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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.

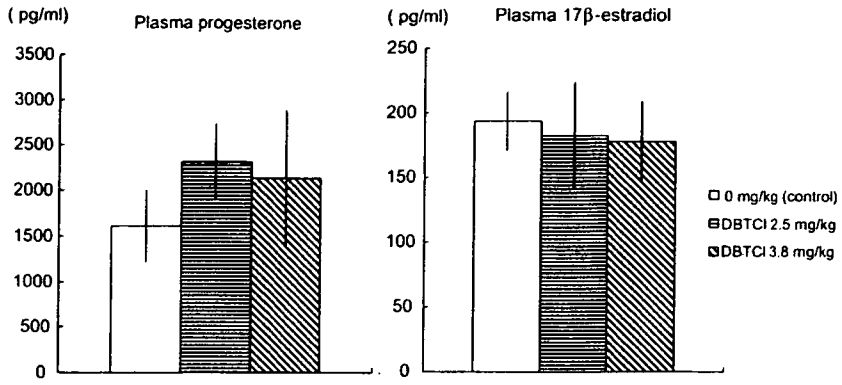

Fig. 1. Plasma progesterone and 17β-estradiol levels in pregnant monkeys given DBTCl on days 20–50 of pregnancy. Blood samples were collected on day 51 of pregnancy, 24 h after the last administration of DBTCl. Values are given as the mean ± S.E.M. of 5–10 monkeys.

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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COMMENTARY

Comments from the Developmental Neurotoxicology Committee of the Japanese Teratology Society on the OECD Guideline for the Testing of Chemicals, Proposal for a New Guideline 426, Developmental Neurotoxicity Study, Draft Document (October 2006 version), and on the Draft Document of the Retrospective Performance Assessment of the Draft Test Guideline 426 on Developmental Neurotoxicity

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ABSTRACT **In October 2006, a new revision of the draft guideline (OECD Guideline for the Testing of Chemicals, Proposal for a New Guideline 426. Developmental Neurotoxicity Study) and Draft Document of the Retrospective Performance Assessment (RPA) of the Draft Test Guideline 426 on Developmental Neurotoxicity were distributed following incorporation of the results of the Expert Consultation Meeting in Tokyo on May 24–26, 2005. The draft guideline consists of 50 paragraphs and an appendix with 102 references; and the draft RPA consists of 37 paragraphs with 109 references. National coordinators were requested to arrange for national expert reviews of these draft documents in their member countries. Members of the Developmental Neurotoxicology (DNT) Committee of the Japanese Teratology Society (JTS) reviewed, discussed, and commented on the draft Test Guideline Proposal. The DNT Committee of the JTS also commented on the draft document of the RPA. These comments were sent to the OECD Secretariat. The DNT Committee of the JTS expects the comments to be useful for the finalization of these draft documents.**

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Key Words: behavior, developmental neurotoxicity, OECD, test guideline

BACKGROUND

In June 1995, the Organization for Economic Co-operation and Development (OECD) Working Group on Reproduction and Developmental Toxicity at Copenhagen recommended that a guideline for developmental neurotoxicity (DNT) should be written (Organisation for Economic Co-Operation and Development 1995). In June 1996, an OECD Consultation Meeting on DNT was held in Copenhagen to provide the Secretariat with a draft report on the outline of a new guideline (Organisation for Economic Co-Operation and Development 1996). Comments on this draft report were provided from the Behavioral Teratology (BT) Committee of the Japanese Teratology Society (JTS), in association with the Meeting of Neurobehavioral Toxicology of the Japanese Society of Toxicology. After this meeting, a draft proposal for Test Guideline 426 on DNT Study was developed, and it was submitted to the Secretariat in February 1998. The draft guideline reflecting these comments was distributed in December 1998. The BT Committee of the JTS commented again on this draft guideline. The draft guideline proposal was extensively revised and distributed in October 1999. In October 2000, an OECD Expert Consultation Meeting and International Life Sciences Institute (ILSI) Risk Science Institute Workshop discussed general issues regarding the design of DNT studies in Washington, D.C. (Organisation for Economic Co-Operation and Development 2003).

A new revision of the guideline was distributed in September 2003. The draft guideline consists of 51 paragraphs and an appendix. The BT Committee of the JTS commented on the draft Test Guideline Proposal (Fukui *et al.* 2004). The BT Committee of the JTS also commented that an International Collaborative Study to validate this protocol should definitely be performed, as indicated in OECD ENV/EHS/HK/mc/2003.49. These comments were sent to the OECD Secretariat through the Japanese national coordinator in January, 2004.

