dinoseb is a high volume chemical with production or importation exceeding 1000 ton/year in Organisation for Economic Cooperation and Development (OECD) member countries and still widely used (OECD, 2004; PAN, 2006). Dinoseb and dinoseb salts are banned in Japan but consented to import (PAN, 2006). It is estimated that the volume of dinoseb imported to Japan is 110 ton from April 2005 to March 2006 (NITE, 2007).

Dinoseb is well absorbed by the oral route in mice (Gibson and Rao, 1973) and the dermal route in rats (Shah et al., 1987; Hall et al., 1992) and can pass through the placenta into the fetus of mice (Gibson and Rao, 1973). In a dermal toxicity study, dinoseb was more absorbed in adult female rats than in young rats (Shah et al., 1987). Dinoseb shows strong acute toxicity with the dermal LD_{50} of 40 mg/kg in rabbits (US EPA, 2003). The inhalation LC_{50} is 33–290 mg/m^3 for 4-h exposure in rats (US EPA, 2003). The basic mechanism of toxicity is thought to be stimulation of oxidative metabolism in cell mitochondria by the uncoupling of oxidative phosphorylation (Leftwich et al., 1982). Toxicity of dinoseb is enhanced by physical activity and high ambient temperature such as in an outdoor agricultural environment (Leftwich et al., 1982; US EPA, 2007). Early symptoms of dinoseb exposure include hyperthermia, sweating, headache, and confusion. Other signs and symptoms include dyspnea, pulmonary edema, nausea, vomiting, abdominal pain, malaise, dehydration, and tachycardia. Severe exposure may result in restlessness, seizures, coma, and death (Leftwich et al., 1982; US EPA, 2006, 2007).

As for developmental toxicity, many studies were conducted in experimental animals in the 1970s and the 1980s. Dinoseb was reported to be teratogenic in mice when administrated intraperitoneally or subcutaneously (Gibson, 1973; Preache and Gibson, 1975a,b), but not by gavage administration (Gibson, 1973). Dietary administered dinoseb was also reported to induce several adverse effects on reproduction and development including teratogenic effects in rats (McCormack et al., 1980; Spencer and Sing, 1982; Giavini et al., 1986; Daston et al., 1988). In a rabbit teratology study, Chinchilla rabbits were exposed by oral gavage to dinoseb at levels of 0, 1, 3, or 10 mg/kg bw/day on days 6–18 of gestation. Teratogenic effects were observed at 10 mg/kg bw/day without maternal toxicity (Research and Consulting Company, 1986). This study, conducted by a laboratory in Switzerland, became a main trigger for the cancellation of dinoseb. A male reproductive toxicity study in rats showed decreased sperm counts and increased atypical spermatozoa when receiving 9.1 mg/kg bw/day and above of dinoseb in feed (Linder et al., 1982). This result is in concordance with a recent study by Takahashi et al. (2004) in which reduced sperm motility and increased incidence of tailless sperm were found.

Exposure to dinoseb may occur by direct contact, ingestion, and inhalation for users and producers, but potential indirect exposure to dinoseb via the environment for consumers is also anticipated. Dinoseb is not strongly adsorbed on most agricultural soils. Microbial breakdown of dinoseb is demonstrated on soils, but dinoseb persists for about 2–4 weeks after application. Dinoseb was reported to be detected in water supplies in Canada and the United States (Health Canada, 1991). The US FDA examined 70 food items in 1985 and 1986 for dinoseb residues. Although no residues were detected in most of crops treated with dinoseb, a positive result was obtained in one cotton meal sample (Health Canada, 1991). Dinoseb is listed in the most recent OECD List of High Production Volume (HPV) chemicals to be investigated for environment and human health effects (OECD, 2004). Although many studies had been conducted for developmental toxicity of dinoseb, these reports could be determined to be inadequate for the initial assessment of the chemical in the OECD HPV Chemicals Programme, because these studies were non-Good Laboratory Practice (GLP) studies or did not totally comply with a specific testing guideline (Klimisch et al., 1997; OECD, 2005). No studies on female reproductive performances were evaluated. Therefore, dinoseb was selected as a target substance for the Safety Examination of Existing Chemicals in Japan (MHLW, 2005) to obtain reliable information on the possible effects on reproduction and development in compliance with the OECD Test Guideline and in accordance with the principles for GLP. The present article reports the result of combined repeated dose and reproductive/developmental toxicity screening test of dinoseb in rats.

