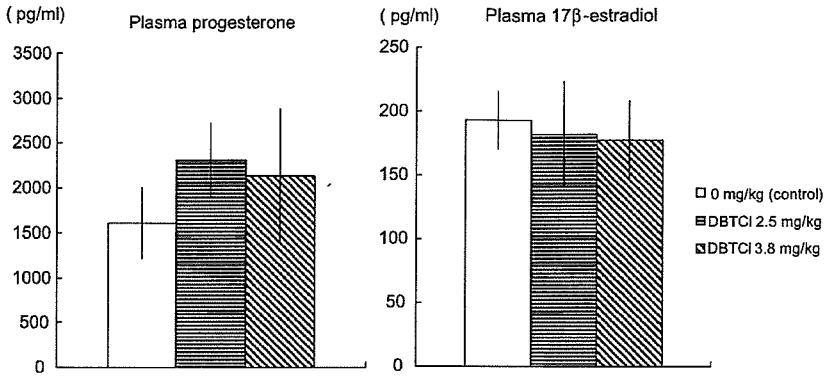

Fig. 1. Plasma progesterone and 17β-estradiol levels in pregnant monkeys given DBTCl on days 20–50 of pregnancy. Blood samples were collected on day 51 of pregnancy, 24 h after the last administration of DBTCl. Values are given as the mean ± S.E.M. of 5–10 monkeys.

had live fetuses at terminal cesarean sectioning. There were no significant differences between the control and DBTCl-treated groups in parameters of fetal growth, such as body weight, crown-rump length and tail length. No significant differences in the head width, chest circumference, paw and foot length, distance between the eyes, umbilical cord length, volume of amniotic fluid and diameters of the primary and secondary placentae were also noted between the control and DBTCl-treated groups (data not shown). No significant differences between the control and DBTCl-treated groups were found in the sex ratio of live fetuses, anogenital distance or placental weight. A single placenta was observed in one dam in the control group, one dam in the 2.5 mg/kg group and three dams in the 3.8 mg/kg group.

Table 3 shows the morphological changes in fetuses of monkeys given DBTCl on days 20–50 of pregnancy. No external, internal or skeletal malformations were found in fetuses in any group. Although internal and skeletal examinations revealed one fetus with an accessory spleen at 3.8 mg/kg, and one fetus with a short supernumerary rib at both 2.5 and 3.8 mg/kg, no difference in the incidence of fetuses with variation was noted between the control and DBTCl-treated groups. There were no differences between the control and DBTCl-treated groups in the number of ossified centers of the vertebral column or length of the humerus, radius, ulna, femur, tibia or fibula.

Although a significant decrease in the absolute weight of the brain and lung, and increase in the relative weight of the spleen were observed in male fetuses at 3.8 mg/kg, no significant difference in the relative weight of the brain and lung or in absolute weight of the spleen was detected between the control and DBTCl-treated groups. There were no differences in absolute and relative weights of the fetal thymus, heart, lung, liver, kidneys, adrenal glands or testes/uterus and ovaries between the control and DBTCl-treated groups (data not shown). Histopathological examinations revealed no abnormalities in single placenta and accessory spleen, and the histological structures of single placenta and accessory spleen were similar to those of normal placenta and spleen.

Plasma progesterone and 17β-estradiol levels are shown in Fig. 1. Although higher levels of plasma progesterone were observed in the DBTCl-treated groups, no statistically significant difference was noted between the control and DBTCl-treated groups. There were no significant differences in the plasma 17β-estradiol levels between the control and DBTCl-treated groups.

4. Discussion

In previous studies, the teratogenic effects of DBT were investigated in rats. The teratogenicity of DBT should be studied using other animal species to gain a better understanding of the developmental toxicity of butyltins. Non-human primates appear to provide an especially appropriate model for teratogenicity testing because of their high ranking on the evolutionary scale [42]. The close phylogenetic relatedness of old world monkeys to humans appears to render them most desirable as models in teratology studies [43]. The similarities in placentation and embryonic development indicate considerable value in the use of monkeys for investigating the developmental toxicity of chemicals [44]. In the present study, we determined the developmental toxicity, particularly the teratogenicity, of DBTCl in monkeys after administration over the entire period of organogenesis.

The doses of DBTCl set in the present study were expected to induce maternal toxicity, such as decreases in maternal body weight gain and food consumption, and were given to monkeys during organogenesis to characterize the effects of DBTCl on embryonic/fetal development. Toxicological sign, as evidenced by the significant increase in the incidence of pregnant females showing soft stool/diarrhea and yellowish stool, was found at 2.5 and 3.8 mg/kg. A significant decrease in the maternal body weight gain accompanied by significantly reduced food consumption was noted at 3.8 mg/kg. A significant decrease in food consumption was also found at 2.5 mg/kg. These maternal findings indicate that more severe adverse effects on pregnant females were noted at 3.8 mg/kg and DBTCl exerts maternal toxicity at 2.5 mg/kg and higher when administered during the entire period of organogenesis in monkeys.

Embryonic/fetal loss was observed in one dam in the control group and eight dams in the 2.5 mg/kg group and four dams in the 3.8 mg/kg group. The increased incidence of pregnant females with embryonic/fetal loss was observed at 2.5 and 3.8 mg/kg, and a significantly increased incidence of these females was found

at 2.5 mg/kg. Embryonic/fetal loss occurred on days 35–64 of pregnancy at 2.5 mg/kg, and on days 38–50 of pregnancy at 3.8 mg/kg. The embryonic mortality during organogenesis in cynomolgus monkeys of 2.4–18.2% has been reported [45]. Binkerd et al. [46] also noted that post-implantation embryonic loss was 5.4% in vehicle control pregnancies in developmental toxicity studies. Average abortion rate in cynomolgus monkeys was 26.1% in control data from 24 teratogenicity studies, and most of the abortions (66.7%) occurred during organogenesis [47]. In the background control data from 1994 to 2004 of the laboratory that performed this study, the post-implantation embryonic loss was 8.8% (29 of the 330 pregnancies). Because the incidence of embryonic/fetal loss in the DBTCl-treated groups was greater than in the historical control values, it was considered to be due to the administration of DBTCl. The data indicate that DBTCl at 2.5 mg/kg was sufficient to induce embryonic/fetal loss and the latter half of organogenesis was more susceptible for DBTCl-induced embryonic loss in cynomolgus monkeys.