In response to the comments, the Expert Consultation Meeting was held to make the final revisions to the draft Test Guideline 426 on DNT in Tokyo on May 24–26, 2005 (Organisation for Economic Co-Operation and Development 2005). Thirteen Japanese, including five persons from the government and eight from the Business and Industry Advisory Committee (BIAC) attended this meeting among the total of 27 participants from the OECD member countries. Participants included the members of the BT Committee of the JTS. Drs Hiroaki Aoyama, Makoto Ema, and Takashi Tanimura. In this Tokyo Meeting, the number of animals used, issues on dose limits and maternal toxicity, the direct dosing of pups, physical development, neuropathological examination, interpretation of results, Table 3, references, and issues on Test Method Performance (motor activity, sensory function, and memory test) were generally discussed (Organisation for Economic Co-Operation and Development 2005). Japan drafted a new Figure 1 (originally prepared by Dr Tanimura) and presented this figure to the meeting for consideration. After the meeting, Japan edited the references in paragraph 35 and recommended to remove some references because they did not correctly refer to the text.

In October 2006, a new revision of the draft guideline (OECD Guideline for the Testing of Chemicals, Proposal for a New Guideline 426. DNT Study) and Draft Document of the Retrospective Performance Assessment (RPA) of the Draft Test Guideline 426 on DNT were distributed following the incorporation of the results of the Tokyo Meeting in 2005. These draft documents are posted on the OECD public web site of the Test guidelines programme at: http://www.oecd.org/document/55/0,2340,en_2649_34377_2349687_1_1_1_1,00.html. The draft guideline consists of 50 paragraphs, one figure, one table, and one appendix with 102 references, and the draft RPA consists of 37 paragraphs and four tables with 109 references. National coordinators were requested to arrange for national expert reviews of these draft documents in their member countries. The deadline for the expert responses to these draft documents was December 15, 2006.

National experts asked the members of the DNT committee (former BT committee) of the JTS and Japanese participants in the Tokyo Meeting to comment on these documents. a new revision of the draft guideline (OECD Guideline for the Testing of Chemicals, Proposal for a New Guideline 426. DNT Study), and the Draft Document of the RPA of the Draft Test Guideline 426 on DNT. The members of the DNT Committee (Chairman: Dr Yoshihiro Fukui, Professor, University of Tokushima School of Medicine) of the JTS reviewed and commented on the draft documents. The national experts received the comments to these draft documents only from the DNT Committee of the JTS. These comments were sent to the OECD Secretariat through the Japanese national coordinator (Ms. Yayoi Sasaki, Director of the Office of Chemical Safety, Pharmaceutical and Food Safety Bureau, Ministry of Health, Labour and Welfare, Japan) before the deadline.

The DNT Committee of the JTS expects the comments to be useful for the finalization of these draft documents.

The comments from the DNT Committee of the JTS are as follows:

COMMENTS

Comments on OECD Guideline for the Testing of Chemicals, Proposal for a New Guideline 426. Developmental Neurotoxicity Study (October, 2006)

General comments

The proposed revised draft of Test Guideline 426 is generally acceptable, except for several minor editorial comments and the need for a uniform style of literature.

Specific comments

A. Editorial remarks

P31	Line 3	Better wording should be used for the sentence: 'If developmental landmarks are . . . measurements should be performed.
P37	Line 18	No references are given for adult rats.
P38	Line 4	'brain' should be central nervous system (CNS). Peripheral Nervous System should be peripheral nervous system.
P40	Line 4	'central (CNS)' should be 'CNS'. See P38 suggestion.
P43	Line 4	'and' before 'the cerebellum' should be deleted for consistency of the document form.
P45	Line 3	Subject (evidence) and verb (are) must agree in number.
P47	Line 7	'be use' is correct? In the third revision (Aug '2005), it was 'be done using'.

Figure 1. 2nd Box, Offspring: Approximately 80/sex/group: Line 4 'bodyweight' should be 'body weight'.

Table 1. Insert a horizontal line between the row m1 + f5 and m2 + f6.

Table 2 and Table 3. Column 1/row 1, 'Pup no' should be 'Pup no.'

Another 13 comments regarding editorial remarks were made.

B. Literature

It is greatly appreciated that many new references have been added. However, their styles are not uniform, especially in the abbreviation of journal titles.

A total of 81 editorial comments were made in LITERATURE.

Comments on the Draft Document of the Retrospective Performance Assessment (RPA) of the Draft Test Guideline 426 on Developmental Neurotoxicity

We feel a strong need to add a description of the collaborative studies of the JTS.