MATERIALS AND METHODS

This study was performed in 2003–2004 at the Nihon Bioresearch (Hashima, Japan) in compliance with OECD Guideline 422 Combined Repeated Dose Toxicity Study with Reproduction/Developmental Toxicity Screening Test (OECD, 1996) and in accordance with the OECD Principles for GLP (OECD, 1998) and Law Concerning the Examination and Regulation of Manufacture, etc., of Chemical Substances (EA, 1984). All animals were treated in accordance with the law governing the protection and management of animals (MOE, 1973), the guidelines for experiments using animals (JALAS, 1987), and the Regulations of the Committee for the Ethical Treatment of Animals (Hashima Laboratory, Nihon Bioresearch).

Animals

International Genetic Standard (Crj: CD(SD)IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in toxicity studies, including reproductive and developmental toxicity studies, and historical control data are available. Males and females at 7 weeks

of age were purchased from Hino Breeding Center, Charles River Japan, (Yokohama, Japan). The rats were quarantined for 5 days and acclimatized to the laboratory for 16 or 17 days before the start of the experiment. Male and female rats found to be in good health were selected for use. The vaginal smears of each female were recorded, and only females showing a 4-day or 5-day estrous cycle were used in the experiment. Male and female rats were distributed into four groups on a random basis. Each group consisted of six males and 12 females as main groups and six males and six females as recovery groups. Rats were housed individually except during the mating period. From day 18 of pregnancy to day 3 of lactation, individual dams and litters were reared using wooden chips as bedding (Sunflake®; Charles River Japan).

Animals were reared on a basal diet (CRF-1; Oriental Yeast Co., Tokyo, Japan) and water *ad libitum* and maintained in an air-conditioned room at a room temperature of 20–26°C, a relative humidity of 40–70%, a 12-h light/dark cycle, and 12 air changes per hour.

Chemicals and Dosing

Dinoseb, yellowish crystals with pungent odour, was obtained from Wako Pure Chemical Industries (Osaka, Japan). The dinoseb (Lot no. RWN9641) used in this study was 96% pure, and was stored under refrigeration before use. The purity and stability of the chemical were verified by analysis before the study. Rats were dosed once daily by gastric intubation with dinoseb at a dose of 0 (control), 0.78, 2.33, or 7.0 mg/kg bw. The dosage levels were determined based on a previous dose-finding study in which no effects were seen at 5 mg/kg bw/day, but deaths and lower body weights were found at 10 and 20 mg/kg bw/day in rats given dinoseb by gavage at 0, 2.5, 5, 10, or 20 mg/kg bw/day for 14 days. The dinoseb was suspended in corn oil, and the control rats were given only corn oil. Twelve males per group were dosed for a total of 42 days beginning 14 days before mating. After the administration period, 6 of 12 males per group were reared for 14 days without administration of dinoseb as the recovery groups. The main group females 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. The recovery group females were given dinoseb for a total of 42 days, followed by a recovery period of 14 days. The first day of dosing was designated as day 0 of administration and the day after the final dose was designated as day 0 of the recovery period. The volume of each dose was adjusted to 5 mL/kg body weight based on the latest body weight. The stability of formulations was confirmed after storage under refrigeration in the dark for 7 days. During use, the formulations were maintained under such conditions for up to 7 days, and the concentration of each preparation was within the acceptable range (91.3–96.4%).

Observations

All rats were observed daily for clinical signs of toxicity. Body weight was recorded twice a week in males and females of the recovery groups, and twice a week during the premating period, on days 0, 7, 14, and 21 of pregnancy and on days 0, 4, 6, and 7 of lactation in females of the main groups. Food consumption was recorded twice a week in males and in the recovery group females, and twice a week during the premating period, on days 2, 9, 16, and 20 of pregnancy and on day 2 of lactation in the main group females. Functional observation battery (FOB) in all animals was recorded once a week during the administration period for 1 h following administration, as follows: (i) posture, biting behaviour, eyelid closure, and convulsion, (ii) ease of removal from cage and handling, muscle tone, fur condition, lacrimation, salivation and respiration, and (iii) rearing frequency, grooming frequency, ambulation, palpebral closure, arousal, behavioural abnormality, and righting reflex.

