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) x 100.
[b]Fecundity index (%) = (no. of pregnant females/no. of females confirmed mating) x 100.
[c]Gestation index (%) = (no. of females with live born/no. of pregnant females) x 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

Table 4: Developmental findings in rats given DCBS by gavage.

Dose (mg/kg bw/day)	0 (control)	6	25	100	400
No. of litters	10	10	10	9	3
No. of corpora lutea[a]	19.7 ± 2.0	18.0 ± 2.3	18.5 ± 2.4	17.6 ± 1.3	16.3 ± 1.2*
No. of implantations[a]	18.8 ± 1.4	16.9 ± 2.3	17.4 ± 1.8	17.4 ± 1.4	15.3 ± 1.7*
Implantation index (%)[b]	95.9	94.0	94.8	99.3	93.7
No. of pups born[a]	17.0 ± 2.9	15.8 ± 1.9	15.5 ± 1.7	16.1 ± 1.9	12.0 ± 3.1*
No. of pups born alive[a]	16.8 ± 2.9	15.4 ± 1.6	14.5 ± 2.0	15.4 ± 1.9	4.0 ± 3.8*
No. of still born[a]	0.2 ± 0.4	0.4 ± 0.7	1.0 ± 2.0	0.7 ± 0.7	8.0 ± 4.0*
Delivery index (%)[c]	100	100	100	90	80
Live birth index (%)[d]	98.8	97.7	94.0	95.9	31.4*
Viability index on postnatal day 4 (%)[e]	97.1	99.3	93.6	88.9	0*
Sex ratio of pups born (males/females)	80/90	69/89	77/78	66/79	30/30
Body weight of male pups during lactation (g)[a]					
Day 0	6.4 ± 0.5	6.5 ± 0.5	6.7 ± 0.5	6.5 ± 0.2	5.6 ± 0.4
Day 4	10.2 ± 1.5	10.4 ± 1.2	10.9 ± 1.1	10.3 ± 0.6	
Body weight of female pups during lactation (g)[a]					
Day 0	6.0 ± 0.5	6.2 ± 0.4	6.4 ± 0.6	6.2 ± 0.2	5.3 ± 0.3
Day 4	9.5 ± 1.3	10.0 ± 1.0	10.6 ± 1.2	10.0 ± 0.8	
External examination of pups[f]					
Total no. of pups (litters) examined	170 (10)	158 (10)	154 (10)[h]	145 (9)	60 (5)
Total no. of pups (litters) with malformations	0	1	0	1	5 (1)
Cleft palate	0	0	0	0	5 (1)
Micromelia and ectrodactyly	0	1	0	0	0
Short tail	0	0	0	1	0
Internal examination of pups[g]					
Total no. of pups (litters) examined	169 (10)[h]	157 (10)[h]	151 (10)[i]	138 (9)[j]	57 (5)[k]
Total no. of pups (litters) with variations	1	6 (4)	2 (1)	4 (2)	1
Thymic remnants in neck	1	3 (3)	2 (1)	1	0
Left umbilical artery	0	3 (2)	0	1	0
Dilatated renal pelvis	0	0	9	2 (1)	1

[a]Values are expressed as Mean ± SD.
[b]Implantation index (%) = (no. of implantations /no. of corpora lutea) x 100.
[c]Delivery index (%) = (no. of pups born/no. of implantations) x 100.
[d]Live birth index (%) = (no. of pups born alive/no. of pups born) x 100.
[e]Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of live pups on postnatal day 0) x 100.
[f]External examinations were performed in all pups born (live born and stillborn) on postnatal day 0.
[g]Internal examinations were performed in all pups (dead pups just after death and live pups on postnatal day 4).
[h]One pup was not examined because of cannibalism.
[i]Four pups were not examined because of cannibalism.
[j]Seven pups were not examined because of cannibalism.
[k]Three pups were not examined because of cannibalism.
*Significantly different from the control, $p < 0.05$.

male and female rats. The data show that DCBS exerts general toxicity and reproductive and developmental toxicity at relatively high doses.

DCBS was given to males during the premating and mating periods and to females during the premating, mating, and pregnancy periods and shortly after parturition. The dosage used in the current study was sufficiently high that it should be expected to induce general toxic effects. As expected, general toxicity, such as death, lowered body weight and food consumption, and toxicologic signs, was observed at 400 mg/kg bw per day. In males, lowered body weight during the whole period of administration and salivation was noted only in two of the 10 males at 400 mg/kg bw per day. In females, however, the lowered body weight was found only on day 20 of pregnancy, and deaths and significantly increased incidences of toxicologic signs, including decreased locomotor activity, soil of the lower abdominal fur, and reddish tears, were noted at 400 mg/kg bw per day. Some toxicologic signs during mid to late pregnancy and deaths during the periparturition period were noted in females. These findings may suggest that female rats have a higher susceptibility to the toxicity of DCBS than male rats. One possible explanation for the higher susceptibility to DCBS toxicity in females may be enhancement of the toxicity of DCBS by the stress of the pregnancy/parturition status in female rats. More precisely, DCBS may be more toxic in females during pregnancy and/or lactation.

Reduced platelet counts at 6 mg/kg bw per day, increased serum levels of total cholesterol at 25 and 100 mg/kg bw per day, and decreased serum levels of sodium at 6 and 400 mg/kg bw per day were found. However, changes in these parameters were thought to have no toxicologic meaning because these changes were relatively slight and were not dose-dependent. Several organ weights were affected by the administration of DCBS. Higher relative weight, but not absolute weight, of the testis and liver in females were observed at 400 mg/kg bw per day. Body weights of male and female rats on the day of scheduled sacrifice were lowered at 400 mg/kg bw per day. The higher relative weights of the testis and liver at the highest dose seem to be due to secondarily lowered body weight, but not due to the direct effects of DCBS on the organs. A decreased weight of the thymus was detected in both sexes at 400 mg/kg bw per day, and these changes were accompanied by atrophy revealed by histopathologic examinations. Atrophy of the spleen was also noted in females at the highest dose. These findings may suggest that one of the target systems of DCBS toxicity is the immune system. The increases in the absolute weights of the kidney at 6, 25, and 100 mg/kg bw per day in male rats are unlikely to be due to the toxic effects of DCBS, because the degree of changes in absolute weight was relatively small and no changes were noted in relative weight. Histopathologic examinations revealed the hyalin droplets in the proximal tubular epithelium in the kidney at 100 and 400 mg/kg bw per day in males. These histopathologic changes are thought to be due to the induction of α2u-globulin accumulation (Hamamura et al., 2006). In the current study,

this type of nephropathy was not detected in female rats. The nephropathy induced by α2u-globulin accumulation is male rat–specific and is unlikely to occur in humans (Hard et al., 1993). Consideration of these findings together suggests that the histopathologic changes in the male kidney are not relevant to human health although 100 mg/kg bw per day was an effect level in male rats.