Efforts for a more unified style of literature are requested.

Addition of the collaborative studies of the JTS

a. P10. Table 1

The following one row is to be added between the rows of 1989 and 1995.

Date	Event	Summary	References
1993–1997	Collaborative studies of the Japanese Teratology Society	Three interlaboratory studies using behavioral teratogens to evaluate a core battery of tests	Fukunishi *et al.* (1998), Tachibana *et al.* (1998), Nishimura *et al.* (2001)

b. P19.

The following new paragraph is to be placed as P19 and the current P19 'The International Programme on Chemical Safety (IPCS) Study' is to be numbered as a New P20.

19. Collaborative Studies of the Japanese Teratology Society – The Japanese Teratology Society established the Behavioral Teratology Meeting (BTM) as a satellite meeting in 1982. Following the small-scale collaborative studies with the unified protocol, workshops were held between 1988 and 1990, with three subgroups: reflexes and sensory function, activity and emotionality, and learning (Tanimura 1992). Subsequently, a core battery test draft for behavioral developmental toxicity was proposed and its utility was examined with three positive behavioral teratogens in 1993–1997. They were phenytoin (Fukunishi *et al.* 1998), retinoic acid (Nishimura *et al.* 2001), and nicotine (Tachibana *et al.* 1998). Participating laboratories were 32, 28, and 18, respectively. It was concluded that the proposed core battery of tests are useful as a screening method to detect postnatal developmental disorders, including behavioral dysfunction, in rats. Activities of the BTM of the Japanese Teratology Society had continued to be presented as the Behavioral Teratology Committee (present DNT Committee) until July 2006. For instance, comments on the OECD Test Guideline 426, Developmental Neurotoxicity Study, Draft Document (September, 2003) were published (Fukui *et al.* 2004).

C. References

The following five references for the New P19 are to be added.

Fukui Y, Ema M, Fujiwara M *et al.* (2004) Comments from the Behavioral Teratology Committee of the Japanese Teratology Society on OECD Guideline for the Testing of Chemicals, proposal for a new guideline 426, developmental neurotoxicity study, draft document (September, 2003). *Congenital Anomalies* **44**: 172–177.

Fukunishi K, Terada Y, Tachibana T, Tanimura T (1998) Collaborative behavioral teratology study of phenytoin: A test battery for neurobehavioral developmental toxicity in rats. *Congenital Anomalies* **38**: 117–141.

Nishimura T, Iwase T, Hashimoto Y, Tanimura T (2001) Evaluation of a core battery of tests for detecting behavioral dysfunction of rat offspring induced by retinoic acid: Collaborative work II of the Japanese Behavioral Teratology Committee. *Congenital Anomalies* **41**: 156–168.

Tachibana T, Narita H, Ogawa T, Tanimura T (1998) Using postnatal age to determine test dates leads to misinterpretations when treatments alter gestation length: Results from a collaborative behavioral teratology study in Japan. *Neurotoxicology and Teratology* **20**: 449–457.

Tanimura T (1992) Update on the activities of the Japanese Behavioral Teratology Meeting. *Congenital Anomalies* **32** (Suppl.): S7–S20.

Note: 'Congenital Anomalies' is the official journal of the Japanese Teratology Society. It has been recently adopted by PubMed and the abbreviated title in PubMed is 'Congenit Anom Kyoto'.

For the Secretariat's convenience, copies of the front page of the five papers are attached.

Uniform style of references

More efforts towards a uniform style of references, especially in the abbreviation of journal titles, are needed. In this document, the majority of journal titles are fully spelled out, while most of the journal titles in Test Guideline 426 are abbreviated. In consideration of the hope that this document may be read not only by academics but also by the public in broader areas, the full spelling of journal titles may have some merits. In any case, the current presentation of references is quite variable, and it is strongly requested that the Secretariat be responsible for the proper amendments.

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Organisation for Economic Co-Operation and Development (OECD) (1995) *Draft Report of the OECD Ad Hoc Working Group on Reproduction and Developmental Toxicity*. Copenhagen, Denmark, 13th–14th June 1995.

Organisation for Economic Co-Operation and Development (OECD) (1996) *Final Report of the Consultation Meeting on Developmental Neurotoxicity*. Copenhagen, Denmark, 17th–18th June 1996.

Organisation for Economic Co-Operation and Development (OECD) (2003) *Report of the Expert Consultation Meeting in Developmental Neurotoxicity Testing*. Washington, US, 23th–25th October 2000.

