Table 1: Maternal findings in rats given DBHCB on days 5–19 of pregnancy.

	Dose (mg/kg)			
	0 (control)	**62.5**	**250**	**1000**
No. of rats	20	20	20	20
No. of pregnant rats	17	18	17	18
No. of dead rats	0	0	0	0
Initial body weight	285 ± 11	280 ± 12	285 ± 18	288 ± 11
Body weight gain during pregnancy (g)[a]				
Days 0–5	30 ± 8	33 ± 5	31 ± 6	30 ± 6
Days 5–14	47 ± 7	44 ± 7	49 ± 5	43 ± 9
Days 14–19	71 ± 9	65 ± 10	67 ± 10	63 ± 12
Days 19–20	16 ± 6	17 ± 4	20 ± 5	18 ± 5
Days 0–20	163 ± 17	159 ± 19	167 ± 14	154 ± 20
Adjusted weight gain[b]	88 ± 9	88 ± 10	91 ± 10	82 ± 18
Feed consumption during pregnancy (g/day)[a]				
Days 0–1	24 ± 3	23 ± 3	23 ± 3	24 ± 4
Days 5–6	27 ± 3	27 ± 3	27 ± 3	27 ± 3
Days 8–9	28 ± 4	28 ± 3	28 ± 3	28 ± 2
Days 11–12	29 ± 4	29 ± 3	28 ± 2	29 ± 3
Days 14–15	28 ± 4	28 ± 3	28 ± 3	28 ± 3
Days 17–18	32 ± 4	30 ± 4	31 ± 3	31 ± 4
Days 19–20	29 ± 4	29 ± 3	31 ± 4	30 ± 3
Weight of gravid uterus (g)[a]	88 ± 9	88 ± 10	91 ± 10	82 ± 18
Weight of ovaries (mg)[a]	149 ± 21	137 ± 14	149 ± 19	139 ± 14

[a]Values are given as the mean ± SD.
[b]Adjusted weight gain refers to maternal weight gain excluding the gravid uterus.

The reproductive findings in rats given DBHCB on days 5–19 of pregnancy are presented in Table 2. No totally resorbed litters were found in any group. No effects of DBHCB were observed on the number of corpora lutea or implantations, incidence of pre- or postimplantation loss, or the number of live fetuses or the sex ratio of live fetuses. There was no difference in the body weight of male and female fetuses between the control and DBHCB-treated groups. No abnormal findings were noted in the placentae of any group.

Morphological findings in the live fetuses of rats given DBHCB on days 5–19 of pregnancy are shown in Table 3. No fetuses with external malformations were observed in any group. Skeletal examination revealed no fetuses with skeletal malformations in any group. Fetuses with skeletal variations were observed in all groups including the control group. The incidence of fetuses with individual skeletal variations was not increased after the administration of DBHCB. The total number of fetuses with skeletal variations was also not increased in the DBHCB-treated groups. The degree of ossification, as evidenced by the numbers of sacral and caudal vertebrae and sternebrae in the DBHCB-treated groups, was not different from that in the control group. No fetuses with internal malformations were detected in any group. The fetuses with internal variations, such as thymic remnants in the neck, dilated renal

Table 2: Reproductive findings in rats given DBHCB on days 5–19 of pregnancy.

	Dose (mg/kg)				
	0 (control)	**62.5**	**250**	**1000**	**Historical control values[d]**
No. of litters	17	18	17	18	652 (48 studies)
No. of litters totally resorbed	0	0	0	0	
No. of corpora lutea per litter[a]	16.9 ± 2.0	16.3 ± 1.1	17.1 ± 1.7	16.6 ± 1.9	13.8–17.5
No. of implantations per litter[a]	16.2 ± 1.4	15.8 ± 1.1	16.6 ± 1.6	15.1 ± 3.4	13.1–16.3
% Preimplantation loss per litter[b]	3.8	3.0	2.3	9.4	0.9–13.6
% Postimplantation loss per litter[c]	4.9	3.3	4.0	6.3	0–11.5
No. of live fetuses per litter[a]	15.4 ± 1.5	15.3 ± 1.3	16.0 ± 1.8	14.2 ± 3.6	12.4–15.5
Sex ratio of live fetuses (male/total)	0.51	0.47	0.48	0.48	0.38–0.59
Body weight of live fetuses (g)[a]					
Male	3.88 ± 0.22	3.87 ± 0.30	3.92 ± 0.19	4.00 ± 0.26	3.56–4.01
Female	3.68 ± 0.19	3.69 ± 0.31	3.70 ± 0.14	3.79 ± 0.29	3.33–3.81

[a]Values are given as the mean ± SD.
[b](No. of preimplantation embryonic loss/no. of corpora lutea) × 100.
[c](No. of resorptions and dead fetuses/no. implantations) × 100.
[d]Historical control values were obtained from the studies performed in SNBL during 1996–2004 using Crj: CD (SD) IGS rats.