Male reproductive parameters, including the histopathology of the reproductive organs, were not affected by the administration of DCBS even at the highest dose. These suggest that DCBS is not toxic to male reproduction in rats. In female rats, no adverse effects on the maternal reproductive parameters, including the mating index, fecundity index, and histopathology of the reproductive organs, were found. However, deaths and toxicologic signs during late pregnancy, a decrease in the gestation index, and total litter loss were noted in females at 400 mg/kg bw per day. These indicate that DCBS possesses toxic effects on female reproduction at 400 mg/kg bw per day.

As for the developmental parameters, decreases in the numbers of corpora lutea, implantations, total pups and live pups delivered, live birth index and viability on PND 4, and an increase in the number of stillborn were detected at 400 mg/kg bw per day. These findings indicate that DCBS is toxic to the survival and growth of offspring and exerts developmental toxicity at 400 mg/kg bw per day in rats.

In the current study, external malformations and variations of the internal organs were found in pups in the DCBS-treated groups. However, incidences of pups with malformations and variations were very low and not significantly different from those in the control group. No consistent tendency was found in the incidence of pups with these morphologic alterations. Furthermore, the external malformations and variations of the internal organs observed in the current study are of the types that occur spontaneously among control rat fetuses reported in the literature (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). Therefore, it seems unlikely that the morphologic changes in pups observed in the current study indicate a teratogenic response and that DCBS possessed teratogenic potential in rats. In the current study, external and internal examinations in the newborn rats were performed, but no skeletal examinations were carried out. To accurately evaluate the prenatal developmental toxicity, including teratogenicity, it is necessary to interrupt pregnancy 12–24 h before the expected term either by hysterectomy or the necropsy of maternal animals (Wilson, 1965, 1973).

The most deleterious effect of DCBS on reproduction and development is the marked decrease in the number of live pups. The most striking adverse effect noted in the current study is a total loss of pups until PND 4 at 400 mg/kg bw per day. The primary effects may be on the gestation index for dams and live birth index for pups, which appear to be affected at multiple points along the female reproductive process, as well as an viability of neonatal pups. The

current study was performed in compliance with the OECD guideline Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test, and this screening test guideline does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the end points. In order to further evaluate the reproductive and developmental toxicity of DCSB in rats, a two-generation reproductive toxicity study is currently in progress.

In conclusion, DCBS caused deaths in females, and decreased body weight and changes in urinalysis, blood chemistry, and/or histopathology in both sexes at 400 mg/kg bw per day. Adverse effects on reproductive and developmental parameters were noted at 400 mg/kg bw per day. At this dose, all dams lost their litters at delivery or by day 4 of lactation. The NOAEL for repeat dose toxicity is considered to be 100 mg kg^{-1} day^{-1} in male and female rats, and the NOAEL for reproductive/developmental toxicity is considered to be 100 mg kg^{-1} day^{-1}.

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ORIGINAL ARTICLE

Evaluation of reproductive and developmental toxicity of the rubber accelerator N,N-dicyclohexyl-2-benzothiazolesulfenamide in rats

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ABSTRACT **Male and female Crl:CD(SD) rats were fed a diet containing the rubber accelerator N,N-dicyclohexyl-2-benzothiazolesulfenamide (DCBS) at 0, 1500, 3000, 6000 or 10 000 p.p.m. (0, 83, 172, 343 or 551 mg/kg bw/day in males and 0, 126, 264, 476 or 707 mg/kg bw/day in females) for a total of 57 days beginning 16 days before mating in males, and a total of 61–65 days from 16 days before mating to day 21 of lactation in females. Body weight gains and food consumption were reduced in males at 6000 p.p.m. and higher and in females at 3000 p.p.m. and higher. The weights of the spleen at 6000 and 10 000 p.p.m. and of the thymus at 10 000 p.p.m. were decreased in females. No changes in estrous cyclicity, copulation index, fertility index, gestation index, delivery index, precoital interval or gestation length were observed at any dose of DCBS. Numbers of implantations at 6000 and 10 000 p.p.m. and pups delivered at 10 000 p.p.m. were reduced. There were no changes in the sex ratio or viability of pups. The body weights of male and female pups were lowered at 6000 p.p.m. and higher. Decreased weight of the spleen in weanlings was also observed in males at 1500 p.p.m. and higher and in females at 3000 p.p.m. and higher. The data indicate that DCBS possesses adverse effects on reproduction and development in rats.**

Key Words: developmental toxicity, N, N-dicyclohexyl-2-benzothiazolesulfenamide, rat, reproductive toxicity, rubber accelerator

INTRODUCTION

Sulfenamide accelerator compounds are widely used in the manufacture of automotive compartments and industrial rubber products such as tires, hoses, conveyer belts, bushings seals, gaskets and windshield wiper blades (EPA 2001). N,N-Dicyclohexyl-2-benzothiazolesulfenamide (DCBS, Fig. 1) is a sulfenamide accelerator. The annual production level of DCBS in Japan was approximately 1000 tons in 1990–1993 and 1900 tons in 2000–2003. Most of this amount was sold and handled domestically (OECD 2007). DCBS is used as an accelerator of vulcanization and is completely reacted in the vulcanizing process (OECD 2007). DCBS is regulated in Germany for use in articles that contact food, but is not regulated by the United States Food and Drug Administration for use in food contact applications (Flexsys 2000). 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 lesser degree during weigh-up activities at the consumer site, there is a possibility of skin and inhalation exposure. Although consumer exposure should be minimal, the most likely route of consumer exposure is skin contact with rubber or latex articles (EPA 2001).