Six animals in each group, with the exception of one surviving female in the 7.0 mg/kg bw/day main group, were subjected to the following observations and examinations unless noted otherwise. Sensory reactions for pupillary reflex, approximation reflex, behavioural abnormality, tactile reflex, auditory reflex and pain reflex, and grip strength of fore and hind limbs were tested in the main group males on day 40 of administration and in the main group females on day 3 of lactation. Spontaneous motor activity, ambulation, and rearing were recorded (Activity Monitor, Med Associate, Vermont) after administration for 1 h at intervals of 10 min on day 39 of administration in the main group males and on day 4 of lactation in the main group females. Fresh urine was sampled from animals using a urine-collecting cage under fasting and watering conditions and collected for 24 h.

The main group rats were euthanized by exsanguination under anesthesia on the day after the final administration in males and on day 7 of lactation in females, and the recovery group rats were euthanized on the day after completion of the recovery period. The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. Blood samples were drawn from the abdominal aorta. The brain (cerebrum, cerebellum, and medula oblongata), pituitary gland, thyroid, thymus, heart, liver, spleen, kidney, adrenal glands, testis, epididymis, tail of the epididymis, ovary, and uterus were isolated and weighed. The lung, trachea, pancreas, salivary gland (sublingual gland and submandibular gland), esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, lymph nodes (mandibular lymph nodes and

mesenteric lymph node), bladder, seminal vesicle, prostate, vagina, parathyroid, spinal cord, sciatic nerve, eye ball, Hardarian gland, sternum, and femur were removed. The numbers of corpora lutea and implantation sites were counted in all the main group females. The testes and epididymides were fixed with Bouin's solution and 90% alcohol. The eye ball was fixed with glutaraldehyde formalin and 20% neutral buffered formalin. Other organs were stored in 20% neutral buffered formalin. Histopathological evaluations were performed on these organs. The caudal epididymis was used for determination of the sperm motility, sperm viability, sperm morphology, and sperm count.

The right epididymal cauda was minced and stored for 5 min in Medium 199 containing 0.5% bovine serum albumin (BSA) at 37°C. This original sperm suspension was used for tests of sperm motility, viability, and morphology. The original sperm suspension was diluted with Medium 199 containing 0.5% BSA and incubated for about 30 min in 5% CO_2 in air at 37°C. After the incubation, the sperm samples were loaded into a chamber (Microslides #HTR1099; VitroCom, Mountain Lakes, NJ), and sperm motility was analyzed using a TOX IVOS (Hamilton Thorne Research, Beverly, MA). The original sperm sample was stained with calcein acetoxy methyl ester (CAM) and ethidium homodimer-1 (EthD-1) and incubated for about 60 min in 5% CO_2 in air. The population of viable and dead spermatozoa was identified by using a fluorescence microscopy according to Kato et al. (2002), and sperm viability and survivability rates were calculated. The original sperm suspension was mounted on a slide glass, fixed with the 10% neutral-buffered formalin and stained with 1% eosin. Sperm morphology was observed by using a microscope. The left epididymal cauda was stored at −80°C and homogenized with 0.1% Triton X-100. Number of sperm was determined with the TOX IVOS (Hamilton Thorne Research).

Urine samples were tested for colour, pH, protein, glucose, ketone body, bilirubin, occult blood, and urobilinogen. Urinary sediment was stained and examined microscopically. Urine volume was calculated from the specific gravity and weight. The collected blood samples were measured for the red blood cell (RBC) count, hemoglobin, platelet count, and white blood cell count. In addition, mean cell volume (MCV), mean cell hemoglobin (MCH), mean corpuscular hemoglobin concentration, reticulocyte rate, and differential leukocyte rates were calculated. Prothrombin time (PT), activated partial thromboplastin time, and fibrinogen were determined. Blood chemistry was tested for aspartate aminotransferase, alkaline phosphatase (ALP), γ-glutamyl transpeptidase, total protein, albumin, albumin/globulin (A/G) ratio, total bilirubin, blood urea nitrogen (BUN), creatinine, glucose, total cholesterol, triglycerides, Na, K, Cl, Ca, and inorganic phosphate.