Table 3: Morphological examinations in fetuses of rats given DBHCB on days 5–19 of pregnancy.

	Dose (mg/kg)				
	0 (control)	62.5	250	1000	Historical control values[b]
External examination					
Total no. of fetuses (litters) examined	262 (17)	275 (18)	272 (17)	255 (18)	9178 (652): 48 studies
Total no. of fetuses (litters) with malformations	0	0	0	0	0–0.8%
Skeletal examination					
Total no. of fetuses (litters) examined	136 (17)	141 (18)	141 (17)	132 (18)	3741 (516): 29 studies
Total no. of fetuses (litters) with malformations	0	0	0	0	0–1.3%
Total no. of fetuses (litters) with variations	18 (7)	12 (10)	11 (8)	17 (11)	3.6–19.2%
Asymmetry of sternebrae	1	1	0	0	0–2.8%
Dumbbell ossification of thoracic centrum	1	3 (3)	2 (1)	2 (2)	0–5.5%
Splitting of thoracic centrum	0	0	0	1	0–3.0%
Full supernumerary ribs	0	0	1	0	0–4.4%
Short supernumerary ribs	16 (6)	8 (6)	9 (7)	14 (8)	0.3–17.1%
Short 13th ribs	0	0	0	1	0%
Degree of ossification[a]					
No. of sacral and caudal vertebrae	8.0 ± 0.4	8.0 ± 0.5	8.2 ± 0.4	8.1 ± 0.3	7.5–8.4
No. of sternebrae	5.4 ± 0.5	5.5 ± 0.6	5.7 ± 0.3	5.4 ± 0.5	4.7–5.7
Internal examination					
Total no. of fetuses (litters) examined	126 (17)	134 (18)	131 (17)	123 (18)	3459 (510): 30 studies
Total no. of fetuses (litters) with malformations	0	0	0	0	0–0.8%
Total no. of fetuses (litters) with variations	2 (2)	5 (4)	8 (6)	10 (6)	0–22.4%
Thymic remnants in neck	1	2 (2)	2 (2)	3 (3)	0–10.0%
Dilated renal pelvis	0	0	3 (2)	3 (2)	0–14.2%
Dilated ureter	1	3 (2)	6 (4)	7 (4)	0–14.2%
Convoluted ureter	0	0	0	1	0–3.8%

[a]Values are given as the mean ± SD.
[b]Historical control values were obtained from the studies performed in SNBL during 1996–2004 using Crj: CD (SD) IGS rats.

pelvis, dilated ureter and/or convoluted ureter, were observed in all groups, including the control group. However, no significant differences in the incidences of the total number of fetuses with internal variations and individual internal variation were found between the control and DBHCB-treated groups.

DISCUSSION

The current study was conducted to determine the prenatal developmental toxicity of DBHCB. The data showed that the prenatal oral administration of DBHCB did not produce any adverse effects, including morphological anomalies in fetuses of rats.

DBHCB was given to pregnant rats during the time of implantation to the term of pregnancy, to characterize the effects of DBHCB on embryonic/fetal development. The number of implantations was slightly reduced, and incidence of preimplantation loss was slightly increased in the high-dosage group, a finding associated with the tendency for reduced maternal body weight gain during the administration period, with an increase in maternal body weight gain after completion of the administration period. These differences were probably associated with the variability in litter sizes in the high-dosage group and unrelated to the administration of the test chemical. No significant changes in any maternal parameters were noted, even at 1000 mg/kg. No significant changes in embryonic/fetal survival or growth parameters were found, even at 1000 mg/kg. These findings indicate that DBHCB is not toxic to maternal animals, embryonic/fetal survival, or fetal growth when administered during the time of implantation to the term of pregnancy.

Morphological examinations in the fetuses of exposed mothers revealed no fetuses with external malformations. However, some fetuses with skeletal and/or internal variations were found in all groups. The variations observed in the current study are of the types that occur spontaneously among the control rat fetuses (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). A skeletal variation (i.e., full supernumerary ribs) has been described as a warning sign of possible teratogenicity and is known to occur in the presence of perturbation of maternal homeostatis. All other variations, short supernumerary ribs, sternebral variations, and bilobed centra of the vertebral column, are frequent variations, which were considered to be normal findings (Kimmel and Wilson, 1973). Although several types of skeletal variations, including full supernumerary ribs, were found in the control and DBHCB-treated groups, no consistent tendency was noted in the incidence of fetuses with these alterations. No significant differences between the control and DBHCB-treated groups were observed in the incidences of the total number of fetuses with skeletal variations or individual types of skeletal variation. Furthermore, these incidences were within the ranges of the background control data in the laboratory-performed current study. As for the internal variations, there was an increasing trend, according to the increasing doses, in the total number of