Only up to 6% biodegradation has been determined for DCBS in a ready biodegradability test, and a measured log Kow value of 4.8 suggests that DCBS may have a high bioaccumulation potential (OECD 2007). The possibility of such a chemical compound entering biological systems has aroused great concern regarding its toxicological potential. Generally, biological effects of chemicals should be studied in laboratory animals to investigate their possible influences on human health, and the results of animal tests of chemical toxicity relevant to humans (Clayson & Krewski 1990). However, very little information on the toxicity of DCBS has been published. The toxic effects of DCBS have been briefly summarized by the European Chemical Bureau (2000) and US EPA (2001). It was reported that the oral LD50 values were 1077–10 000 mg/kg bw in rats, the oral NOAEL for 44-day repeated dose toxicity was higher than 100 mg/kg bw/day in rats, and no effects on reproduction were observed at doses up to 400 mg/kg bw/day in rats (EPA 2001). The oral LD50 value was 8500 mg/kg bw in male mice, and repeated daily inhalation exposure of male rats for 15 days at 2 h/day and 350–400 mg/m^3 caused mucous membrane irritation (Vorobera 1969).

The Japanese Government (MHW 1998) conducted toxicity studies for DCBS, including acute toxicity, *in vitro* genotoxicity and repeat dose toxicity combined with reproductive/developmental toxicity as a part of the Safety Examination of Existing Chemical Substances and Chemical Safety Programmes. These toxicity studies are summarized in the IUCLID Data Sets (EPA 2006), OECD Screening Information Data Sets (OECD 2007) and the Hazard Assessment Sheet (CERI 2002). We previously reported the results of a screening test for repeat dose toxicity combined with a reproductive/developmental toxicity in rats, where DCBS at 400 mg/kg bw/day had a deleterious effect on reproduction and development and caused a marked decrease in the number of live pups as well as a total loss of pups by postnatal day (PND) 4 (Ema *et al.* 2007). The primary effects may be on the gestation index for dams and live birth index for pups, both of which appear to be affected at multiple points along the female reproductive process. The viability of neonatal pups may also be affected. To examine the adverse effect of dietary DCBS on survival and growth of pups, a reproductive and developmental toxicity study was performed in rats given DCBS during an extended administration period up to the weaning of pups.

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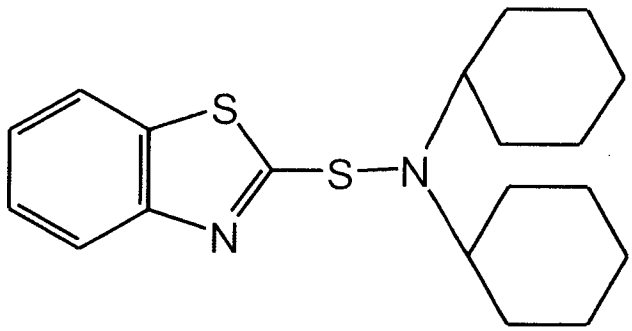

Fig. 1 Structural formula of N,N-dicyclohexyl-2-benzothiazolesulfenamide.

MATERIALS AND METHODS

This study was performed in 2005–2006 at the Safety Research Institute for Chemical Compounds (Sapporo, Japan) in compliance with *Law for the Humane Treatment and Management of Animals* (Law no. 105, October 1, 1973, revised December 22, 1999, Revised Law no. 221; revised June 22, 2005, Revised Law no. 68), *Standards Relating to the Care, Management and Refinement of Laboratory Animals* (Notification no. 88 of the Ministry of the Environment, Japan, April 28, 2006) and *Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in the Testing Facility under the Jurisdiction of the Ministry of Health, Labour and Welfare* (Notification no. 0601005 of the Health Sciences Division, Ministry of Health, Labour and Welfare, Japan, June 1, 2006).

Chemical and dosing

DCBS (CAS no. 4979-32-2) was obtained from Ouchishinko Chemical Industrial (Tokyo, Japan). DCBS in the form of off-white to tan granules is very slightly soluble in water and methanol but soluble in oil. Its melting point is 100–105°C, density is 1230 kg/m^3 and molecular weight is 347 (Flexsys 2000). DCBS (Lot no. 508001) used in this study was 99.7% pure and was kept in a sealed container under cool (1–8°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 given dietary DCBS at a concentration of 0 (control), 1500, 3000, 6000 or 10 000 p.p.m. Males were fed a diet containing DCBS for a total of 57 days beginning 16 days before mating. Females were fed a diet containing DCBS for a total of 61–65 days from 16 days before mating to day 21 of lactation throughout the mating, gestation and lactation periods. Control rats were fed a basal diet only.

The dosage levels were determined based on the results of a previous study in rats that were given DCBS by gavage at 0, 6, 25, 100, or 400 mg/kg bw/day for a total of 44 days from 14 days before mating in males and a total of 40–51 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation periods in females (Ema *et al.* 2007). In that study, toxicologically significant changes were observed only at 400 mg/kg bw/day. Three of 10 females died during parturition. An increased incidence of females showing decreased locomotor activity, soil of the lower abdominal fur and reddish tears was observed. Decreased body weights were found in males and females. Decreased weight of the thymus in both sexes was noted. Decreases in the gestation index, numbers of corpora lutea, implantations, pups born and pups born alive, live birth index and viability index were detected.

Dosed diet preparations were formulated by mixing DCBS into an appropriate amount of a powdered basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) for each dietary concentration. Chemical analysis showed that DCBS in the diet was stable for at least 21 days at room temperature and the formulations were maintained in a room temperature for no more than 21 days. Generally, the diet was replaced once a week.