Organisation for Economic Co-Operation and Development (OECD) (2005) *Draft Meeting Report of the Expert Consultation Meeting for the Revision of draft Test guideline 426 on 'Developmental Neurotoxicity Study'*. 24–16 May, 2005, Tokyo, MHLW, Japan.

Tachibana T, Narita H, Ogawa T, Tanimura T (1998) Using postnatal age to determine test dates leads to misinterpretations when treatments alter gestation length: Results from a collaborative behavioral teratology study in Japan. *Neurotoxicol Teratol* **20**: 449–457.

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Screening Study for Repeated Dose and Reproductive/ Developmental Toxicity of Rubber Accelerator, *N,N*-Dicyclohexyl-2-benzothiazolesulfenamide, in Rats

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A screening study for a vulcanization accelerator *N,N*-dicyclohexyl-2-benzothiazolesulfenamide (DCBS) was performed in rats. Rats were given DCBS by gavage daily at 0, 6, 25, 100, or 400 mg/kg. Males were dosed for a total of 44 days beginning 14 days before mating. Females were dosed for a total of 40–51 days beginning 14 days before mating to day 3 of lactation. Toxicologic changes were significantly noted only at 400 mg/kg. Three females died. An increased incidence of females showing decreased locomotor activity, soil of the lower abdominal fur, and reddish tears was observed. A lowered body weight was found in males and females. Increased urinary ketones and serum inorganic phosphorus and decreased serum glutamate pyruvate transaminase in males were found. Increased absolute and relative weights of the kidneys in males and decreased absolute weight of the thymus in both sexes were noted. Significant fatty degeneration of the renal tubular epithelia, vacuolation of the adrenocortical cells, and atrophy of the spleen were observed in females. Significant decreases in the gestation index, numbers of corpura lutea, implantations, pups born and pups born alive, live birth index, and viability index were detected. It is concluded that the No

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Observed Adverse Effect Levels (NOAELs) for repeat dose and reproductive/developmental toxicity are 100 mg kg^{-1} day^{-1} in this screening study.

Keywords *N,N*-Dicyclohexyl-2-benzothiazolesulfenamide, Repeated dose toxicity, Reproductive and developmental toxicity, Vulcanization accelerator.

INTRODUCTION

N,N-Dicyclohexyl-2-benzothiazolesulfenamide (CAS no. 4979-32-2; DCBS) is one of the sulfonamide accelerators, and the sulfenamide accelerators have been manufactured in the United States for more than 60 years (EPA, 2001). The sulfenamide accelerator materials are shipped extensively throughout the world from manufacturing plants located in North America, South America, Europe, Asia, and Africa (EPA, 2001). DCBS is produced with an annual production level of about 1000 tons/year in 1990–1993 and 1900 tons in 2000–2003 in Japan, and most of this amount was sold and handled in Japan (OECD, 2006). The main application area of DCBS is in large tires, but it is also employed in conveyor belts, driving belts, shock absorbers, mountings, and other intricately shaped molded goods requiring extremely long flow periods in the molding process (Akrochem, 2006). DCBS is regulated for use in articles in contact with food in Germany, but this compound is not regulated for use in U.S. FDA food contact applications (Flexsys, 2006). Only up to 6% of biodegradation of DCBS was determined in a ready biodegradability test (OECD, 2006). A measured log K_{ow} value of 4.8 suggests that DCBS is suspected to have a high bioaccumulation potential (OECD, 2006). Exposure of workers handling sulfenamide accelerator materials is likely to be highest in the area of materials packaging. During material packout at the manufacturing site and, to a somewhat lesser degree during weigh-up activities at the consumer site, there is a potential for skin and inhalation exposure. Although consumer exposure is minimal, the most likely route of consumer exposure is skin contact from rubber or latex articles (EPA, 2001).

The possibility of these compounds entering into biological systems has aroused great concern about its toxicologic potential. It is generally assumed that the biological effects produced by chemicals should be studied in laboratory animals to investigate their possible influences on human health, and the results of animal tests of chemical toxicity are relevant to humans (Clayson et al., 1990). However, very little information on the toxicity of this compound has been published. Vorobera (1969) reported that the oral LD_{50} values were 8500 mg/kg in male mice and repeated inhalation exposure of male rats for 15 days, daily, 2 h/day, at 350–400 mg/m^3 caused mucous membrane irritation. The results of toxicity studies on DCBS were briefly summarized by the European Chemical Bureau (2000) and the U.S. EPA (2001), but the descriptions regarding the toxicity of DCBS were insufficient to evaluate the toxic effects of this compound. The EPA (2001) noted that the oral No Observed