fetuses with internal variations and the number of fetuses with dilated renal pelvis or ureter. In the current study, the incidences of fetuses with internal variations, with dilated renal pelvis, and with dilated ureter at 1000 mg/kg were 7.5%, 2.1%, and 5.4%, respectively. In the background control data in the current study, these values were 0–22.4%, 0–14.2%, and 0–14.2% (Table 3). Because the incidences of fetuses with internal variations were within the range of the historical control data, and there were no statistically significant differences between the control and DBHCB-treated groups, these findings were considered unrelated to DBHCB and simply expression of the normal background incidence of such findings. Chahoud et al. (1999) noted that variations are unlikely to adversely affect the survival or health, and this might result from a delay in growth or morphogenesis that has otherwise followed a normal pattern of development. The alterations observed in the current study are not thought to be due to the administration of DBHCB, because they have occurred at a very low incidence and are of types that occur sporadically among control rat fetuses. Consideration of these findings together suggests that the morphological changes in fetuses observed in the current study do not indicate a teratogenic response and that DBHCB possesses no teratogenic potential in rats.

There was no available data for human exposure to this chemical. Actual human exposure to DBHCB may be estimated to be very low, because this chemical was not detected from polyethyleneterephthalate bottles in Brazil (Monteiro et al., 1998) and from polyethylene products in Japan (Kawamura et al., 1997). Consideration of these findings and the results of the current study together suggests that the risk of adverse effects of DBHCB on prenatal development of offspring is very low.

CONCLUSION

The current results showed that the administration of DBHCB to pregnant rats during the time of implantation to the term of pregnancy had no adverse effects on maternal rats and embryonic/fetal development, even at 1000 mg/kg no observed adverse effect levels. Based on these findings, it is concluded that the (NOAELs) of DBHCB for both dams and fetuses were 1000 mg kg^{-1} day^{-1} in rats.

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Developmental toxicity of dibutyltin dichloride in cynomolgus monkeys

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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

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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.

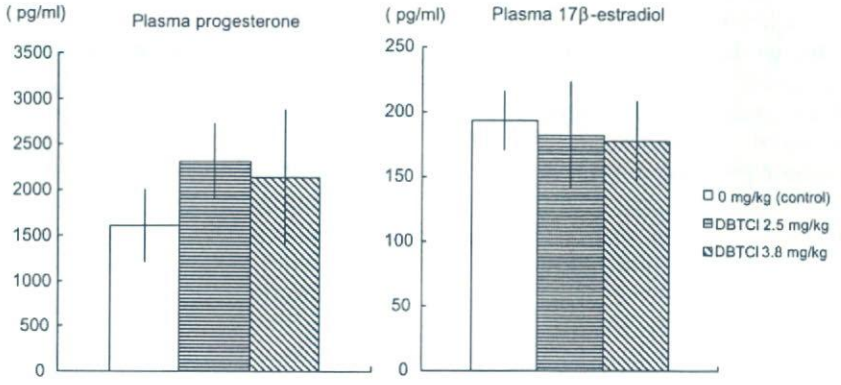

Fig. 1. Plasma progesterone and 17β-estradiol levels in pregnant monkeys given DBTCl on days 20–50 of pregnancy. Blood samples were collected on day 51 of pregnancy, 24 h after the last administration of DBTCl. Values are given as the mean ± S.E.M. of 5–10 monkeys.

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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Evaluation of developmental neurotoxicity of polysorbate 80 in rats

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Abstract

The developmental neurotoxicity of polysorbate 80 (PS80) was evaluated in rats. Crl:CD(SD) rats were given drinking water containing PS80 at 0, 0.018, 0.13, 1.0, or 7.5% (0, 0.035, 0.245, 1.864, or 16.783 ml/kg bw/day) on day 0 of pregnancy through day 21 after delivery. Pregnant rats were allowed to deliver spontaneously. Potential adverse effects of pre- and post-natal exposure on the development and function of the nervous system in offspring of rats given PS80 were examined. Maternal body weight was lowered at 7.5%. Number of pups born was lowered at 7.5%. There were no compound-related effects on locomotor activity of offspring on postnatal days (PNDs) 14–15, 17–18, 20–21 and 33–37. No compound-related changes were found in developmental landmarks, sexual maturation, or reflex responses. Although decreased rate of avoidance responses was noted on PNDs 23–27 in male and female offspring at 7.5%, no compound-related changes were found in performance in the conditioned avoidance response on PNDs 60–67. Histopathological examinations of the brain revealed no toxicological changes. Lowered body weight was observed in male and female offspring at 7.5%. The NOAEL in this study was considered to be 1.0% (1.864 ml/mg/kg bw/day).