Animals and housing conditions

Sprague–Dawley (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 and female rats at nine weeks of age were purchased from the Tsukuba Breeding Center (Charles River Laboratories Japan, Yokohama, Japan). The rats were acclimated to the laboratory for six days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Rats (F0) were randomly distributed into five groups of six males and six females each, and all animals were assigned a unique number and tattooed on the ear prior to the start of the experiment. Animals were housed individually in suspended aluminum/stainless steel cages except during the acclimation, mating and nursing periods. From day 17 of pregnancy to the day of weaning, individual dams and litters were reared using wood chips as bedding (White Flake; Charles River Laboratories Japan.).

Animals were reared on a basal diet or a diet containing DCBS and filtered tap water *ad libitum* and maintained in an air-conditioned room at 22 ± 3°C with a humidity of 50 ± 20% and a 12-h light (8:00–20:00)/dark (20:00–8:00) cycle. The room was ventilated 10–15 times/h.

Observations

All rats were observed twice a day for clinical signs of toxicity. The body weight was recorded once a week for males and once a week during the premating period, on days 0, 7, 14 and 20 of pregnancy, and on days 0, 4, 7, 14 and 21 of lactation for females. Food consumption was recorded once a week for males, and once a week during the premating period, on days 0, 7, 14 and 20 of pregnancy and on days 0, 7, 14 and 21 of lactation for females.

Rats were euthanized by exsanguination under ether anesthesia. Males were euthanized at 17 weeks and females at 18 weeks on day 21 of lactation. The external surfaces of the rats were examined for abnormalities. The abdomen and thoracic cavities were opened and gross internal examination was performed. In females, the number of implantation sites was recorded. The brain, pituitary, thymus, thyroid, liver, kidney, spleen, adrenal gland, testis, epididymis, seminal vesicle, ventral prostate, ovary and uterus were weighed. The thyroid and seminal vesicle were weighed after fixation with 10% neutral buffered formalin.

Daily vaginal lavage samples from each female were evaluated for estrous cyclicity for two weeks of the premating period. Females with repeated 4–6 day estrous cycles were judged to be normal. 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 sperm in the vaginal smear and/or a vaginal plug was considered evidence of successful mating (day 0 of pregnancy). Copulated females were checked for signs of parturition three times a day on days 21–23 of pregnancy.

The females were allowed to deliver spontaneously and nurse their pups until PND 21. The day on which parturition was

completed by 13:00 was designated as PND 0. Total litter size and the numbers of live and dead pups were recorded. Live pups were counted, sexed, examined grossly and individually weighed on PND 0, 4, 7, 14 and 21. On PND 4, litters were randomly adjusted to eight pups comprised of four males and four females. No adjustment was made for litters with fewer than 8 pups. Selected pups were assigned a unique number and tattooed on a limb on PND 4. Unselected pups were necropsied on PND 4. Weanlings were necropsied on PND 21 and the brain, thymus, liver, spleen and uterus were weighed.

Statistical analysis

Statistical analysis of the offspring was carried out using the litter as the experimental unit.

Body weight, body weight gain, food consumption, length of estrous cycle, precoital interval, gestation length, number of implantations and pups delivered, delivery index, organ weight, organ/body weight ratio (relative organ weight) and the viability of pups were analyzed for statistical significance in the following way. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances. If the variances were equivalent, the groups were compared by one-way analysis of variance (ANOVA). If significant differences were found, Dunnett's multiple comparison test was performed. If the groups did not have equivalent variances, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pairwise comparisons were made by Mann–Whitney *U*-test. The incidence of females with normal estrous cycles, copulation index, fertility index, gestation index and neonatal sex ratio was analyzed by the χ^2 test or Fisher's exact test.

The 0.05 level of probability was used as the criterion for significance.

RESULTS

Clinical observations, body weight and food consumption (F0 males and females)

No deaths were found in F0 males and females. In males, there were no compound related clinical signs of toxicity at any doses. Hematuria and soil of perigenital fur were each observed at 10 000 p.p.m. in one female.

Table 1 shows body weight gain in F0 males and females during dosing. In males, body weight gain on days 0–7 of the dosing period at 6000 p.p.m. and higher was significantly lowered. In females,

Table 1 Body weight gains of F0 parental male and female rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	0 (Control)	1500	3000	6000	10 000
No. males	6	6	6	6	6
Initial body weight (g)†	367 ± 7	367 ± 6	366 ± 7	366 ± 8	366 ± 7
Body weight gain during dosing period (g)†					
Days 0–7	48.0 ± 10.4	36.8 ± 14.5	36.3 ± 4.8	26.7 ± 8.5**	25.2 ± 6.5**
Days 7–14	38.2 ± 9.2	33.7 ± 13.4	34.5 ± 8.7	35.2 ± 5.6	29.5 ± 5.5
Days 14–21	21.7 ± 9.2	27.3 ± 7.1	24.3 ± 5.2	23.0 ± 12.6	21.8 ± 3.1
Days 21–28	26.8 ± 10.2	25.7 ± 8.3	22.3 ± 10.5	23.5 ± 6.7	25.2 ± 5.0
Days 28–35	21.2 ± 7.7	20.8 ± 6.5	28.5 ± 12.6	24.0 3.7	19.2 ± 4.1
Days 35–42	14.8 ± 6.9	15.3 ± 6.5	20.3 ± 6.9	17.3 ± 6.3	20.5 ± 5.2
Days 42–49	13.8 ± 8.3	19.5 ± 4.2	13.5 ± 2.7	19.8 ± 5.5	17.2 ± 2.9
Days 49–56	14.8 ± 7.6	19.5 ± 6.3	16.7 ± 5.0	20.5 ± 6.2	17.0 ± 3.5
No. females	6	6	6	6	6
Initial body weight (g)†	238 ± 6	239 ± 7	237 ± 5	238 ± 6	237 ± 7
Body weight gain during premating period (g)†					
Days 0–7	6.5 ± 7.7	8.8 ± 8.8	6.8 ± 4.6	−6.5 ± 9.7*	−19.3 ± 9.3**
Days 7–14	15.7 ± 8.5	16.3 ± 6.9	14.2 ± 5.9	12.2 ± 8.0	13.0 ± 8.7
Body weight gain during pregnancy (g)†					
Days 0–7	45.3 ± 6.5	42.5 ± 4.2	32.8 ± 5.4*	31.2 ± 8.6*	19.5 ± 12.0**
Days 7–14	38.3 ± 6.0	35.7 ± 5.4	36.8 ± 7.0	35.2 ± 6.2	31.2 ± 8.6
Days 14–20	76.7 ± 14.6	68.3 ± 4.3	75.8 ± 12.4	68.7 ± 7.7	62.5 ± 12.2
Body weight gain during lactation (g)†					
Days 0–4	28.0 ± 15.7	10.8 ± 24.3	28.0 15.7	8.2 8.7	−2.5 ± 14.6*
Days 4–7	6.5 ± 2.7	12.0 ± 9.0	10.0 ± 10.2	5.3 ± 7.3	0.5 ± 10.9
Days 7–14	1.3 ± 10.7	10.2 ± 7.3	4.2 ± 8.1	14.2 ± 11.1	6.0 ± 12.5‡
Days 14–21	−19.0 ± 14.7	−31.7 ± 9.9	−17.0 ± 8.3	−8.8 ± 9.8	2.6 ± 14.3‡*