We previously reported that DBTCl during early pregnancy caused pre- and post-implantation embryonic loss in pregnant rats [19,20] and that DBTCl suppressed uterine decidualization and reduced the levels of serum progesterone in pseudopregnant rats at doses that induced implantation failure [48]. We also showed that the suppression of uterine decidualization was reversed by administration of progesterone in pseudopregnant rats [48], and that progesterone protected against DBTCl-induced implantation failure [21]. Based on these findings, we hypothesized that the decline in serum progesterone levels was a primary factor for the implantation failure due to DBTCl in rats. However, no significant changes in plasma progesterone levels were noted in monkeys after the administration of DBTCl during organogenesis. The peripheral serum progesterone levels during the first 8 days of pseudopregnancy were essentially similar to those found in pregnant rats, and the serum progesterone levels rose steadily to a peak on day 4 and remained at a plateau of approximately 70 ng/ml until day 8 of pseudopregnancy [49]. In cynomolgus monkeys, plasma progesterone levels had distinct two peaks, one about 15 days postbreeding and another at about days 23–25, the progesterone decline which followed the second peak reached minimal levels (1–2 ng/ml) by about day 45 of pregnancy, and progesterone levels increased gradually throughout the rest of pregnancy with average levels of approximately 4 ng/ml [50]. In our previous study [48], rat blood samples were obtained on day 4 or 9 of pseudopregnancy. At these stages, progesterone levels could be steadily rising or remained at a plateau in pseudopregnant rats. In the present study, blood samples were collected from pregnant monkeys that were carrying their offspring and had not suffered from miscarriage on day 51 of pregnancy. At this stage, progesterone levels could be remained at a nadir in pregnant cynomolgus monkeys. The discrepancy in the effect of DBTCl on serum progesterone levels between rats and monkeys may be explained by the differences in the status and stage of pregnancy. Further studies are required to characterize more precisely the relationship between embryonic loss and maternal progesterone levels in monkeys given DBTCl.

Decreases in the absolute weights of the brain and lung, and an increase in the relative weight of the spleen, which were observed in male fetuses at 3.8 mg/kg, were not thought to de due to the toxic effects of DBTCl on fetal development, because these changes were not found in female fetuses and differences were not detected in the relative weight of the brain and lung or the absolute weight of the spleen in male fetuses. Any adverse effects on the parameters of fetal growth were also not detected in the surviving fetuses of dams given DBTCl. These findings indicate that DBTCl is not toxic to fetal growth at up to 3.8 mg/kg when administered over the entire period of organogenesis. Placental examinations revealed single placenta in all groups. In the background control data of the laboratory that performed the present study, the incidence of single placenta over a period of 10 years was 0–66.7% (mean = 13.0%, 26 of the 213 pregnancies). Histopathological examinations of single placenta revealed no changes, and the histological structure of single placenta was similar to that of normal placenta. These findings indicate that the single placenta observed in the present study was of no toxicological significance.

In the morphological examinations of the fetuses of exposed dams, a few fetuses with morphological changes were found in the DBTCl-treated groups. An accessory spleen was observed in one fetus at 3.8 mg/kg, and a short supernumerary rib was found in one fetus at both 2.5 and 3.8 mg/kg. In the background control data of the laboratory that performed the present study, the accessory spleen over the last 10 years was not observed. Leemans et al. [51] noted that the exact frequency of accessory spleen is not known, but is estimated to be between 10 and 30% in humans, and the immunohistological structure of the accessory spleen was similar to that of the normal spleens. In the present study, histopathological examinations of the accessory spleen revealed no changes, and the histological structure of accessory spleen was similar to that of the normal spleen. The accessory spleen observed in the present study contained only a minute amount of accessory tissue, and it was not considered to be a malformation. Short supernumerary rib is classified as skeletal variation [52], and the incidence of this change in the historical control data of the laboratory that performed the present study was 13.3% (31 of the 240 fetuses). DBTCl caused no skeletal retardation, as evidenced by no significant changes in the number of ossified centers of the vertebral column or the length of the humerus, radius, ulna, femur, tibia or fibula. Chahoud et al. [53] noted that variations are unlikely to adversely affect survival or health, and might result from a delay in growth or morphogenesis; the fetuses otherwise following a normal pattern of development. Furthermore, morphological examinations of aborted or dead embryos/fetuses in the DBTCl-treated groups revealed no anomalies. Considered collectively, these findings suggest that the morphological changes observed in the fetuses in the present study do not indicate a teratogenic response, and that DBTCl possesses no teratogenic potential in cynomolgus monkeys.

In conclusion, the administration of DBTCl to pregnant cynomolgus monkeys throughout organogenesis had an adverse effect on embryonic/fetal survival, but had no adverse effects on fetal morphological development, even at a maternal toxic

dose level. The data from the present study indicate that DBTCl shows embryonic/fetal lethality in monkeys.

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Early Pregnancy Failure Induced by Dibutyltin Dichloride in Mice

Makoto Ema,[1] Sakiko Fujii,[2] Tsuguo Ikka,[2] Mariko Matsumoto,[1] Akihiko Hirose,[1] Eiichi Kamata[1]

[1]Division of Risk Assessment, Biological Safety Center, National Institute of Health Sciences, Tokyo 185-8501, Japan

[2]Safety Research Institute for Chemical Compounds, Co., Ltd., Sapporo 004-0839, Japan

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ABSTRACT: In this study, we examined the adverse effects of dibutyltin on initiation and maintenance of pregnancy after maternal administration during early pregnancy in mice. Following successful mating, female ICR mice were given dibutyltin dichloride (DBTCl) at 0, 7.6, 15.2, or 30.4 mg/kg bw/day by gastric intubation on days 0–3 or days 4–7 of pregnancy. Female mice were sacrificed on day 18 of pregnancy, and the pregnancy outcome was determined. After administration of DBTCl on days 0–3, the rate of non-pregnant females and the incidence of preimplantation embryonic loss were significantly increased at 30.4 mg/kg bw/day. The incidences of postimplantation embryonic loss in females given DBTCl on days 0–3 at 15.2 mg/kg and higher and on days 4–7 at 7.6 mg/kg bw/day and higher were increased. No increase in the incidence of fetuses with external malformations was observed after the administration of DBTCl on days 0–3 or days 4–7. A decline in the serum progesterone levels was detected in mice given DBTCl at 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The data show that DBTCl adversely affects the initiation and maintenance of pregnancy when administered during early pregnancy in mice and suggest that the decline in serum progesterone levels is responsible for pregnancy failure. Environ Toxicol 22: 44–52, 2007.