Keywords: Polysorbate 80; Tween 80; Developmental neurotoxicity; Behavior; Developmental landmarks; Rat

1. Introduction

Polysorbate 80 (PS80, CAS No. 9005-65-6, polyoxyethylene (20) sorbitan monooleate, commercially also known as Tween® 80) is a mixture of polyoxyethylene ethers of mixed partial oleic acid esters of sorbitol anhydrides and related compounds [1]. PS80 is very soluble in water and soluble in ethanol. PS80 is widely used in biochemical applications including, solubilizing proteins, isolating nuclei from cells in cell culture, growing tubercule bacilli, and emulsifying and dispersing substances in medicinal and food products [2]. PS80 is often used in foods as an emulsifier in ice cream, frozen custard, ice milk, fruit sherbet, and nonstandardized frozen desserts. PS80 is also used in yeast-defoamer formulations and as a solubilizing and depersing agent in pickles and pickle products [1]. Exposure of the general population to PS80 is mainly through its use as a food additive.

Several reports on neurobehavioral toxicity of PS80 are available. Varma et al. [3] reported that PS80 caused a decreased locomotor activity and hyperthermia at 2 ml/kg, and exhibition of paralytic activity at 10 ml/kg after oral administration, and decreased locomotor activity, depression and potentiation of the penobarbitone sleeping time at 2 ml/kg after intraperitoneal administration in mice. They concluded that intraperitoneal doses generally showed more pronounced effects than oral doses, and PS80 did not show any neuropharmacological effects in a dose not more than 1 ml/kg when given either intraperitoneally or orally [3]. PS80 also caused behavioral and neurochemical changes in cats after intraperitoneal administration [4,5]. Intraperitoneal injection of 0.1% saline solution of PS80 in a volume of 3 ml/kg three times every 12 h decreased the carbachol-induced growing response and increased the content of 5-hydroxyindoleacetic acid in the hypothalamus in cats [4]. As for the developmental neurotoxicity of PS80, Brubaker et al. [6] reported that locomotor activity was enhanced in pre-weaning male offspring of rats received drinking water containing PS80 at 1.25 ml/l (0.125%) during the pre-mating, mating, pregnancy and lactation periods. However, their study did not provide enough information on all aspects of developmental neurotoxicity due to the use of one dose group and the

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selection of endpoints. Only pre-weaning locomotor activity in male offspring was determined, and no other parameters were evaluated in their study. The present study was therefore conducted to further evaluate the developmental neurotoxicity of PS80, including locomotor activity, in rats using a study design similar to the OECD Draft Proposal for New Guideline 426, Developmental Neurotoxicity Study [7].

2. Materials and methods

This study was performed in accordance with the principles for Good Laboratory Practice [8]. This study was conducted also in compliance with the "Law of Humane Treatment and Management of Animals" [9] and "Guidance for Animal Care and Use" of Ina Research Inc. and in accordance with the protocol reviewed by the Institutional Animal Care and Use Committee of Ina Research Inc. fully accredited by AAALAC International [Accredited Unit No. 001107].

2.1. Animals and housing conditions

Crl:CD(SD) rats were used throughout this study. Rats of this strain were chosen because they are the most commonly used in reproductive and developmental toxicity studies and historical control data are available. Male rats at 10 weeks of age and female rats at 9 weeks of age were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The males and females were acclimated to the laboratory for 7 days, prior to the start of the experiment, and rats found to be in good health were selected for use. Vaginal smears of each female rat were recorded, and rats showing regular estrous cycles were used in the experiment. Animals were reared on a basal diet (NMF; Oriental Yeast Co. Ltd., Tokyo, Japan) and water *ad libitum*, and were maintained in an air-conditioned room at 21.0–25.0 °C, with a relative humidity of 40–70%, a 12-h light (7:00–19:00)/dark (19:00–7:00) cycle, and ventilation of 16 air changes/h. Virgin female rats were mated overnight with male rats. The day when sperm was detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats, weighing 215–324 g, were distributed into five groups of 22 females to equalize the body weights among groups. Rats were housed individually, except during the acclimation, mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared on sterilized wooden chips (Sun Flake: Charles River Laboratories Japan, Inc.).

2.2. Chemical and dosing

Polysorbate 80 (PS80) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The PS80 used in this study was a technical grade (Lot no. EWP7301/code no. 162-21771, saponification value: 49.3, hydroxyl value: 70.1), and was kept in a dark and cool place. The stability of the PS80 was verified by analysis before and after the study. Rats were given PS80 in their drinking water at a concentration of 0 (control), 0.018, 0.13, 1.0, or 7.5% on day 0 of pregnancy through day 21 after delivery. The dosage levels were determined based on the results of our previous dose-finding study, in which decreased body weight gain and food and water consumption at 10.0% and higher, slight decrease in the body weight gain and food consumption at 7.5%, and no adverse effects at 5.0% and below were observed in female rats given PS80 in their drinking water for 14 days (data not shown). Dosed water preparations were formulated by mixing and dissolved PS80 into an appropriate amount of distilled water (Otsuka Pharmaceutical Factory, Inc., Naruto, Japan) for each water concentration. Rats were given PS80 at a constant water concentration. The control rats were given only water. The stability of formulations at room temperature has been confirmed for up to 7 days. During use, formulations were maintained at room temperature for not more than 5 days, and were 100.4–108.6% of the target concentration.