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

†Values are given as mean ± SD. ‡data were obtained from five females because one female was excluded (total litter loss on day 9 of lactation).

body weight gains were decreased on days 0–7 of the premating period at 6000 p.p.m. and higher, on days 0–7 of pregnancy at 3000 p.p.m. and higher, and on days 0–4 of lactation at 10 000 p.p.m. Body weight gain on days 14–21 of lactation was significantly increased at 10 000 p.p.m.

In F0 males, food consumption was significantly decreased during the first week at 6000 p.p.m. and higher and during the second week at 10 000 p.p.m. In F0 females, food consumption was significantly decreased throughout the premating, pregnancy and lactation periods at 6000 and 10 000 p.p.m., except on days 7–14 and 14–20 of pregnancy at 6000 p.p.m. A tendency towards decreased food consumption was observed on days 0–7 of pregnancy at 3000 p.p.m.

The mean daily intakes of DCBS were 83, 172, 343 and 551 mg/kg bw in F0 males, and 126, 264, 476 and 707 mg/kg bw in F0 females for 1500, 3000, 6000 and 10 000 p.p.m., respectively.

Estrous cyclicity (F0 females)

All F0 females showed normal estrous cycles in all groups, and the length of the estrous cycles was not significantly different between the control and DCBS-treated groups.

Reproductive and developmental effects (F0 parents/F1 offspring)

The reproductive and developmental parameters for F0 parents/F1 offspring are presented in Table 2. In F0 parent animals in all groups, all pairs copulated, all male and female rats were fertile and all females delivered live pups. All rats of all groups mated within four days. There were no significant differences between control and DCBS-treated groups in copulation index, fertility index, gestation index, precoital interval, gestation length, delivery index, sex ratio of F1 pups, or viability of F1 pups during lactation. Significantly lower numbers of implantations at 6000 and 10 000 p.p.m. and pups delivered at 10 000 p.p.m. were observed. Body weights of male pups were significantly lowered on PND 4, 7 and 21 at 6000 p.p.m. and on PND 7, 14 and 21 at 10 000 p.p.m. In female pups, significantly lower body weights were observed on PND 7, 14 and 21 at 6000 p.p.m. and higher. No malformed pups were detected in any groups.

Necropsy and organ weights (F0 males and females)

Atrophy of the thymus was found in two females at 10 000 p.p.m. No compound-related gross lesions of the reproductive organs were noted in F0 males and females. In males, significantly increased relative weights of the liver and kidney were observed at 10 000 p.p.m.

The organ weights of F0 females are shown in Table 3. The body weight at the scheduled terminal sacrifice was significantly lowered at 6000 and 10 000 p.p.m. The absolute weight of the ovary was significantly lowered at 10 000 p.p.m. Significantly increased relative weights were found for the pituitary at 3000 p.p.m., the liver at 6000 p.p.m., and the brain, kidney and adrenal gland at 10 000 p.p.m. The absolute and relative weights of the thymus at 10 000 p.p.m. and the spleen at 6000 p.p.m. and higher were significantly decreased.

Necropsy and organ weights (F1 weanlings)

No compound related gross lesions were observed in F1 weanlings.

The organ weights of F1 male weanlings are presented in Table 4. The body weight at the scheduled sacrifice was significantly reduced at 6000 and 10 000 p.p.m. The absolute weights of the brain at 6000 and 10 000 p.p.m. and the liver at 10 000 p.p.m. were also significantly reduced. The relative weights of the liver at 1500 and 6000 p.p.m. and of the brain at 10 000 p.p.m. were significantly increased. Significantly decreased absolute and relative weights of the spleen, except for the relative weight at 3000 p.p.m., were noted at 1500 p.p.m. and higher.

The organ weights of F1 female weanlings are presented in Table 5. The body weight at the scheduled sacrifice was significantly reduced at 6000 p.p.m. and higher. Significantly reduced absolute weights of the brain at 6000 and 10 000 p.p.m., the liver at 10 000 p.p.m., and the uterus at 3000 p.p.m. and 10 000 p.p.m. were also observed. The relative weight of the brain was significantly increased at 10 000 p.p.m. The absolute and relative weights of the spleen were significantly reduced at 3000 p.p.m. and higher.

DISCUSSION

This study was designed to assess the effects of DCBS on continuous parameters such as body weight and food consumption, as well as endpoints for reproductive and developmental toxicity.