Adverse Effect Level (NOAEL) for 44 days repeated dose toxicity was higher than 100 mg/kg bw per day in rats, and no effects on reproduction were observed at doses up to 400 mg/kg bw per day in rats. Toxicity studies of DCBS including acute toxicity, *in vitro* genotoxicity and repeat dose toxicity combined with reproductive/developmental toxicity studies, were performed as a part of the Safety Examination of Existing Chemical Substances and Chemical Safety Programmes by the Japanese government (MHW, 1998). The results of these toxicity studies are summarized in the IUCLID Data Sets (EPA, 2006), OECD Screening Information Data Sets (OECD, 2006), and Hazard Assessment Sheet (CERI, 2002). However, detailed data have not been published in the scientific journals. Although the testing for reproductive and developmental toxicity in animal models is an important part of the overall toxicology, we cannot obtain the detailed information on the reproductive and developmental toxicity of DCBS. In this paper, therefore, we reevaluated the data of the repeat dose toxicity combined with reproductive/developmental toxicity screening test of DCBS and prepared the manuscript to be published in the scientific literature.

MATERIALS AND METHODS

Animals

Crj:CD (SD) rats were used throughout this study. This strain was chosen because it is the most commonly used in toxicity studies, including reproductive and developmental toxicity studies, and historical control data are available. Males at 7 weeks of age and females at 6 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimatized to the laboratory for 12 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Male and female rats were distributed on a random basis into five groups of 10 males and 10 females each. The 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 using wooden chips as bedding (White flake; Charles River Japan, Inc.). Animals were reared on a basal diet (CE-2; CLEA Japan Inc., Tokyo, Japan) and filtered tap water available *ad libitum* and maintained in an air-conditioned room at 22 ± 3°C, with a relative humidity of 55 ± 10%, a 12-h light/dark cycle, and ventilation with 10 and more air changes/hour.

Chemicals and Dosing

DCBS was obtained from Ouchishinko Chemical Industrial Co., Ltd. (Tokyo, Japan). The DCBS, in the form of light-gray granules, is very slightly

soluble in water and methanol and soluble in oil, and its melting point is 100–105°C, specific gravity is 1.2, and molecular weight is 346.6 (Flexsys, 2006). The DCBS (lot no. 307021) used in this study was 99.2% pure, and it was kept in a sealed container under cool (4°C) and dark conditions. The purity and stability of the chemical were verified by analysis using high-performance liquid chromatography before and after the study. Rats were dosed once daily by gastric intubation with DCBS at a dose of 0 (control), 6, 25, 100, or 400 mg/kg body weight (bw). The dosage levels were determined based on the results of our previous dose-finding study, the 14-day repeated dose toxicity study in rats given DCBS by gavage at 0, 3, 10, 30, 100, or 300 mg/kg bw per day, at which the tendency to decrease the gain in body weight and to increase the weight of the kidney and adrenal gland were found at 300 mg/kg bw per day (data not shown). DCBS was dissolved or suspended in sesame oil (lot no. A113, Miyazawa Yakuhin Co., Ltd., Tokyo, Japan). Males (10 rats/group) were dosed for a total of 44 days beginning 14 days before mating. Females (10 rats/group) were dosed for a total of 40–51 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation period. The volume of each dose was adjusted to 5 mL/kg bw based on the latest body weight during the premating and mating period in males and females. The control rats were given only sesame oil. The stability of formulations has been confirmed for up to 7 days in a cool (4°C) and dark place. During use, the formulations were maintained under such conditions for less than 7 days, and the target concentration was 96.0% to 99.1%.

Experimental Design

This study was performed in 1994 at the Research Institute for Animal Science in Biochemistry and Toxicology (Sagamihara, Japan) in compliance with the OECD guideline Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (OECD, 1990; Tobe et al, 1991) and in accordance with the principles for Good Laboratory Practice (OECD, 1981; EA, MHW and MITI, 1988) and the Guidance for Animal Care and Use of the Research Institute for Animal Science in Biochemistry and Toxicology.

All rats were observed daily for clinical signs of toxicity. The body weight was recorded once a week in males during the administration period, and once a week during the premating and mating periods, on days 0, 7, 14, and 20 of pregnancy, and on days 0 and 4 of lactation in females. The food consumption on days of measurement of body weight in both sexes and on day 3 of lactation in females were measured.

On day 42 of the administration period, urine was collected and analyzed for dipstick parameters, such as pH, glucose, occult blood, protein, ketones, bilirubin, and urobilinogen, in 10 male rats per group.