2.3. Observations of dams

All pregnant rats were observed daily for clinical signs of toxicity. Maternal body weight and water consumption were recorded daily, and food consumption was recorded every 3 or 4 days. Female rats were checked for signs of parturition before and after noon from days 20 to 25 of pregnancy to determine the time of delivery. The day on which parturition was completed by 16:00 was designated as day 0 after delivery. The females were allowed to deliver spontaneously and nurse their pups until day 21 after delivery. Parental female rats were euthanized by exsanguination under isoflurane anesthesia on day 21 after delivery. The external surfaces of rats were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. For each female, the number of uterine implantation sites was recorded, and the weights of the brain, liver, kidney, spleen, and adrenal were determined.

2.4. Observations of offspring

The day of birth was designated as postnatal day (PND) 0. On PND 0, total litter size and the numbers of live and dead pups were recorded, and pups were counted, sexed, and examined grossly on PND 0. All pups were observed daily for clinical signs of toxicity, and individually weighed on PNDs 0, 4, 7, 14 and 21. On PND 4, each of the litters was randomly adjusted to eight pups comprising of four males (1m, 2m, 3m and 4m) and four females (1f, 2f, 3f and 4f). Litters of less than eight pups were not used in the experiment. All pups were observed daily for pinna unfolding beginning on PND 2, fur appearance and incisor eruption beginning on PND 8, and eye opening beginning on PND 12. Body weights of pups were recorded on the day of completion of these developmental landmarks. Pups were weaned on PND 21.

2.4.1. Functional/behavioral observations during the pre-weaning period

One male (1m) and one female (1f) pup selected from all dams in each group was evaluated for surface righting reflex on PND 5, and negative geotaxis reflex on PND 8. Locomotor activity of offspring (1m and 1f) on PNDs 14–15, 17–18, and 20–21 at 20:00, 2:00, 8:00 and 14:00 was determined in the open field. Subject rats were placed individually in a box (26 cm in length and width, and 20 cm height) in a 3 × 3 matrix, consisting of a black acrylic plate, with a camera directly overhead and were allowed to explore freely for 10 min. The distance traveled by each monitored rat was recorded with video-based tracking software (BigBrother, Actimetrics, Inc., Wilmette, IL). Locomotor activity was determined under white noise (60 dB) to attenuate external sound, and light at 166–300 lx during the diurnal period and an infrared lamp during the nocturnal period.

2.4.2. Functional/behavioral observations during the adolescent and young adult periods

All remaining male (2m, 3m and 4m) and female (2f, 3f and 4f) pups of each dam were observed daily for clinical signs of toxicity, and individually weighed on PNDs 28, 35, 42, 49, 56 and 70.

Male (2m) and female (2f) pups selected from all dams in each group were evaluated for pupillary reflex, Preyer's reflex, pain response and mid-air righting on PNDs 23–26 and 62–64, and locomotor activity was determined on PNDs 33–37 and 60–66. An open-field with a box (39 cm in length and width, and 30 cm height) in a 2 × 2 matrix was used to evaluate locomotor activity in post-weaning offspring. Other procedures for the determination of locomotor activity were the same as described above for pre-weaning pups. Offspring (2m and 2f) were observed daily for male preputial separation beginning on PND 35 or female vaginal opening beginning on PND 25. The body weight of the respective rats was recorded on the day of preputial separation or vaginal opening.

Conditioned avoidance response was determined on PNDs 23–27 in male (3m) and female (3f) pups of half of the dams in each group, and on PNDs 60–67 in male (3m) and female (3f) pups of the other half of the dams in each group. The shuttle box (40 cm in length, 20 cm width, and 20 cm height), which consisted of transparent acrylic plastic panels, was divided into two equal compartments by a roller (40 and 55 mm in diameter for pups on PNDs 23–27 and PNDs 60–67, respectively). A rat placed in one compartment could get over the roller and cross to the other side. The grid floor of each compartment consisted of stainless steel rods spaced at 10 mm (for pups on PNDs 23–27) or 13 mm (for pups on PNDs 60–67) center to center. An electric shock could be delivered through the grid floor of the occupied compartment from a shock generator/scrambler (MU Co., Chino, Japan). A subject rat was given 2 min to adapt to the shuttle box after its introduction into one compartment. The trial began with a warning buzzer

(2000 Hz, 25 dB) as the conditioned stimulus (CS) for 5 s. A rat crossing to the opposite side of the shuttle box during the buzzer period would successfully avoid the electric shock (3 mA) that followed the buzzer. If the rat had not yet crossed to the opposite compartment of the shuttle box after the 5 s buzzer period, an electric shock was applied for 10 s, as the unconditioned stimulus. A 30 s intertrial interval preceded the next presentation of the CS. Each rat was tested for 60 min a day for three consecutive days. The rate of successfully conditioned responses for every 10 and 60 min was calculated.