Significant decreases in body weight gain and food consumption were observed at 6000 p.p.m. and higher in F0 males and females. In females at 3000 p.p.m., body weight gain was significantly decreased during early pregnancy. Food consumption also decreased, but not significantly. The data indicate that changes in body weight gain were associated with changes in food consumption and that DCBS adversely affects body weight gain and food consumption at 6000 p.p.m. in male rats and 3000 p.p.m. in female rats. The higher relative weights of the liver and kidney at the highest dose in F0 males seem to be due to secondary effects of lowered body weight rather than direct effects of DCBS on the organs. More pronounced effects on organ weights were noted in females. Lower absolute and relative weights of the thymus at 10 000 p.p.m. and spleen at 6000 p.p.m. and higher were detected. In our previous study, histopathological examination revealed atrophy of the thymus and spleen at 400 mg/kg bw/day (Ema *et al.* 2007). Other changes in female organ weight such as the relative weights of the brain, pituitary, liver, kidney and adrenal gland, as well as the absolute weight of the ovary are unlikely to be due to the toxic effects of DCBS because the degree of changes was relatively small, no dose-dependency was shown and no changes were noted in absolute or relative weight. These findings suggest that the immune system may be a target of DCBS toxicity, and that female rats have a higher susceptibility to the toxicity of DCBS than male rats. These findings are consistent with our previous study (Ema *et al.* 2007). The higher susceptibility to DCBS toxicity in females may be explained by the stress of pregnancy and lactation. DCBS is likely to be not reproductively toxic in male rats because DCBS caused neither pathological changes in male reproductive organs nor changes in male reproductive parameters.

In our previous study, DCBS given by gavage to rats at 400 mg/kg bw/day from 14 days before mating to day 3 of lactation caused significant decreases in the gestation index, number of corpora lutea, implantations, pups born and pups born alive, live birth index and viability index (Ema *et al.* 2007). This dose also caused severe maternal toxicity and a total loss of pups by PND 4. No maternal or reproductive/developmental toxicity was detected at 100 mg/kg bw/day in our previous study. In the present study, no serious reproductive difficulties were noted even at the highest dose of 10 000 p.p.m., and necropsy of the reproductive organs revealed no evidence of reproductive failure. Although decreased numbers of implantations and pups delivered were noted at the highest dose, the viability of pups until weaning was not significantly decreased. In the present feeding study, the mean daily intakes of DCBS at the

Table 2 Reproductive and developmental findings for F0 parents/F1 offspring of rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. pairs	6	6	6	6	6
Copulation index[b]					
Male/female (%)	100/100	100/100	100/100	100/100	100/100
Pre-coital interval (days)[a]	2.2 ± 0.8	2.3 ± 1.2	3.2 ± 0.8	3.0 ± 0.9	2.7 ± 1.2
Fertility index[c]					
Male/female (%)	100/100	100/100	100/100	100/100	100/100
Gestation index (%)[d]	100	100	100	100	100
Gestation length (days)[a]	22.2 ± 0.4	22.2 ± 0.4	22.2 ± 0.4	22.0 ± 0.0	22.2 ± 0.4
No. implantations[a]	16.0 ± 1.8	15.0 ± 0.9	16.3 ± 1.2	13.5 ± 2.0*	12.8 ± 1.2**
Delivery index (%)[a,e]	95.8 ± 8.0	96.7 ± 3.7	95.8 ± 5.3	95.6 ± 8.1	86.7 ± 21.1
No. pups delivered[a]	15.3 ± 2.2	14.5 ± 1.0	15.7 ± 1.8	13.0 ± 2.6	11.2 ± 3.1*
Sex ratio of F1 pups[f]	0.467	0.448	0.564	0.526	0.463
Viability index (%)[a]					
PND 0[g]	100 ± 0	100 ± 0	100 ± 0	100 ± 0	91.2 ± 12.9
PND 4[h]	99.1 ± 2.3	97.9 ± 3.3	95.9 ± 5.3	90.6 ± 12.2	72.1 ± 40.8
PND 21[i]	97.9 ± 5.1	97.9 ± 5.1	100.0 ± 0.0	89.6 ± 25.5	83.3 ± 40.8
Male pup body weight during lactation (g)[a]					
PND 0	6.8 ± 0.4	6.7 ± 0.7	6.3 ± 0.4	6.2 ± 0.6	6.5 ± 0.7
PND 4	10.6 ± 0.9	10.3 ± 0.8	9.6 ± 0.6	9.1 ± 0.7**	9.1 ± 2.2[j]
PND 7	18.7 ± 1.3	17.7 ± 1.3	17.6 ± 1.3	14.5 ± 2.2**	13.3 ± 3.7[j]**
PND 14	39.2 ± 3.0	36.2 ± 3.0	37.3 ± 2.9	33.0 ± 4.0	26.3 ± 7.2[j]**
PND 21	67.0 ± 4.6	61.1 ± 6.1	62.8 ± 3.2	55.7 ± 7.6*	44.1 ± 9.9[j]**
Female pup body weight during lactation (g)[a]					
PND 0	6.4 ± 0.4	6.4 ± 0.5	6.0 ± 0.3	5.8 ± 0.6	6.2 ± 0.5
PND 4	10.1 ± 1.1	9.9 ± 0.7	9.0 ± 0.6	8.7 ± 0.7	8.5 ± 1.9
PND 7	18.2 ± 2.0	17.4 ± 0.7	16.0 ± 1.2	13.8 ± 1.3**	11.7 ± 4.2*
PND 14	38.6 ± 3.5	36.1 ± 2.1	35.0 ± 2.4	31.5 ± 4.9*	25.3 ± 7.2[k]**
PND 21	65.1 ± 5.2	60.1 ± 3.7	58.2 ± 3.3	53.5 ± 9.0*	42.5 ± 9.9[k]**

*Significantly different from the control. $P < 0.05$: **significantly different from the control. $P < 0.01$.