Prior to scheduled terminal necropsy on the next day of the last administration, while male rats were under ether anesthesia, blood samples for hematologic and blood biochemical evaluations were collected from the abdominal aorta of 10 fasted male rats per group. Blood samples were analyzed for the following hematologic parameters, using K_2-EDTA as anticoagulant: red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), total white blood cell count (WBC), and platelet count (Automated Blood Cell Counter, E-4000; Toa Medical Electronics Corp., Kobe, Japan). The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were calculated from Hb, Ht, and RBC.

Serum samples, obtained from centrifuged whole blood, were analyzed following biochemistry parameters: total protein, albumin, albumin–globulin ratio, glucose, triglycerides, total cholesterol, total bilirubin, urea nitrogen, creatinine, glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, gamma-glutamyl transpeptidase, alkaline phosphatase, calcium, and inorganic phosphorus (Automated Biochemical Analyzer, JCA-VX-1000 Clinalyzer; Japan Electron Optics Laboratory Co., Ltd., Akishima, Japan) and sodium, potassium, and chloride (Automated Electrolytes Analyzer, NAKL-1, Toa Medical Electronics Corp.).

At the scheduled terminal necropsy (males on day 45; females on day 4 of lactation), all rats were euthanized by exsanguination under ether anesthesia. All rats were subjected to gross necropsy, which included an external examination of all body orifices and surfaces, and examinations of all cranial, thoracic, and abdominal organs. Samples of tissues and organs were removed preserved in neural phosphate-buffered 10% formaldehyde solution. The testis and epididymis were fixed in Bouin's solution. The liver, kidney, and thymus in females and testis and epididymis in males were weighed. Relative organ weights (g/100 g body weight) were calculated on the basis of the terminal body weight of rats. In females, the numbers of corpora lutea and implantation sites were recorded. Histopathologic evaluations were performed on the tissues specified below after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin: brain, heart, thymus, liver, kidney, spleen, adrenal, and cecum in both sexes, testis in males, and ovary and mammary gland in females in the control and 400 mg/kg groups, and kidney and thymus in both sexes, liver, spleen, and adrenal gland in females at 6, 25, and 100 mg/kg, and brain, heart, thymus, liver, kidney, spleen, adrenal, cecum, pituitary and testis, epididymis, seminal vesicle and prostate/ovary, mammary gland, uterus and vagina in infertile male and female rats and in females with total litter loss, respectively in all groups.

Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of the sperm in the vaginal smear and/or a vaginal plug was considered evidence for

successful mating. The day of successful mating was designated as day 0 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal day (PND) 4. The day on which parturition was completed was designated as PND 0. Litter size and numbers of live and dead pups were recorded, and live pups were sexed and individually weighed on PNDs 0 and 4. Dead pups were examined grossly. On PND 4, the pups were euthanized by exsanguinations under ether anesthesia, and gross internal examinations were performed. Live and dead pups were examined for external and internal anomalies.

Data Analysis

The statistical analysis of pups was carried out using the litter as the experimental unit. The body weight, food consumption, hematology and blood chemistry data, organ weight, numbers of corpora lutea, implantations, and pups were analyzed with Bartlett's test for homogeneity of distribution. When homogeneity was recognized, the groups were compared by one-way analysis of variance. If a significant difference was found, Dunnett's test or Scheffe's test was conducted for comparison between the control and each dosage group. The data without homogeneity or nonparametric data were analyzed using the Kruskal–Wallis rank sum test. If significant differences were found, mean rank test of Dunnett's type or Scheffe's type was conducted for comparison between the control and each dosage group. The mortality of parent animals, incidences of toxicologic signs and histopathologic changes, mating index, fecundity index, gestation index, incidences of females with live born and with total litter loss, the sex ratio of pups, and the incidence of pups with anomalies were analyzed with the chi-square test. The 5% level of probability was used as the criterion for significance.

RESULTS

Two males showed salivation at 400 mg/kg bw per day. Three females died on the expected day of parturition or on the following day. A significantly increased incidence of females showing decreased locomotor activity, soil of the lower abdominal fur, and reddish tears were observed at 400 mg/kg per day. Emaciation and hair loss in one female each, reddish tears in two females, and decreased locomotor activity in three females appeared on days 39–52 of the administration period at 400 mg/kg bw per day.

The body weights on days 7, 14, 21, 28, 35, 42, and 43 of the administration period and food consumption on the first day of administration was significantly lowered at 400 mg/kg bw per day in male rats. In female rats, the body weight on day 20 of pregnancy and food consumption on the first day of administration and day 20 of pregnancy were significantly reduced at 400 mg/kg bw per day.