Keywords: dibutyltin dichloride; organotin; pregnancy failure; early embryonic loss; progesterone

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 the 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). Wide-spread use of organotin compounds has caused increasing amounts to be released into environment. The most important route of entry of organotin compounds as nonpesticides into the environment is through the leaching of organotin-stabilized PVC by water (Quevauviller et al., 1991), and its use in antifouling agents resulting in the entry of organotin into the aquatic environment (Maguire, 1991). 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) has been reported. TBT is degraded spontaneously and biochemically via a debutylation pathway to DBT in the environment (Seligman et al., 1988; Stewart and de Mora, 1990). Food chain bioaccumulation of butyltin in oysters (Waldock and Thain, 1983), mud crabs (Evans and Laughlin, 1984), marine mussels (Laughlin et al., 1986), Chinook salmon (Short and Thrower, 1986), and dolphin, tuna, and shark (Kannan et al., 1996) has been reported. These findings indicate that butyltins accumulate in the

Correspondence to: M. Ema; e-mail: ema@nihs.go.jp

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food chain and are bioconcentrated, and that humans can be exposed to butyltins via food.

Organotins possesses toxic effects on reproduction and development in experimental animals (Ema and Hirose, 2006). We previously reported that dibutyltin dichloride (DBTCl) by gavage throughout the period of organogenesis resulted in a significant increase in the incidence of fetal malformations in rats (Ema et al., 1991) and that rat embryos were highly susceptible to the teratogenic effects of DBTCl when administered on day 7 and day 8 of pregnancy (Ema et al., 1992). Tetrabutyltin (TeBT) is metabolized to TBT, DBT, and monobutyltin (MBT) derivatives (Fish et al., 1976; Kimmel et al., 1977). The TBT compound is metabolized to DBT and MBT derivatives and DBT is metabolized to MBT derivatives (Iwai et al., 1981). The developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from those of TeBT, TBT, and MBT in its mode of action, because the susceptible period for teratogenicity and types of malformations induced by DBT are different from those induced by TeBT, TBT, and MBT (Ema et al., 1995, 1996). Tributyltin chloride (TBTCl) (Harazono et al., 1996, 1998ab) and DBTCl (Ema and Harazono, 2000ab) during early pregnancy produced pregnancy failure in rats. In rats, the predominant adverse effects on reproduction and development of TBTCl and DBTCl on days 0–3 of pregnancy were a decrease in the pregnancy rate and an increase in the incidence of preimplantation embryonic loss, and TBTCl and DBTCl on days 4–7 of pregnancy mainly caused postimplantation embryonic loss (Harazono et al., 1998b; Ema and Harazono, 2000ab). The doses of DBTCl that caused early embryonic loss were lower than those of TBTCl (Ema and Harazono, 2000b). Thus, the possibility exists that DBTCl and/or metabolites participate in the induction of early embryonic loss due to TBTCl.

The reproductive and developmental effects of organotin compounds, including DBT, were extensively investigated in rats (Ema and Hirose, 2006). We are unaware of any studies in which the adverse effects of DBT on initiation and maintenance of pregnancy have been assessed in mice. Studies in mice would be of great value in evaluating the reproductive and developmental toxicity of DBT. The present study was therefore conducted to determine the adverse effects on the initiation and maintenance of pregnancy of maternal exposure to DBTCl during early pregnancy in mice.

MATERIALS AND METHODS

Animal Husbandry and Maintenance

Male and female Crlj:CD1(ICR) mice at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, (Yokohama, Japan). The mice were acclimatized to the laboratory for 11 days prior to the start of the experiment. Male and female mice found to be in good health were selected for use. Female mice were caged with male mice and checked the following morning for signs of successful mating by examining vaginal plugs. The day when vaginal plugs were detected was considered to be day 0 of pregnancy. Successfully mated females were distributed into eight groups of 12 mice each and housed individually. Animals were reared on a γ-irradiated basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) and filtered tap water *ad libitum*, and maintained in an air-conditioned room at 22°C ± 3°C, with a relative humidity of 50% ± 20%, under a controlled 12 h light/dark cycle, and ventilation with 10–15 air changes/hour. This study was performed in 2005 at the Safety Research Institute for Chemical Compounds. (Sapporo, Japan) in compliance with the "Law for the Humane Treatment and Management of Animals" (Ministry of the Environment, Japan, 1973), "Standards Relating to the Care and Management, etc. of Experimental Animals" (Prime Minister's Office, Japan, 1980) and "Guidance for Animal Care and Use of the Safety Research Institute for Chemical Compounds, Co."

Chemicals and Dosing

DBTCl was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The DBTCl used in this study was 99.5% pure, and it was kept in a dark and cool place. DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Osaka, Japan). The female mice were dosed once daily by gastric intubation with DBTCl at a dose of 7.6, 15.2, or 30.4 mg/kg bw (25, 50 or 100 μmol/kg bw) on days 0–3 of pregnancy or on days 4–7 of pregnancy. The dosage levels were determined based on the results of our previous studies, in which increases in the incidence of pre- and postimplantation embryonic loss were caused in female rats gavaged with DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 and days 4–7 of pregnancy, respectively (Ema and Harazono, 2000ab) and our dose-finding study in which no adverse effects on embryonic survival at 15.2 mg/kg bw/day and lower, increased embryonic loss at 30.4 mg/kg bw/day, and one death and three pregnancy failure in four females at 60.8 mg/kg bw/day were found in mice gavaged with DBTCl on days 0–3 of pregnancy. The volume of each dose was adjusted to 5 mL/kg of body weight based on the daily body weight. The control mice received olive oil only on days 0–3 or days 4–7 of pregnancy. All DBTCl solutions were prepared fresh daily.