[a]Values are given as mean ± SD; [b]copulation index (%) (number of animals with successful copulation/number of animals paired) × 100; [c]fertility index (%) (number of animals that impregnated a female or were pregnant/number of animals with successful copulation) × 100; [d]gestation index (%) (number of females that delivered live pups/number of pregnant females) × 100; [e]delivery index (%) (number of pups delivered/number of implantations) × 100; [f]sex ratio (total number of male pups/total number of pups delivered); [g]viability index on PND 0 (number of live pups on PND 0/number of pups delivered) × 100; [h]viability index on PND 4 (number of live pups on PND 4/number of live pups on PND 0) × 100; [i]viability index on PND 21 (number of live pups on PND 21/number of live pups selected on PND 4) × 100; [j]data were obtained from five litters because one female experienced total male litter loss by day 1 of lactation; and [k]data were obtained from five litters because one female experienced total litter loss by day 9 of lactation.

PND, post natal day.

highest dose were 551 and 707 mg/kg bw in F0 males and females, respectively. One possible explanation for the discrepancy in the degree of reproductive and developmental toxicity between the present and previous studies may be the difference in administration method. Some studies have shown that gavage and feed administration result in different toxicokinetics for various chemicals (Yuan *et al.* 1994, 1995). Further studies are needed to clarify the difference in DCBS toxicokinetics between gavage and feed administrations.

Regarding the development of offspring, decreases in the numbers of implantations and pups delivered and lowered body weights of male and female pups were noted at 6000 p.p.m. and higher. These findings indicate that the dose level of 6000 p.p.m. used in this study was potent enough to adversely affect the survival and growth of pups. Reduced weight of the spleen was also observed in male and female weanlings. These findings also suggest that the immune system may be a target of DCBS toxicity. Other changes in the weights of organs, such as the brain and liver in male weanlings and the brain, liver and uterus in female weanlings are unlikely to be due to the toxic effects of DCBS because the degree of changes was relatively small, no dose dependency was shown, no changes were noted in the absolute or relative weight, and also

Table 3 Absolute and relative organ weights of F0 female rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. females	6	6	6	6	5
Body weight (g)†	331 ± 18	316 ± 16	320 ± 11	306 ± 14*	274 ± 20**
Brain (g)†	2.10 ± 0.05‡	2.11 ± 0.08	2.10 ± 0.05	2.06 ± 0.10	2.06 ± 0.03
	0.63 ± 0.03§	0.67 ± 0.04	0.66 ± 0.02	0.67 ± 0.04	0.76 ± 0.05**
Pituitary (mg)†	13.3 ± 1.6‡	13.4 ± 2.4	15.4 ± 0.9	13.9 ± 1.9	12.9 ± 2.6
	4.03 ± 0.44§	4.24 ± 0.65	4.81 ± 0.32*	4.53 ± 0.46	4.70 ± 0.66
Thyroid (mg)†	18.3 ± 3.6‡	17.6 ± 3.5	17.7 ± 4.3	18.8 ± 2.7	17.5 ± 3.6
	5.52 ± 0.87§	5.55 ± 0.99	5.51 ± 1.18	6.15 ± 0.94	6.39 ± 1.02
Thymus (mg)†	255 ± 47‡	205 ± 63	237 ± 45	186 ± 89	116 ± 60**
	77.1 ± 14.4§	65.0 ± 19.6	74.2 ± 13.1	60.1 ± 26.5	41.7 ± 19.9*
Liver (g)†	13.03 ± 0.83‡	12.51 ± 0.71	13.42 ± 1.18	13.69 ± 0.68	12.18 ± 1.60
	3.94 ± 0.21§	3.97 ± 0.23	4.20 ± 0.27	4.48 ± 0.09**	4.46 ± 0.59
Kidney (g)†	2.34 ± 0.16‡	2.38 ± 0.13	2.35 ± 0.10	2.20 ± 0.12	2.51 ± 0.41
	0.71 ± 0.04§	0.75 ± 0.05	0.74 ± 0.04	0.72 ± 0.03	0.92 ± 0.18**
Spleen (mg)†	682 ± 74‡	589 ± 68	600 ± 89	493 ± 24**	459 ± 46**
	206 ± 20§	187 ± 19	188 ± 31	161 ± 5**	168 ± 15**
Adrenal (mg)†	75.5 ± 11.0‡	81.8 ± 12.9	77.0 ± 8.8	72.0 ± 8.8	88.2 ± 8.3
	22.9 3.2§	26.0 ± 3.9	24.1 ± 2.7	23.5 ± 2.8	32.4 ± 3.8**
Ovary (mg)†	109 ± 18‡	113 ± 17	101 ± 5	101 ± 10	75 ± 23**
	32.9 ± 3.8§	36.1 ± 6.8	31.6 ± 2.4	32.9 ± 3.9	27.1 ± 6.4
Uterus (mg)†	513 ± 68‡	465 ± 73	489 ± 101	414 ± 71	369 ± 183
	156 ± 24§	148 ± 26	153 ± 32	135 ± 22	132 ± 56

*Significantly different from the control, $P < 0.05$; **significantly different from the control, $P < 0.01$.
†Values are given as the mean ± S.D; ‡absolute organ weight; §relative organ weight (organ weight [g or mg]/100 g body weight).

Table 4 Absolute and relative organ weights for F1 male weanlings of rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. males	6	6	6	6	5
Body weight (g)†	67.1 ± 6.7	62.5 ± 4.5	63.8 ± 4.2	55.3 ± 8.9*	43.8 ± 10.6**
Brain (g)†	1.70 ± 0.05‡	1.63 ± 0.12	1.59 ± 0.04	1.51 ± 0.05**	1.45 ± 0.11**
	2.55 ± 0.21§	2.61 ± 0.24	2.50 ± 0.15	2.78 ± 0.42	3.44 ± 0.74*
Thymus (mg)†	257 ± 44‡	219 ± 33	265 ± 45	246 ± 36	190 ± 65
	382 ± 50§	351 ± 57	415 ± 59	449 ± 60	424 ± 50
Liver (g)†	2.56 ± 0.35‡	2.65 ± 0.29	2.69 ± 0.38	2.37 ± 0.38	1.72 ± 0.49**
	3.80 ± 0.17§	4.22 ± 0.20*	4.20 ± 0.37	4.30 ± 0.33*	3.90 ± 0.22
Spleen (mg)†	372 ± 63‡	276 ± 53**	296 ± 32*	250 ± 45**	148 ± 36**
	556 ± 84§	442 ± 80*	466 ± 56	452 ± 32*	337 ± 31**

*Significantly different from the control, $P < 0.05$; **significantly different from the control, $P < 0.01$.
†Values are given as mean ± S.D; ‡absolute organ weight; §relative organ weight (organ weight [g or mg]/100 g body weight).

because the changes seem to be secondary effects of the lowered body weight. In the present study, external and internal morphological examinations of offspring were performed, but no skeletal examinations were conducted. To accurately evaluate prenatal developmental toxicity including teratogenicity, it is necessary to interrupt pregnancy 12–24 h before the expected term either by hysterectomy or the necropsy of maternal animals (Wilson 1965).