Daily vaginal lavage samples of each female were evaluated for estrous cyclicity throughout the premating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the 2-week mating period had elapsed. 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 as evidence for successful mating. Once insemination was confirmed, the females were checked for signs of parturition before 10:00 from day 21 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal day (PND) 6. The day on which parturition was completed by 10:00 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. Pups were inspected for external malformations and malformations within the oral cavity on PND 0. On PND 4, the pups were euthanized by exsanguination under anesthesia, and gross internal examinations were performed.

Data Analysis

The statistical analysis of pups was carried out using the litter as the experimental unit. Mean and standard deviation in each dose group were calculated for the body weight gain, food consumption, grip strength, spontaneous motor activity, urine volume, urinary specific gravity, hematological test results, blood biochemical test results, absolute and relative organ weights, sperm test results, numbers of estrous cases and conceiving days, length of gestation, numbers of corpora lutea and implantations, implantation index, total number of pups born, number of live and dead pups, sex ratio, delivery index, birth index, live birth index, live pups and viability index on day 4 of lactation, and body weight of pups. These were analyzed with Bartlett's test (Bartlett, 1937) for homogeneity of variance. If it was homogeneous, the data were analyzed using Dunnett's multiple comparison test (Dunnett, 1955) to compare the mean of the control group with that of each dosage group, and if it was not homogeneous, the Dunnett's rank test (Miller, 1981) was applied. The copulation index, fertility index, and gestation index were analyzed with Fisher's exact test (Fisher, 1973). The 5% levels of probability were used as the criterion for significance.

RESULTS

General Findings

Table 1 shows the general findings in male rats given dinoseb. No deaths were observed in any of the groups. Transient salivation was observed immediately after administration in one male in the 0.78 mg/kg bw/day dose group on day 14, in 11 males on day 8 or later in the 2.33 mg/kg bw/day dose group and in all 12 males on day 2 or later in the T1

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TABLE I. General findings in male rats given dinoseb

Dose (mg/kg bw/day)	0 (Control)	0.78	2.33	7.0
No. of male rats	12	12	12	12
No. of deaths	0	0	0	0
Initial body weight (g)[a]	362 ± 13	363 ± 11	361 ± 13	360 ± 16
Body weight gain (g)				
The administration period				
Days 0–7	18 ± 9	23 ± 6	17 ± 8	7 ± 7**
Days 7–14	18 ± 7	18 ± 4	16 ± 6	14 ± 6
Days 14–21	18 ± 7	17 ± 5	17 ± 8	9 ± 10*
Days 21–28	22 ± 8	18 ± 6	21 ± 6	16 ± 7
Days 28–35	20 ± 4	22 ± 5	19 ± 5	19 ± 5
Days 35–41	10 ± 8	11 ± 6	11 ± 6	9 ± 8
The recovery period				
Days 0–7	13 ± 6	18 ± 4	14 ± 10	26 ± 7**
Days 7–14	6 ± 9	11 ± 3	13 ± 7	18 ± 3*
Food consumption (g/day/rat)				
The administration period				
Days 0–1	20 ± 2	20 ± 2	17 ± 3*	17 ± 2*
Days 3–4	21 ± 3	21 ± 2	20 ± 2	23 ± 2
Days 7–8	21 ± 3	21 ± 2	21 ± 3	23 ± 4
Days 10–11	20 ± 3	21 ± 2	21 ± 2	24 ± 2**
Days 28–29	21 ± 2	20 ± 2	21 ± 2	23 ± 3
Days 31–32	21 ± 2	21 ± 3	21 ± 2	24 ± 3**
Days 35–36	20 ± 2	21 ± 3	19 ± 2	23 ± 2*
Days 38–39	22 ± 2	21 ± 2	22 ± 3	24 ± 2*
The recovery period				
Days 0–1	22 ± 2 (6)	22 ± 2 (6)	21 ± 2 (6)	25 ± 2* (6)
Days 3–4	24 ± 2 (6)	24 ± 2 (6)	21 ± 2 (6)	25 ± 2* (6)
Day 7–8	26 ± 3 (6)	24 ± 3 (6)	25 ± 4 (6)	27 ± 2 (6)
Days 10–11	22 ± 3 (6)	25 ± 3 (6)	27 ± 2** (6)	27 ± 1** (6)

Figures in parentheses indicate number of males.
[a]Values are given as the mean ± SD.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