Observations

All mice were observed for clinical signs of toxicity twice a day during the administration period and daily during the nonadministration period. Females showing a moribund condition were euthanized under ether anesthesia. Maternal

TABLE I. Maternal findings in mice given DBTCl by gastric intubation on days 0–3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	1	0	0
Moribund condition (euthanized)	0	1	1	1
Vaginal discharge	0	1	0	0
Jaundice	0	2	7*	10*
Decreased locomotor activity	0	2	1	1
Hypothermia	0	1	1	1
Soil of perigenital fur	0	0	1	0
Initial body weight (g)[a]	27.4 ± 2.0	27.2 ± 2.1	27.2 ± 2.4	27.2 ± 2.1
Body weight gain (g)[a]				
Days 0–4	1.7 ± 1.1	0.6 ± 1.2	1.2 ± 1.6	0.3 ± 0.9*
Days 4–8	2.9 ± 1.5	2.5 ± 2.6	2.1 ± 2.0	1.6 ± 1.5
Days 8–18	20.1 ± 9.1	21.3 ± 12.4	13.6 ± 12.2	8.6 ± 12.2
Adjusted weight gain[b]	8.9 ± 3.4	9.9 ± 3.8	7.9 ± 4.8	5.3 ± 5.0
Food consumption (g)[a]				
Days 0–4	18.2 ± 1.8	15.0 ± 1.9*	16.7 ± 3.2	14.8 ± 2.3*
Days 4–8	22.9 ± 4.9	22.0 ± 2.7	21.7 ± 3.5	20.9 ± 3.5
Days 8–18	71.7 ± 10.1	71.0 ± 12.5	64.6 ± 13.3	57.8 ± 13.4*

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

body weight was recorded daily, and food consumption was recorded on days 0, 4, 8, 12, and 18 of pregnancy. The females were euthanized by exsanguination under ether anesthesia on day 18 of pregnancy. The uterus was weighed and the number of corpora lutea was recorded. The numbers of implantations, live and dead fetuses, and of resorptions were counted. The uteri were placed in 10% ammonium sulfide for confirmation of the dam's pregnancy status (Salewski, 1964). The live fetuses removed from the uterus were sexed, weighed, and inspected for external malformations and malformations within the oral cavity. The placental weight was also measured.

Analysis of Serum Steroids Hormone Levels

Blood samples were collected from the abdominal aorta under ether anesthesia on day 4 or day 8 of pregnancy, 24 h after the last administration of DBTCl at 0 or 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The serum was separated and stored at −80°C for later assay of steroid hormones. Serum progesterone and 17β-estradiol were measured by Teizo Medical (Kawasaki, Japan) using the liquid chromatography-electrospray ionization Tandem Mass Spectrometry (LC-MS/MS, Applied Biosystems/MDS SCIEX). The detection limits of serum progesterone and 17β-estradiol were 10.0 and 0.25 pg/mL, respectively. The intra- and interassay coefficients of variation for 17β-estradiol were below 6.4% and 8.9%, respectively. The intra- and interassay coefficients of variation for progesterone were below 9.0% and 7.9%, respectively.

Statistical Analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. Maternal body weight, body weight gain, adjusted weight gain, food consumption, numbers of corpora lutea, implantations, embryonic/fetal loss and live fetuses, fetal weight, and placental weight were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the groups were not equivalent, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann–Whitney U test. The incidences of pre- and postimplantation embryonic loss and fetuses with external malformations were analyzed using Wilcoxon's rank sum test. The incidence of clinical signs in dams, pregnancy, nonpregnancy, and litters with fetal malformations, and the sex ratio of live fetuses were analyzed using Fisher's exact test. The levels of serum progesterone and 17β-estradiol were analyzed by Student's *t*-test. The 0.05 level of probability was used as the criterion for significance.

TABLE II. Reproductive and developmental findings in mice given DBTCl by gastric intubation on days 0–3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of nonpregnant females	1	3	4	7*
No. of pregnant females	11	9	8	5*
No. of implantations per female[a,b]	9.5 ± 5.1	9.8 ± 7.1	8.3 ± 7.0	5.4 ± 6.7
Pre-implantation loss per female (%)[a,b]	9.7	29.7[c]	34.0	58.3*
No. of pregnant females surviving until scheduled sacrifice	11	8	7	4
No. of litters totally resorbed	0	0	1	1
No. of corpora lutea per litter[a,d]	10.5 ± 4.3	13.1 ± 4.9	12.4 ± 4.4	13.3 ± 1.3
No. of implantations per litter[a,d]	10.4 ± 4.3	12.6 ± 4.9	12.3 ± 4.4	13.3 ± 1.3
Pre-implantation loss per litter (%)[d,e]	1.5	3.3	1.1	0
No. of post-implantation loss per litter[a,d]	1.0 ± 1.0	1.1 ± 1.5	4.1 ± 3.2	4.0 ± 5.4
Post-implantation loss per litter (%)[d,f]	10.1	14.1	41.3*	32.2
No. of live fetuses per litter[a,d]	9.4 ± 4.2	11.5 ± 5.3	8.1 ± 5.0	9.3 ± 6.2
Sex ratio of live fetuses (male / female)	50/53	47/45	30/27	21/16
Body weight of live fetuses (g)[a]				
Male	1.54 ± 0.19	1.30 ± 0.12*	1.14 ± 0.22*	1.12 ± 0.10*
Female	1.42 ± 0.15	1.28 ± 0.20	1.08 ± 0.26*	1.01 ± 0.11*
External examinations of fetuses				
No. of fetuses (litters) examined	103 (11)	92 (8)	57 (6)	37 (3)
No. of fetuses (litters) with anomalies	1 (1)	0	1 (1)	0
Cleft palate	1	0	1	0
Kinked tail	0	0	1	0
Placental weight (mg)[a]	125 ± 56	116 ± 15	120 ± 17	119 ± 16

[a] Values are given as mean ± SD.
[b] Values obtained from females successfully mated.
[c] Value obtained from 11 females, because corpora lutea were indistinguishable in one female.
[d] Values obtained from pregnant females surviving until scheduled sacrifice.
[e] [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.
[f] (No. of resorptions and dead fetuses/no. of implantations) × 100.
* Significantly different from the control, $P < 0.05$.

RESULTS

Administration of DBTCl on Days 0–3 of Pregnancy

Table I shows the maternal findings in mice given DBTCl on days 0–3 of pregnancy. One death was observed at 7.6 mg/kg bw/day, and one female each showed a moribund condition at 7.6, 15.2, and 30.4 mg/kg bw/day, and was euthanized. The female mice in the DBTCl-treated groups showed vagina discharge, jaundice, decreased locomotor activity, hypothermia and/or soiled perigenital fur, and the incidence of females showing jaundice was significantly increased at 15.2 mg/kg bw/day and higher. A significantly decreased body weight gain on days 0–4 was noted at 30.4 mg/kg bw/day. Food consumption on days 0–4, days 4–8, and days 8–18 in the DBTCl-treated groups were reduced, and significantly decreased food consumptions on days 0–4 at 7.6 and 30.4 mg/kg bw/day and on days 8–18 at 30.4 mg/kg bw/day were observed.