The adverse effects of DCBS on reproduction and development noted in the present feeding study are almost consistent with the findings of our previous gavage study (Ema *et al.* 2007), which showed decreased numbers of implantations and pups delivered and decreased body weight of the pups at higher doses. These endpoints appear to be affected at multiple points of the female reproductive and developmental process. The decreased number of implantations

Table 5 Absolute and relative organ weights for F1 female weanlings of rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. females	6	6	6	6	5
Body weight (g)†	65.7 ± 7.2	61.1 ± 3.4	59.9 ± 4.6	54.0 ± 9.6*	42.8 ± 9.6**
Brain (g)†	1.60 ± 0.09‡	1.56 ± 0.07	1.53 ± 0.03	1.50 ± 0.05*	1.37 ± 0.08**
	2.46 ± 0.25§	2.56 ± 0.16	2.57 ± 0.18	2.84 ± 0.39	3.34 ± 0.78**
Thymus (mg)†	272 ± 46‡	253 ± 33	252 ± 27	243 ± 51	216 ± 82
	415 ± 56§	415 ± 57	422 ± 37	456 ± 101	491 ± 92
Liver (g)†	2.58 ± 0.31‡	2.47 ± 0.27	2.42 ± 0.42	2.27 ± 0.43	1.71 ± 0.49**
	3.93 ± 0.14§	4.03 ± 0.22	4.02 ± 0.41	4.19 ± 0.13	3.96 ± 0.29
Spleen (mg)†	360 ± 57‡	296 ± 16	267 ± 60*	247 ± 50**	163 ± 59**
	548 ± 66§	484 ± 9	442 ± 72*	456 ± 37*	371 ± 58**
Uterus (mg)†	44.7 ± 6.6‡	41.3 ± 6.1	35.7 ± 2.1*	42.0 ± 6.9	32.4 ± 4.8**
	68.9 ± 14.0 Temp.§	67.7 ± 9.8	60.0 ± 7.4	78.5 ± 10.8	77.3 ± 10.3

*Significantly different from the control, $P < 0.05$; ** significantly different from the control, $P < 0.01$.
†Values are given as the mean ± S.D; ‡absolute organ weight; §relative organ weight (organ weight [g or mg]/100 g body weight).

was the most striking effect in the present study. In our previous study, a decreased number of corpora lutea was noted in female rats given DCBS (Ema *et al.* 2007). Therefore, it is likely that the decreased number of implantations can be attributed to the decreased number of corpora lutea. The present study does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the endpoints. To further evaluate the reproductive and developmental toxicity of DCBS in rats, a two-generation reproductive toxicity study should be performed.

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Combined Repeated Dose and Reproductive/ Developmental Toxicity Screening Test of the Nitrophenolic Herbicide Dinoseb, 2-*sec*-Butyl-4,6-Dinitrophenol, in Rats

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ABSTRACT: In a combined repeated dose toxicity study with reproduction/developmental toxicity screening test, Crj:CD(SD)IGS rats were dosed with dinoseb, 2-*sec*-butyl-4,6-dinitrophenol, by gavage at 0 (vehicle), 0.78, 2.33, or 7.0 mg/kg bw/day. Six males per group were dosed for a total of 42 days beginning 14 days before mating. Twelve females per group were dosed for a total of 44–48 days beginning 14 days before mating to day 6 of lactation throughout the mating and gestation period. Recovery groups of six males per group and nonpregnant six females per group were dosed for 42 days followed by a 14-day recovery period. No deaths were observed in males of any dose group or in females of the recovery groups. At 7.0 mg/kg bw/day, eight females died and two animals were moribund during late pregnancy, and a significant decrease in body weight gain was found in both sexes. Hematocrit was significantly higher at 0.78 mg/kg bw/day and above in the main group males at the end of administration period. Reduction in extramedullary hematopoiesis in the spleen was significant at 2.33 mg/kg bw/day in the main group females. Sperm analysis revealed a decrease in sperm motility and an increase in the rates of abnormal sperm, abnormal tail, and abnormal head at 7.0 mg/kg bw/day. A number of dams delivered their pups and of dams with live pups at delivery was significantly lowered in the 7.0 mg/kg bw/day group. Based on these findings, the LOAEL for males and NOAEL for females were 0.78 mg/kg bw/day, and the NOAEL for reproductive/developmental toxicity was considered to be 2.33 mg/kg bw/day. © 2007 Wiley Periodicals, Inc. Environ Toxicol 00: 000–000, 2007.

Keywords: dinoseb; nitrophenolic herbicide; 2-*sec*-butyl-4,6-dinitrophenol; repeated dose toxicity; reproductive and developmental toxicity; screening test; testis toxicity; rat

INTRODUCTION

Dinoseb, 2-*sec*-butyl-4,6-dinitrophenol (CAS No. 88-85-7), was approved for sale as a nitrophenolic herbicide in the United States in 1948, and it is used in soybeans, vegetables, fruits, nuts, citrus, and other field crops for the selective control of grass and broadleaf weeds. It is also used as an insecticide in grapes and as a seed crop drying agent (EXTOXNET, 1996). Although the use of dinoseb as a pesticide was banned in the United States in 1986 and in Europe in 1991, based on the potential risk of birth defects and other adverse health effects in humans (EXTOXNET, 1996; Rotterdam Convention, 2006), it is reported that

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