Urinalysis revealed a significant increase in urinary ketones in males at 400 mg/kg bw per day. Other values in urinalysis were not significantly changed by the administration of DCBS in males.

Although the platelet count was significantly decreased at 6 mg/kg bw per day, any other hematologic parameters were not changed by the administration of DCBS. In blood chemistry, significantly lower levels of glutamate pyruvate tramsaminase (GPT) and chloride at 400 mg/kg bw per day and sodium at 6 and 400 mg/kg bw per day, and higher levels of total cholesterol at 25 and 100 mg/kg bw per day and phosphorus at 400 mg/kg bw per day were noted.

The absolute and relative weights of the organs in male and female rats given DCBS are shown in Table 1. In males, a significant increase in the absolute weight of the kidneys at 25, 100, and 400 mg/kg bw per day and in the relative weight of the kidneys and testis at 400 mg/kg bw per day and a decrease in the absolute weight of the thymus at 400 mg/kg bw per day were observed. In females, a significantly higher relative weight of the liver and decreased absolute weight of the thymus was found at 400 mg/kg bw per day.

Table 1: Absolute and relative organ weights of male and female rats given DCBS by gavage.

Dose (mg/kg bw/day)	0 (control)	6	25	100	400
No. of males	10	10	10	10	10
Terminal body weight (g)[a]	467 ± 30	469 ± 33	478 ± 17	476 ± 27	411 ± 18*
Liver (g)[a]	13.63 ± 1.27[b]	14.00 ± 1.96	15.09 ± 0.92	15.01 ± 0.92	12.99 ± 1.19
	2.92 ± 0.11[c]	2.97 ± 0.22	3.16 ± 0.14	3.14 ± 0.23	3.16 ± 0.20
Kidney (g)[a]	3.06 ± 0.27	2.98 ± 0.30	3.09 ± 0.19*	3.14 ± 0.15*	3.19 ± 0.19*
	0.66 ± 0.06	0.64 ± 0.04	0.65 ± 0.04	0.66 ± 0.04	0.78 ± 0.04*
Thymus (g)[a]	0.43 ± 0.07	0.37 ± 0.08	0.36 ± 0.08	0.40 ± 0.10	0.31 ± 0.09*
	0.09 ± 0.01	0.08 ± 0.02	0.08 ± 0.02	0.08 ± 0.02	0.07 ± 0.02
Testis (g)[a]	3.30 ± 0.24	3.25 ± 0.21	3.27 ± 0.23	3.25 ± 0.20	3.25 ± 0.15
	0.71 ± 0.07	0.70 ± 0.06	0.69 ± 0.06	0.68 ± 0.05	0.80 ± 0.05*
Epididymis (g)[a]	1.42 ± 0.09	1.36 ± 0.16	1.35 ± 0.09	1.35 ± 0.13	1.28 ± 0.11
	0.30 ± 0.03	0.29 ± 0.03	0.28 ± 0.03	0.28 ± 0.04	0.31 ± 0.03
No. of females	10	10	10	9	5
Terminal body weight (g)[a]	323 ± 26	313 ± 23	320 ± 23	308 ± 26	263 ± 59*
Liver (g)[a]	13.16 ± 1.18[b]	13.72 ± 1.26	12.88 ± 0.95	13.14 ± 1.51	12.48 ± 3.38
	4.08 ± 0.32[c]	4.38 ± 0.30	4.03 ± 0.24	4.26 ± 0.36	4.72 ± 0.35*
Kidney (g)[a]	1.98 ± 0.14	1.89 ± 0.16	1.94 ± 0.20	2.01 ± 0.19	1.96 ± 0.22
	0.62 ± 0.06	0.60 ± 0.03	0.61 ± 0.05	0.66 ± 0.11	0.77 ± 0.17
Thymus (g)[a]	0.22 ± 0.07	0.21 ± 0.07	0.21 ± 0.08	0.18 ± 0.05	0.10 ± 0.04*
	0.07 ± 0.02	0.07 ± 0.02	0.06 ± 0.02	0.06 ± 0.01	0.04 ± 0.01

[a]Values are expressed as Mean ± SD.
[b]Absolute organ weight.
[c]Relative organ weight = organ weight (g)/100 g body weight.
*Significantly different from the control, $p < 0.05$.