The reproductive and developmental findings in mice given DBTCl on days 0–3 of pregnancy are shown in Table II. The total absence of any implantation site, i.e., nonpregnancy, was found in one, three, four, and seven of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively. In the successfully mated females, the pregnancy rate was significantly decreased, and the incidence of preimplantation embryonic loss per females was significantly increased at 30.4 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, the number of corpora lutea per litter, implantations per litter, live fetuses per litter, the incidence of litters totally resorbed and of preimplantation loss per litter, and the sex ratio of live fetuses were not significantly different between the control and DBTCl-treated groups. The incidence of postimplantation loss per litter was increased in the DBTCl-treated groups, and a significant increase was observed at 15.2 mg/kg bw/day. A significantly lower fetal weight was found in males at 7.6 mg/kg bw/day and in both sexes at 15.2 and 30.4 mg/kg bw/day. One fetus with cleft palate in the control group and one fetus with a cleft palate and kinked tail in the 15.2 mg/kg bw/day group were observed. The placental weight in the DBTCl-treated

TABLE III. Maternal findings in mice given DBTCl by gastric intubation on days 4–7 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	0	1	0
Moribund condition (euthanized)	0	0	0	1
Vaginal discharge	0	0	4	4
Jaundice	0	0	2	6*
Decreased locomotor activity	0	0	0	1
Hypothermia	0	0	0	1
Initial body weight (g)[a]	28.1 ± 1.8	28.1 ± 1.8	28.1 ± 1.8	28.2 ± 1.7
Body weight gain (g)[a]				
Days 0–4	1.6 ± 1.0	1.9 ± 0.8	1.2 ± 1.2	1.6 ± 0.9
Days 4–8	3.1 ± 1.1	1.9 ± 1.6	0.5 ± 1.8*	−0.3 ± 2.1*
Days 8–18	24.9 ± 9.1	14.9 ± 8.9*	2.9 ± 6.3*	2.4 ± 2.4*
Adjusted weight gain[b]	8.3 ± 3.5	8.1 ± 4.3	3.2 ± 5.3*	3.8 ± 3.2*
Food consumption (g)[a]				
Days 0–4	18.5 ± 1.9	18.9 ± 2.4	18.4 ± 2.7	18.8 ± 1.3
Days 4–8	21.8 ± 1.9	19.2 ± 2.6	16.4 ± 3.3*	15.6 ± 3.5*
Days 8–18	74.5 ± 12.1	67.7 ± 9.9	55.2 ± 12.6*	57.2 ± 6.2*

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

groups was not significantly different from that in the control group.

Administration of DBTCl on Days 4–7 of Pregnancy

Table III shows the maternal findings in mice given DBTCl on days 4–7 of pregnancy. One death was observed at 15.2 mg/kg bw/day, and one female that showed a moribund condition at 30.4 mg/kg bw/day was euthanized. The female mice in the DBTCl-treated groups showed vaginal discharge, jaundice, decreased locomotor activity, and/or hypothermia, and the incidence of females with jaundice was significantly increased at 30.4 mg/kg bw/day. The body weight gain on days 4–8 and adjusted weight gain, which indicates the net weight gain of female mice, at 15.2 mg/kg bw/day and higher, and on days 8–18 at 7.6 mg/kg bw/day and higher were significantly decreased. Food consumption on days 4–8 and days 8–18 was significantly lowered at 15.2 mg/kg bw/day and higher.

The reproductive and developmental findings in mice given DBTCl on days 4–7 of pregnancy are presented in Table IV. Although nonpregnancy was found in one, two, and one of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively, no significant decrease in the pregnancy rate was noted in the DBTCl-treated groups. In the successfully mated females, the number of implantations per female was significantly decreased at 15.2 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, totally resorbed litters were found in 2 of the 11 females at 7.6 mg/kg bw/day, 8 of the 9 females at 15.2 mg/kg bw/day, and 10 of the 10 females at 30.4 mg/kg bw/day. At 30.4 mg/kg bw/day, no live fetuses were obtained. The numbers of corpora lutea per litter, implantations per litter, and preimplantation loss per litter, and the sex ratio of live fetuses in the DBTCl-treated groups were not significantly different from those in the control group. A significant increase in the number and incidence of postimplantation loss per litter, and a decrease in the number of live fetuses were found in the DBTCl-treated groups. The weights of male and female fetuses were significantly lowered at 7.6 mg/kg bw/day. One fetus with omphalocele, and one fetus with exencephaly and open eyelids were observed at 7.6 mg/kg bw/day. The placental weight was not significantly different between the control and the DBTCl-treated groups.

Serum Progesterone and 17β-Estradiol Levels

The serum progesterone and 17β-estradiol levels are shown in Figure 1. A significant reduction in the serum progesterone levels was noted in female mice given DBTCl on days 0–3 or days 4–7 of pregnancy. Although higher levels of serum 17β-estradiol were observed after the administration of DBTCl on days 4–7 of pregnancy, no statistically significant difference in 17β-estradiol levels were detected between the control and DBTCl-treated groups.