7.0 mg/kg bw/day dose group. Body weight gain was significantly decreased on days 0–7 and 14–21 at 7.0 mg/kg bw/day during the administration period, and it was significantly increased at 7.0 mg/kg bw/day during the recovery period. During the administration period, food consumption was significantly low on days 0–1 at 2.33 and 7.0 mg/kg bw/day, and it was significantly higher than controls on days 10–11, 31–32, 35–36, and 38–39 at 7.0 mg/kg bw/day. During the recovery period, no significant changes were observed in general condition and body weight gain at 0.78 and 2.33 mg/kg bw/day. Food consumption was significantly higher than controls on recovery days 10–11 at 2.33 and 7.0 mg/kg bw/day and on recovery days 0–1 and 3–4 at 7.0 mg/kg bw/day.

T2 Table II presents the general findings in the main group female rats given dinoseb. In the 7.0 mg/kg bw/day main group, seven animals died on gestation day 19 and one on gestation day 21, and one animal was moribund on each of gestation days 19 and 20. Transient salivation was observed immediately after administration in five females on day 10 or later in the 2.33 mg/kg bw/day dose group and in 11 females on day 3 or later in the 7.0 mg/kg bw/day dose group. During the gestation period, transient salivation was observed immediately after administration in three animals at 2.33 mg/kg bw/day and in all females at 7.0 mg/kg bw/day. In addition, females in the highest dose group that died or were moribund showed a decrease in spontaneous motor activity, prone position, low body temperature, dyspnea, cyanosis, and bradypnea. In the 7.0 mg/kg bw/day main group, body weight gain was significantly lowered on administration days 0–7 compared with controls. During the gestation and lactation, no significant change was observed in body weight gain in any of the dinoseb-treated groups. There was significantly higher food consumption compared with controls on days 7–8 of the premating period and on gestation days 1–2, 8–9, and 15–16 at 7.0 mg/kg bw/day in the main groups.

In females of the recovery groups, no deaths were observed. Food consumption was significantly increased during the administration period at 7.0 mg/kg bw/day and

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TABLE II. General findings in female rats given dinoseb

Dose (mg/kg bw/day)	0 (Control)	0.78	2.33	7.0
No. of female rats	12	12	12	12
No. of deaths during premating period	0	0	0	0
No. of deaths during pregnancy	0	0	0	10
Initial body weight (g)[a]	251 ± 9	250 ± 8	250 ± 9	251 ± 9
Body weight gain (g)				
Days 0–7	12 ± 9	8 ± 11	10 ± 6	2 ± 5**
Days 7–14	12 ± 7	9 ± 6	11 ± 4	8 ± 7
Days 0–7 of gestation	35 ± 9 (11)	35 ± 6 (11)	34 ± 6	37 ± 6
Days 7–14 of gestation	34 ± 4 (11)	34 ± 2 (11)	37 ± 4	38 ± 5
Days 14–21 of gestation	91 ± 12 (11)	79 ± 32 (11)	85 ±13	65 (2)
Days 0–6 of lactation	6 ± 11 (11)	10 ± 9 (10)	14 ± 11	9 (1)
Food consumption (g/day/rat)				
Days 0–1	13 ± 2	15 ± 3	15 ± 1	14 ± 2
Days 3–4	15 ± 3	16 ± 4	15 ± 2	16 ± 3
Days 7–8	13 ± 2	14 ± 3	13 ± 3	17 ± 3**
Days 10–11	16 ± 4	16 ± 3	17 ± 3	18 ± 3
Days 1–2 of gestation	18 ± 2	18 ± 2	18 ± 1	22 ± 3**
Days 8–9 of gestation	19 ± 2	19 ± 2	19 ± 2	23 ± 2**
Days 15–16 of gestation	19 ± 2	20 ± 2	21 ± 2	23 ± 3*
Days 15–16 of gestation	19 ± 2	17 ± 5	20 ± 3	19 ± 5 (4)
Days 1–2 of lactation	23 ± 3	22 ± 4	21 ± 4	13 (1)

Figures in parentheses indicate number of dams.
[a] Values are given as the mean ± SD.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

during the recovery period at 2.33 mg/kg bw/day and above (data not shown).

Functional Observation

No treatment-related effects on the detailed clinical observations, FOB, sensory reactivity such as pupillary reflex, approximation reflex, behavioural abnormality, tactile reflex, auditory reflex and pain reflex, grip strength, and spontaneous motor activity were observed in males and females in the test and recovery groups (data not shown).