One male (4m) and one female (4f) pups selected from each dam were maintained as reserve animals for replacements or additional tests.

2.4.3. Necropsy of offspring

Humane sacrifice was performed on PND 22 for pups (1m and 1f), on PND 70 for pups (2m, 3m, 2f and 3f), and on PNDs 103–126 for pups (4m and 4f) of each dam. The external surfaces of pups were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. For histopathological examinations, half of pups in each group (10–11/sex/group) killed on PNDs 22 or 70 were perfused with heparinized phosphate-buffered solution and paraformaldehyde-phosphate buffered solution, and the brain, spinal cord in the thoracic and lumbar regions, and sciatic nerve were removed and stored in 10% neutral buffered formalin. Histopathological evaluations were performed on the cerebrum, cerebellum, medulla oblongata, pons, spinal cord and sciatic nerve of male and female pups in the control and highest dose groups after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin. The remaining pups (8–11/sex/group) killed on PNDs 22 or 70 were subjected to weighing of the brain, liver, kidney, spleen and adrenal.

2.5. Statistical analysis

Statistical analysis of offspring before weaning was carried out using the litter as the experimental unit. The initial body weight, body weight gain and food and water consumptions of maternal rats, numbers of implantations and pups per litter, organ weight, pup weight, day of completion of developmental landmarks, latency of reflex response, distance traveled by pups, rate of avoidance response were analyzed with Bartlett's test [10] for homogeneity of variance at the 5% level of significance. If it was homogeneous, the data were analyzed using Dunnett's multiple comparison test [11] to compare the mean of the control group with that of each dosage group, and if it was not homogeneous, the mean rank of the PS80-treated groups was compared with that of the control group with the Dunnett-type test which was used for the gestation length, delivery index, incidence of pups with malformations, viability index of pups and rate of pups that completed reflex responses to compare the mean rank of groups treated with PS80 and the control group. The fecundity index and gestation index were analyzed by chi-square test. The 0.05 level of probability was used as the criterion for significance.

3. Results

3.1. Findings in dams

No feces were found in one female on days 20–21 of pregnancy and on day 0 of lactation at 7.5%. During lactation, loose stools in 18 females on days 2–21, and scattering of offspring on days 0–1, scant or no feces on days 1–2, reddish brown soiled perianal region on days 12 and 20 and death of all offspring on day 2 in one female each were observed at 7.5%. No clinical signs of toxicity were noted at 1.0% and below (data not shown).

The body weights of maternal rats during pregnancy and lactation are shown in Fig. 1. A significantly lower body weight was observed on days 3, 10, 12–20 of pregnancy and days 0, 2–18 of lactation at 7.5%. In this group, body weight gain was also significantly decreased during pregnancy.

A significant decrease in food consumption on all measuring days during pregnancy and lactation was noted at 7.5%. No

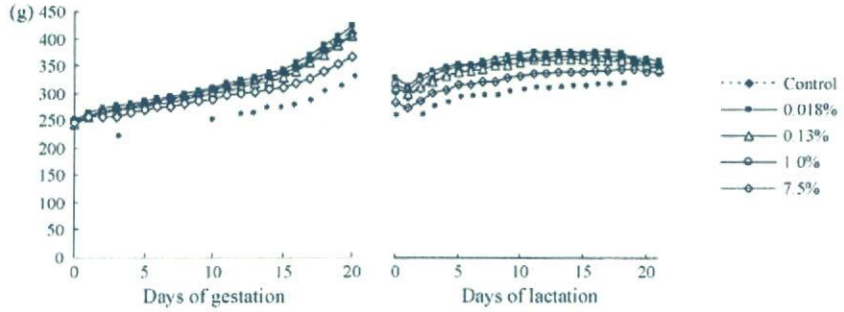

Fig. 1. Body weight of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

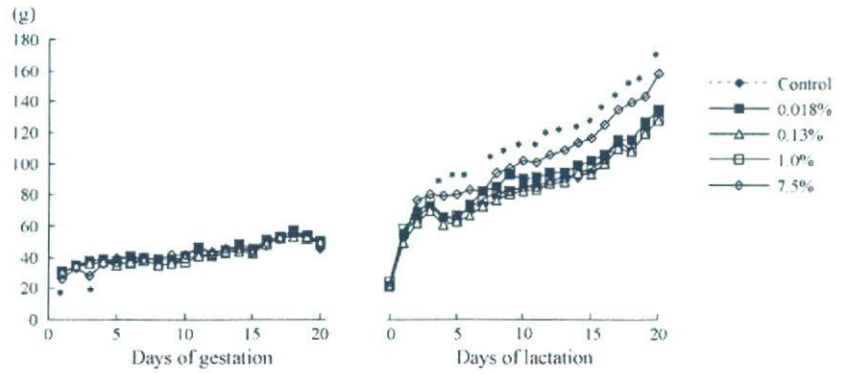

Fig. 2. Water consumption of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

significant changes in food consumption were found at 1.0% and below (data not shown).