DISCUSSION

The present study was designed to evaluate the adverse effects of DBTCl on the initiation and maintenance of

TABLE IV. Reproductive and developmental findings in mice given DBTCl by gastric intubation on days 4–7 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of nonpregnant females	1	1	2	1
No. of pregnant females	11	11	10	11
No. of implantations per female[a,b]	12.6 ± 4.4	13.2 ± 4.6	7.5 ± 5.7*	11.1 ± 5.4
Pre-implantation loss per female (%)[a,b]	8.9	8.9	24.7	18.3[c]
No. of pregnant females surviving until scheduled sacrifice	11	11	9	10
No. of litters totally resorbed	0	2	8*	10*
No. of corpora lutea per litter[a,d]	13.8 ± 2.1	14.5 ± 2.3	10.6 ± 5.2	13.9 ± 2.8
No. of implantations per litter[a,d]	13.7 ± 2.1	14.4 ± 2.2	9.4 ± 5.1	12.7 ± 4.1
Pre-implantation loss per litter (%)[d,e]	0.6	0.6	10.7	10.2
No. of postimplantation loss per litter[a,d]	0.6 ± 1.0	7.2 ± 6.1*	8.7 ± 4.8*	12.7 ± 4.1*
Post-implantation loss per litter (%)[d,f]	4.3	48.3*	94.4*	100*
No. of live fetuses per litter[a,d]	13.1 ± 2.0	7.2 ± 5.6*	0.8 ± 2.3*	0
Sex ratio of live fetuses (male/female)	82/62	50/29	4/3	
Body weight of live fetuses (g)[a]				
Male	1.45 ± 0.10	1.23 ± 0.10*	1.27	
Female	1.39 ± 0.10	1.18 ± 0.14*	1.18	
External examinations of fetuses				
No. of fetuses (litters) examined	144 (11)	79 (9)	7 (1)	
No. of fetuses (litters) with anomalies	0	2 (2)	0	
Omphalocele	0	1	0	
Exencephaly and open eyelids	0	1	0	
Placental weight (mg)[a]	102 ± 10	99 ± 12	114	

[a] Values are given as mean ± SD.
[b] Values obtained from females successfully mated.
[c] Value obtained from 11 females, because corpora lutea were indistinguishable in one female.
[d] Values obtained from pregnant females surviving until scheduled sacrifice.
[e] [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.
[f] (No. of resorptions and dead fetuses/no. of implantations) × 100.
* Significantly different from the control, $P < 0.05$.

pregnancy following maternal exposure during early pregnancy in mice. The most striking finding in the present study is pregnancy failure, decrease in the pregnancy rate, and litters totally resorbed, in females given DBTCl during early pregnancy.

Death and/or moribund condition were observed after the administration of DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 of pregnancy and at 15.2 mg/kg bw/day and higher on days 4–7 of pregnancy, and significant increased incidence of females showing clinical signs of toxicity were found after the administration of DBTCl at 15.2 mg/kg bw/day and higher on days 0–3 of pregnancy and at 30.4 mg/kg bw/day on days 4–7 of pregnancy. These findings indicate that more severe general toxicity was induced by DBTCl on days 0–3 of pregnancy than that on days 4–7 of pregnancy. However, adverse effects on body weight gain were detected after the administration of DBTCl at 30.4 mg/kg bw/day on days 0–3 of pregnancy and at 7.6 mg/kg bw/day and higher on days 4–7 of pregnancy. Although the recovery of body weight gain was observed after the administration of DBTCl on days 0–3 of pregnancy, recovery by the end of the study was not found in females given DBTCl at 7.6 mg/kg bw/day and higher after the administration on days 4–7 of pregnancy. Following the administration on days 4–7 of pregnancy, a significantly lower adjusted weight gain was also noted in females given DBTCl at 15.2 mg/kg/day and higher. These findings indicate that more severe adverse effects on body weight gain were induced by DBTCl on days 4–7 of pregnancy than that on days 0–3 of pregnancy. More severe effects of DBTCl on body weight gain following the administration on days 4–7 may be attributable to the significant decrease in the number of live fetuses.

The earlier administration period, days 0–3 of pregnancy, corresponds to the period before implantation, and the later administration period, days 4–7 of pregnancy, corresponds to the period when implantation is in progress and the period shortly after implantation in mice (Rugh, 1968). We expected that DBTCl insult on days 0–3 of pregnancy might result in preimplantation loss of embryos; i.e., the absence or decrease of implantation sites, and DBTCl insult on days 4–7 of pregnancy might result in postimplantation loss of embryos; i.e.,

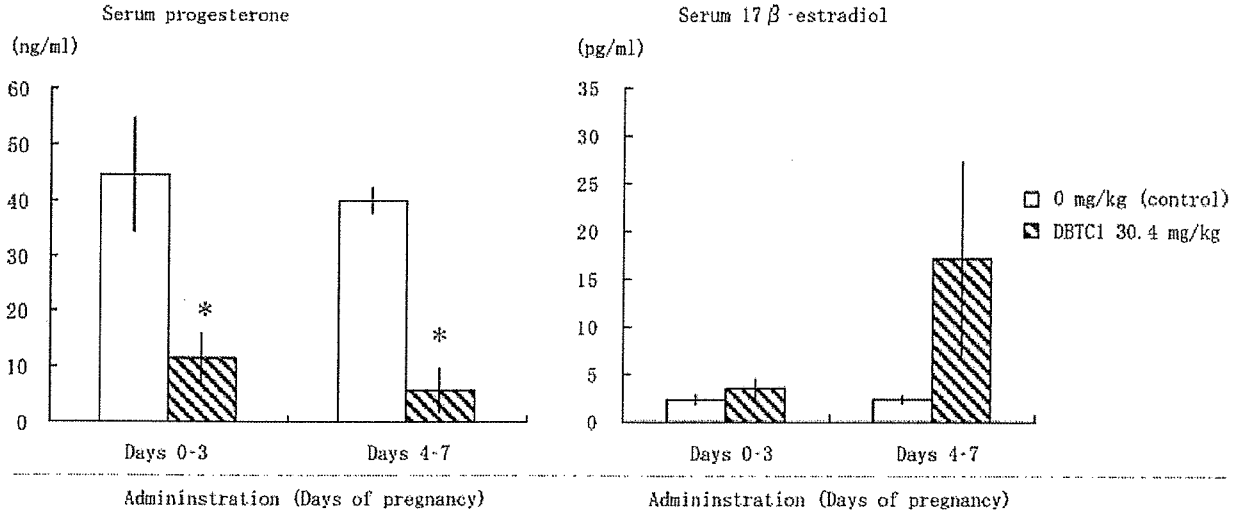

Fig. 1. Serum progesterone and 17β-estradiol levels in female mice given DBTCl on days 0–3 or days 4–7 of pregnancy. Blood samples were collected on day 4 or day 8 of pregnancy, 24 h after the last administration of DBTCl. Values are given as the mean ± SEM of seven or eight mice. *Significantly different from the control group, $P < 0.05$.

the resorption of implantation sites. The most striking adverse effects of DBTCl on reproduction and development were a decrease in pregnancy rate, complete implantation failure, when DBTCl was given to mice on days 0–3 of pregnancy. The findings of an increased incidence of preimplantation embryonic loss in successfully mated females, and an increased incidence of postimplantation embryonic loss and low fetal weight in pregnant females survived until scheduled sacrifice after the administration of DBTCl on days 0–3 of pregnancy may suggest that DBTCl adversely affects preimplantation embryos and also the later survival and growth of embryos/fetuses when administered during the preimplantation period. On the other hand, the predominant adverse effects of DBTCl on reproduction and development were postimplantation loss, complete litter loss, when DBTCl was given to mice on days 4–7 of pregnancy. The findings of an increase in the incidence of postimplantation embryonic loss and a decrease in the fetal weight after administration of DBTCl on days 4–7 of pregnancy may suggest that DBTCl has effects on the later survival and growth of embryos/fetuses when administered during the peri-implantation period. Considered collectively, these findings indicate that the manifestation of adverse effects of DBTCl on reproduction and development varies with the stages of pregnancy at the time of maternal exposure.