The histopathologic findings in male and female rats given DCBS are presented in Table 2. In males, significantly higher incidences of rats with hyalin droplets in the proximal tubular epithelium in the kidney at 100 and 400 mg/kg bw per day and with atrophy of the thymus at 400 mg/kg bw per day were noted. No administration-related changes were observed in the testis at 400 mg/kg bw per day, and in the pituitary, testis, epididymis, seminal vesicle, and prostate of infertile males. In females, the incidences of rats with fatty degeneration of the proximal tubular epithelium in the kidney, cortical cell vacuolization in the adrenal, and atrophy of the spleen were significantly higher at 400 mg/kg bw per day. No administration-related changes were noted in the ovary and mammary gland at 400 mg/kg bw per day and pituitary ovary, mammary gland, uterus, and vagina in infertile females and females showing total litter loss.

The reproductive findings in rats given DCBS are presented in Table 3. All pairs were copulated and all females were impregnated in all groups. No effects of DCBS were observed on the mating index and gestation length. A lower, but not significantly lower, fecundity index was noted at 100 and 400 mg/kg bw per day. The gestation index at 400 mg/kg bw per day was significantly lower than that in the control group. Poor maternal

Table 2: Histopathological findings in male and female rats given DCBS by gavage.

Dose (mg/kg bw/day)	0 (control)	6	25	100	400
No. of males	10	10	10	10	10
Kidney					
Eosinophilic bodies in proximal tubule	3	2	2	2	1
Focal tubular basophilic change	3	1	3	3	2
Distal tubular dilatation	1	0	0	0	0
Focal tubular dilatation with or without hyaline casts	2	1	1	2	1
Hyalin droplets in proximal tubular epithelium	0	0	0	4*	8*
Thymus: Atrophy	0	1	1	0	4*
No. of females	10	10	10	10	10
Liver					
Congestion	0	0	0	0	3
Hepatocellular fatty acid change	0	0	0	1	1
Kidney					
Congestion	0	0	0	0	1
Focal tubular basophilic change	3	0	1	2	1
Focal tubular dilatation with hyaline casts	1	0	1	0	0
Fatty degeneration of proximal tubular epithelium	0	0	0	3	4*
Adrenal					
Congestion	0	0	0	0	1
Cortical cell vacuolization	0	0	0	1	9*
Thymus: Atrophy	2	2	3	3	7
Spleen: Atrophy	0	0	0	1	5*

*Significantly different from the control, $p < 0.05$.

Table 3: Reproductive findings in rats given DCBS by gavage.

Dose (mg/kg bw/day)	0 (control)	6	25	100	400
No. of pairs cohabitated	10	10	10	10	10
No. of pairs with confirmed mating	10	10	10	10	10
Mating index (%)[a]					
Male	100	100	100	100	100
Female	100	100	100	100	100
No. of pregnant females	10	10	10	9	8
No. of non-pregnant females	0	0	0	1	2
Fecundity index (%)[b]	100	100	100	90	80
No. of dead pregnant females	0	0	0	0	3
No. of females with live born	10	10	10	9	3*
Gestation index (%)[c]	100	100	100	90	38*
Gestation length (day)	21.2 ± 0.4	21.4 ± 0.5	21.5 ± 0.5	21.2 ± 0.4	21.0 ± 1.2
No. of females with totally litter loss	0	0	0	0	3

[a]Mating index (%) = (no. of rats confirmed mating/no. of rats cohabitated) × 100.
[b]Fecundity index (%) = (no. of pregnant females/no. of females confirmed mating) × 100.
[c]Gestation index (%) = (no. of females with live born/no. of pregnant females) × 100.
*Significantly different from the control, $p < 0.05$.

behavior or nursing and total litter loss was found in three females at 400 mg/kg bw per day.

The developmental findings in rats given DCBS are shown in Table 4. Significant decreases in the numbers of corpora lutea, implantations and pups born, and in the live birth index were detected at 400 mg/kg bw per day. A significantly higher number of stillborn was also observed at 400 mg/kg bw per day. At this dose, no live pups were obtained on PND 4. No parameters for developmental toxicity at 6, 25, and 100 mg/kg bw per day were significantly different from the control values. No fetuses with external malformations were observed in the control group or the group given DCBS at 25 mg/kg bw per day. External examination revealed one pup with micromelia and ectrodactyly at 6 mg/kg bw per day, one pup with a short tail at 100 mg/kg bw per day, and five pups, in one litter, with cleft palate at 400 mg/kg bw per day. A few pups with internal variations, such as thymic remnants in the neck, left umbilical artery, and dilatated renal pelvis, were found in all groups, including the control group. There were no significant differences in the incidences of pups with external malformations and internal variations between the control and DCBS-treated groups.

DISCUSSION

The current study was conducted to obtain initial information about the possible general toxicity and reproductive and developmental toxicity of DCBS in