The water consumption of maternal rats during pregnancy and lactation are shown in Fig. 2. At 7.5%, water consumption was significantly decreased on days 1 and 3 of pregnancy and increased on days 5–7 and 9–21 of lactation. No significant changes in water consumption were found at 1.0% and below. The least water consumption was observed on day 0 of lactation and the most water consumption was observed on day 21 of lactation in all groups.

The average daily intakes of PS80 during pregnancy were 0.024, 0.171, 1.314 and 10.576 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The average daily intake of PS80 during lactation were 0.046, 0.321, 2.521 and 23.908 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The average daily intakes of PS80 throughout the administration period were 0.035, 0.245, 1.864 and 16.783 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The least intake of PS80 was noted on day 0 of lactation and the most intake of PS80 was noted on day 21 of lactation in all groups. The ranges of average daily intakes of PS80 based on each day of the administration period were 0.012–0.071, 0.093–0.499, 0.837–3.765 and 5.869–36.431 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively.

At necropsy of dams, dilatation of the cecum in seven females and a significant increase in the relative weight, but not in the absolute weight, of the kidney, were observed at 7.5%. No changes in gross pathology or in absolute and relative weights were detected in any organs at 1.0% and below (data not shown).

3.2. Reproductive/developmental findings

The reproductive findings in maternal rats are presented in Table 1. One female at 7.5% showed total litter loss by day 2 of lactation, one female at 0.018 and 1.0% and two females at 0.13% were not impregnated, and one pregnant female at 1.0%, died on day 22 of pregnancy; however, no significant differences were noted in the fecundity index, gestation index and length of gestation between control and PS80-treated groups.

The developmental findings are shown in Table 2. The number of pups born was significantly reduced at 7.5%. There were no significant effects of treatment of PS80 on the numbers of implantations, pups born alive and dead pups, delivery index and sex ratio of pups and the viability index of pups before weaning. A fetus with acaudate and anal atresia was observed at 0.018%, but no fetuses with external malformations were found in other groups. Although no significant changes in the body weight of male and female pups were observed on PNDs 0, 4 and 7 in PS80-treated groups, significantly reduced body weights were noted on PNDs 14 and 21 at 7.5%. No PS80-related clinical signs of toxicity were found during the pre-weaning period.

The body weights after weaning in male and female offspring of rats given PS80 during pregnancy and lactation are shown in Table 3. At 7.5%, a significantly reduced body weight was noted on PNDs 28, 35, 42, 49 and 56 in males and on PNDs 28 and 35 in females.

One male at 1.0% died on PND 23; however, there were no compound-related clinical signs of toxicity or adverse effects on the survival rate in male and female weaned rats (data not shown).

3.3. Developmental landmarks in offspring

Physical development in male and female pups is presented in Table 4. There was no significant difference in the age of male and female pups that displayed pinna unfolding, fur appearance, incisor eruption, or eye opening. Body weight at the age of fur appearance in males and eye opening in both sexes was significantly reduced at 7.5%.

Data on sexual development in male and female pups is shown in Table 5. No significant differences in age at preputial separation in males or vaginal opening in females, or body weight at the age of preputial separation or vaginal opening were found between control and PS80-treated groups.

Examination of reflex ontogeny revealed no significant difference between control and PS80-treated groups in the latency of response, i.e., the time taken by the subject to complete reflex response, or the incidence of pups completing reflex response. All male and female pups in all groups, except for one male pup at 1.0%, showed completion of the reflex response when testing surface righting reflex on PND 5 and negative geotaxis reflex on

Table 1
Reproductive findings in rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of females copulated	22	22	22	22	22
No. of pregnant females	22	21	20	21	22
No. of non-pregnant females	0	1	2	1	0
Fecundity index (%)[a]	100	95.5	90.9	95.5	100
No. of deaths during pregnancy	0	0	0	1	0
Gestation length (days)[b]	21.9 ± 0.4	21.8 ± 0.4	21.9 ± 0.4	21.9 ± 0.4	21.7 ± 0.5
No. of females with live born	22	21	20	20	22
Gestation index (%)[c]	100	100	100	95.2	100
No. of females with totally litter loss	0	0	0	0	1

[a] Fecundity index (%) = (no. of pregnant females/no. of females confirmed mating) × 100.
[b] Values are given as the mean ± S.D.
[c] Gestation index (%) = (no. of females with live pups born/no. of pregnant females) × 100.