The corpora lutea are essential up to the end of pregnancy in mice (Deansely, 1966). The embryo transport process in mice is triggered by progesterone and requires progesterone activity for its maintenance (Kendle and Lee, 1980). In mice, 24 h of progesterone priming is not only adequate for implantation, but this priming has a long-term effect on implantation (Huet-Hudson and Dey, 1990). In our previous studies in rats, increases in the incidences of early embryonic loss were observed after the administration of DBTCl during early pregnancy (Ema and Harazono, 2000ab). The suppression of uterine decidualization and reduced levels of serum progesterone were found in female rats given DBTCl on days 0–3 or days 4–7 of pseudopregnancy (Harazono and Ema, 2003), and lowered reproductive parameters in female rats given DBTCl were recovered by the administration of progesterone (Ema et al., 2003). Based on these findings, we hypothesized that the decline in serum progesterone levels in pregnant animals was a primary mechanism for the implantation failure due to DBTCl in rats. In the present study in mice, a decline in serum progesterone levels was detected after the administration of DBTCl during early pregnancy. These findings are in good agreement with previous findings that DBTCl induced early embryonic loss and decreased serum progesterone levels in pregnant rats. There is a similarity in the effects of DBTCl on progesterone levels in early pregnancy in rats and mice, and these suggest that the decline in the serum progesterone levels is also the factor responsible for the DBTCl-induced pregnancy failure in mice. Early pregnancy failure was also caused by systemic activation of the CD-40 immune costimulatory pathway in mice (Erlebacher et al., 2004). They noted that pregnancy failure resulted from impaired progesterone synthesis by the corpus luteum of the ovary, an endocrine defect in turn associated with ovarian resistance to the gonadotropic effects of prolactin and that pregnancy failure also required the proinflammatory cytokine TNF-a and correlated with the luteal induction of the prolactin receptor signaling inhibitors suppressor of cytokine signaling 1

(Socs1) and Socs3. Our results of the present study may support their argument. To further evaluate the adverse effects of DBTCl during early pregnancy, determination of the gene expression profile in the uterus of mice and rats is currently in progress.

In conclusion, DBTCl adversely affects the initiation and maintenance of pregnancy when administered during early pregnancy in mice, and the present data suggest that the decline in progesterone is the responsible factor for the early pregnancy failure in mice.

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妊娠とくすり

7．生殖発生毒性試験の役割

江馬 眞*
国立医薬品食品衛生研究所安全性生物試験研究センター総合評価研究室*

Key Words/生殖発生毒性，催奇形性，動物実験

要旨

サリドマイド事件を契機として，各国における薬事制度の見直し，強化がはかられ，実験動物を用いた生殖発生毒性試験に関する資料の提出が新有効成分，新投与経路の承認申請時に必要となっている．本稿では医薬品によるヒトにおける生殖発生障害の例と動物実験との関わりについて述べ，実験動物を用いた生殖発生毒性試験の特徴および試験結果を評価する際の留意点について概説した．

生殖発生毒性試験

生殖（Reproduction）とは，種を存続させるための生物学的過程をいい，生殖毒性（reproductive toxicity）には，成熟動物の生殖能に対する有害作用と子孫における発生毒性（developmental toxicity）が含まれる．生殖能に対する有害影響とは，雌雄の生殖器や内分泌系の変化に起因する有害影響（春期発動，配偶子形成・輸送，生殖周期，性行動，繁殖，分娩，生殖系の統合性に依存するそのほかの機能に対する影響等）であり，発生毒性とは，親の妊娠前から児の性成熟までの曝露による正常な発生の障害（死亡，形態異常，成長の変化，機能障害）を指す[1]．すなわち，生殖毒性は親の世代を中心にとらえたときの環境要因による不妊や次世代の発生障害を指し，次世代を中心にとらえた発生毒性とほぼ同義である．生殖発生毒性試験の目的は，哺乳類の生殖発生に対するあらゆる影響を明らかにすることである[2]．薬物の即時的および遅発的影響を検出するために，親の世代の受精から次世代の受精までの完全な生殖周期（図1）に薬物を投与して，その間の観察を行う．新薬の申請時に必要とされている生殖発生毒性試験のうち，着床から硬口蓋閉鎖までの期間中の雌動物に投与を行う「胚・胎児発生に関する試験」，いわゆる催奇形性試験は，最も重要視されており，2種の動物を用いた試験が課せられている．動物の生殖発生の特定の段階に

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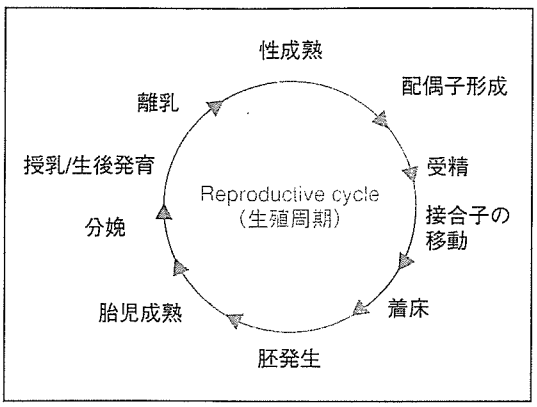

図1 生殖周期

生殖発生毒性試験においては，成熟動物および受精から性成熟までの発生の全過程にわたって薬物に曝露し，薬物の即時的および遅発的な影響を検出するため，完全な生殖周期，すなわち，一世代での受精から次の世代での受精までの観察を継続して行う．

被験物質を投与してその影響を観察することにより，どの生殖発生段階に障害を生じるかを明確にすることができる．このような試験方法は，大半の医薬品では亜急性的な服用が想定されることから，ヒトでの曝露状況をよく反映している．長期間曝露が想定される医薬品では，1世代または2世代試験が有用である．生殖発生毒性試験で得られた結果を，ヒトにおける生殖発生への危険性が他の毒性試験の結果から予見される危険性の程度と比較検討する．

生殖発生障害にかかわる出来事

生殖発生障害に関する主な出来事を表1に示

表1 生殖発生に関わる主な出来事

1744年	ヒドラの切断による多頭体
1870年	キニーネによるヒト児の難聴
1902年	妊娠モルモットへのブドウ球菌投与による児の白内障
1905年	妊娠ウサギへのX線処置による児の眼異常
1907年	妊娠ウサギへのコリン投与による実験（最初の化学物質の催奇形実験）
1911年	妊娠ウサギへのナフタリン投与による児の白内障等の眼異常
1913年	東北医専眼科教授小玉龍蔵，わが国最初の催奇形実験
1933年	ビタミンA欠乏食によるブタ児の無眼（近代実験奇形学のはじまり）
1941年	ヒト先天性風疹症候群
1950年	ストレプトマイシンによるヒト児の難聴
1952年	ヒト胎児性水俣病，アミノプテリンによるヒト胎児の髄膜脳瘤
1953年	男性ホルモンによるヒト女児の偽半陰陽
1956年	ベンデクチン発売開始
1957年	サリドマイド発売（鎮静薬「コンテルガン」西独グリュネンター社）
1961年	サリドマイド事件（米FDAフランシス・ケルシー承認与えず）
1963年	「胎児に及ぼす影響に関する動物試験法」厚生省薬務局長通知（わが国の最初の試験法ガイドライン）
1967年	「医薬品の製造承認等の基本方針」厚生省薬務局長通知（急性，亜急性，慢性，胎児及びその他の特殊毒性データの要求）
1968年	カネミ油症「コーラベビー」
1971年	ジエチルスチルベストロール（DES）服用の母親から生まれた女児の腟がん
1982年	「医薬品の安全性試験の実施に関する基準」（GLP）制定
1983年	ベンデクチン発売中止
1983年	イソトレチノイン（13-cis-retinoic acid）によるヒト児の小耳
1992年	ヒト精子の減少をスキャケベックが報告
1997年	薬審第316号「医薬品の生殖発生毒性に係わるガイドライン改定について」
2000年	薬審第1834号「医薬品の生殖発生毒性についてのガイドラインの改定について」：本ガイドラインの一部改訂

した．ヒトに関しては，1870年に小児の難聴とキニーネ（Quinine）の妊娠中の摂取との関係を疑った報告があり，その後，先天性風疹症候群，ストレプトマイシンによる難聴，胎児性水俣病，男性ホルモンによる女児の偽半陰陽，サリドマイド（Thalidomide）事件，カネミ油症，ジエチルスチルベストロール（DES:Diethystilbestrol）による女児の膣癌，イソトレチノイン（Isotretinoin）による小耳などが報告されているが，なかでも特筆すべきはサリドマイド事件である．1954年に西ドイツのグリュネンタール社においてサリドマイドが合成され，1957年10月にコンテルガン®という商品名で，睡眠薬，精神安定薬として発売され始め，世界の46カ国で発売された．睡眠薬としては即効性，持ち越し作用がなく，致死的作用もなく，当時の西ドイツおよび諸外国で大衆薬として広く使われた[3]．その後，サリドマイドを妊娠初期に服用した妊婦の出産児が先天異常を有することが報告され，1961年頃からサリドマイド禍として認識され始めた．このサリドマイド事件を契機として，医薬品の催奇形作用が問題視され，各国における薬事制度の見直し，強化がはかられた．わが国では1963年4月に「医薬品の胎児に及ぼす影響に関する動物試験法」が厚生省薬務局長から通知された．この通知はわが国で最初の具体的な毒性試験ガイドラインであり，2種類の動物を用いて行う器官形成期投与試験が示された．その後，何回かの改正を経て，現行の生殖発生毒性試験法が2000年12月に医薬審第1834号[2]として通知され，このガイドラインにしたがって新薬の承認申請のための生殖発生毒性試験が行われている．医薬品の承認申請には，医薬品の品質，有効性，安全性を評価するために，規格および試験方法，安定性，毒性，薬理作用，吸収・分布・代謝・排泄，臨床試験についての資料の提出が求められている．申請内容に応じて必要な資料が定められており，生殖発生毒性に関する資料の提出は新有効成分，新投与経路の承認申請時に必要とされている[4]．

医薬品によるヒトにおける発生障害と動物実験との関わりの例

1．サリドマイド（Thalidomide）

サリドマイドに関しては，動物実験で催奇形性が証明される前に不幸にもヒトでの薬害が起こってしまった．サリドマイド禍当時の世界中のどこの国においても，医薬品や化学物質について発生中の生物に対する影響に関する試験は要求されておらず，食物中の重要な化学物質または生殖器官に選択的な反応を示すと推定される化学物質についてのみ生殖毒性試験が推奨されていたにすぎなかった．これらの生殖毒性試験では，数世代にわたって妊娠率，出生児数，児の成長などについて重点的に調べられていたが，胎児についての検査は十分に行われていなかった．したがって，当時の試験枠組みではサリドマイド禍は避けられなかったのかもしれない[5]．サリドマイドの催奇形性に対して，ラットおよびマウスはほとんど感受性を示さず（胚死亡は惹起されるとする報告はある），ハムスター，ブタ，ネコ，イヌ，フェレット，アルマジロおよびニワトリでは感受性を示すが，特異的な奇形は惹起されない[6][7]．ウサギおよび非ヒト霊長目では感受性を示し，特異的な奇形が惹起される．ウサギでは胎児致死作用が強く発現するような高用量でのみ四肢奇形およびその他の奇形が認められるが，奇形発現率は低く，系統間で感受性に差がある．9種中8種の非ヒト霊長目でヒトと同様の用量および感受期の投与により，特徴的な四肢奇形が観察されている[6]．非ヒト霊長目または最も鋭敏な系統のウサギを催奇形性スクリーニング試験に汎用することは現実的には困難である．Kalter（2003）[6]は，あ