Table 2
Developmental findings in rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of litters	22	21	20	21[a]	22
No. of implantations[b]	16.1 ± 1.7	15.4 ± 2.7	15.1 ± 1.6	15.8 ± 1.4	15.1 ± 2.1
Total no. of pups born[b]	15.4 ± 1.8	14.8 ± 2.4	14.2 ± 2.1	14.6 ± 1.2	13.9 ± 1.7*
No. of pups born alive[b]	14.7 ± 1.8	14.6 ± 2.3	13.7 ± 2.0	14.5 ± 1.2	13.6 ± 2.0
No. of dead pups[b]	0.7 ± 1.2	0.1 ± 0.4	0.5 ± 0.7	0.2 ± 0.7	0.3 ± 0.8
Delivery index (%)[c]	95.8	96.0	94.1	92.9	92.8
Proportion of male pups (%)[d]	54.2	49.2	53.6	47.7	44.7
Viability index before weaning (%)					
Postnatal day 0[e]	95.5	99.1	96.6	99.0	97.6
Postnatal day 4[f]	98.6	98.2	98.5	98.7	93.8
Postnatal day 21[g]	100	100	100	100	100
Body weight of male pups before weaning (g)[b]					
Postnatal day 0	6.4 ± 0.5	6.5 ± 0.5	6.5 ± 0.5	6.5 ± 0.4	6.1 ± 0.8
Postnatal day 4	10.1 ± 1.0	10.6 ± 1.3	10.4 ± 1.3	10.4 ± 0.8	9.8 ± 1.1
Postnatal day 7	16.9 ± 1.4	17.6 ± 1.5	17.1 ± 1.9	17.4 ± 1.1	16.2 ± 1.8
Postnatal day 14	35.3 ± 1.6	36.5 ± 2.4	34.8 ± 2.5	35.6 ± 1.8	32.4 ± 3.3*
Postnatal day 21	58.0 ± 3.6	59.0 ± 4.1	57.0 ± 3.5	57.1 ± 3.0	50.4 ± 5.1*
Body weight of female pups before weaning (g)[b]					
Postnatal day 0	6.0 ± 0.5	6.2 ± 0.5	6.2 ± 0.5	6.1 ± 0.3	5.8 ± 0.7
Postnatal day 4	9.6 ± 1.0	10.2 ± 1.1	9.9 ± 1.3	9.9 ± 0.8	9.5 ± 1.0
Postnatal day 7	16.1 ± 1.4	17.0 ± 1.6	16.3 ± 1.9	16.6 ± 1.1	15.7 ± 1.8
Postnatal day 14	34.0 ± 1.9	35.2 ± 2.3	33.5 ± 2.3	34.4 ± 1.4	31.5 ± 3.1*
Postnatal day 21	55.0 ± 3.7	56.4 ± 4.0	54.5 ± 3.1	55.1 ± 2.0	49.2 ± 4.7*
External examination of pups[h]					
No. of pups (litters) examined	339 (22)	310 (21)	284 (20)	278 (19)	306 (22)
No. of pups with malformations	0	1[i]	0	0	0

[a] One female, who delivered four live pups on day 23 of pregnancy, was euthanized on day 0 after delivery, and her data were excluded.
[b] Values are expressed as the mean ± S.D.
[c] Delivery index (%) = (no. of pups born/no. of implantations) × 100.
[d] Proportion of male pups = (no. of male pups/total no. of pups) × 100.
[e] Viability index on postnatal day 0 (%) = (no. of pups born alive/total no. of pups born) × 100.
[f] Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of pup born alive) × 100.
[g] Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live on postnatal day 4 after cull) × 100.
[h] External examinations were performed on all pups born on postnatal day 0.
[i] One live pup had acaudate and anal atresia.
* Significantly different from the control, $p < 0.05$.

PND 8. As for the sensory function of offspring, all male and female pups in all groups completed pupillary reflex, Preyer's reflex, pain response and mid-air righting reflex when tested on PNDs 23–26 and 62–64 (data not shown).

3.4. Locomotor activity in offspring

Locomotor activity of male and female pups during the pre-weaning period is presented in Fig. 3. No significant differences in the distance traveled by male and female pups during the nocturnal period (20:00 and 2:00) and diurnal period (8:00 and 14:00) were found between control and PS80-treated groups when locomotor activity was determined on PNDs 14–15. Although a significantly higher activity was observed in male pups in the 1.0% group at 2:00 on PND 18, no significant changes in activity were noted in males and females at any other test time on PNDs 17–18. There were no significant differences between control and PS80-treated groups in locomotor activity of male and female offspring at any test time on PNDs 20–21.

After weaning, no significant differences in the distance traveled by male and female offspring were detected at any test time when activity was determined on PNDs 33–37 and PNDs 60–66 (data not shown).

3.5. Conditioned avoidance response in offspring

The rate of successfully conditioned responses for every 10 min test period on PNDs 23–27 is presented in Fig. 4. On the first day of the test, the rate of successful responses for 60 min was lower in males and females at 7.5%, and a significantly decreased rate was noted in males during the last 10 min and in females during the first and third 10 min test periods. However, there were no significant changes in the rate of successful responses in any 10 min test periods in males and females of any PS80-treated groups on the second- and third-day of the test. No significant changes in the total rate of successfully responses for 60 min were found in male and female pups in any PS80-treated groups on any test days.