Urinalysis

Urinalysis revealed no significant differences in any parameters between the control and dinoseb-treated groups in males and females in the test and recovery groups (data not shown).

Hematology

T3 Table III shows hematological findings in male and female rats. In males of the main groups, RBC count was significantly higher at 0.78 mg/kg bw/day and hematocrit was significantly higher at 0.78 mg/kg bw/day and above. Hemoglobin and PT were significantly higher than controls at 2.33 and 7.0 mg/kg bw/day. In the 7.0 mg/kg bw/day dose group, MCV was significantly higher than controls. As for females in the main groups, RBC count was significantly higher, and MCV and MCH were significantly lower at 0.78 mg/kg bw/day. Fibrinogen was significantly lower at 2.33 mg/kg bw/day. RBC count was significantly lower, and MCV was significantly higher in the 7.0 mg/kg bw/day recovery dose group males.

Blood Biochemistry

Blood biochemical findings are shown in Table IV. Total cholesterol was significantly lower at 2.33 mg/kg bw/day, and creatinine was significantly higher at 7.0 mg/kg bw/day in the main group males. Albumin and A/G ratio were significantly higher in females of the main groups at 2.33 mg/kg bw/day. In the recovery group males, A/G ratio and Cl were significantly lower at 0.78 mg/kg bw/day. A/G ratio was significantly lower, and total cholesterol was significantly higher at 2.33 mg/kg bw/day. In the 7.0 mg/kg bw/day group, ALP and albumin were significantly lower and BUN was significantly higher. T4

Organ Weight

Table V shows organ weight of male and female rats given dinoseb. At 7.0 mg/kg bw/day, relative brain weight was significantly higher in the main group males. At the end of T5

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TABLE III. Hematological findings in male and female rats given dinoseb

	Main Group				Recovery Group			
Dose (mg/kg bw/day)	0 (Control)	0.78	2.33	7.0	0 (Control)	0.78	2.33	7.0
No. of male rats	6	6	6	6	6	6	6	6
RBC ($10^4/\mu L$)[a]	801 ± 13	844 ± 30*	833 ± 31	834 ± 29	858 ± 21	831 ± 22	852 ± 38	817 ± 22*
Hemoglobin (g/dL)	14.9 ± 0.5	15.5 ± 0.7	15.7 ± 0.5*	16.2 ± 0.5**	15.9 ± 0.4	15.3 ± 0.4	15.6 ± 1.1	15.8 ± 0.3
Hematocrit (%)	43.9 ± 0.8	46.4 ± 1.9*	46.6 ± 1.4**	47.7 ± 1.2**	46.8 ± 0.9	45.2 ± 1.2	46.6 ± 3.4	46.0 ± 1.0
MCV (fL)	54.8 ± 1.3	55.0 ± 1.1	56.0 ± 1.6	57.2 ± 1.5*	54.5 ± 0.8	54.4 ± 1.5	54.6 ± 1.8	56.3 ± 1.0
MCH (pg)	18.6 ± 0.7	18.4 ± 0.6	18.8 ± 0.6	19.4 ± 0.7	18.6 ± 0.5	18.4 ± 0.6	18.3 ± 0.7	19.3 ± 0.4*
MCHC (g/dL)	33.8 ± 0.5	33.5 ± 0.7	33.6 ± 0.4	33.9 ± 0.7	34.0 ± 0.4	33.9 ± 0.6	33.6 ± 0.4	34.3 ± 0.4
Platelet count ($10^4/\mu L$)	91.2 ± 10.2	95.0 ± 15.4	88.1 ± 11.9	78.2 ± 7.5	91.2 ± 10.2	95.0 ± 15.4	88.1 ± 11.9	78.2 ± 7.5
Reticulocyte (‰)	28 ± 3	24 ± 3	25 ± 4	24 ± 3	24 ± 6	23 ± 4	24 ± 6	22 ± 3
PT (s)	13.2 ± 0.3	13.6 ± 0.5	13.8 ± 0.3*	14.1 ± 0.4**	19.1 ± 3.1	19.5 ± 3.5	19.9 ± 3.6	16.3 ± 1.5
APTT (s)	27.6 ± 1.1	29.8 ± 1.6	28.3 ± 3.2	28.2 ± 1.8	37.1 ± 4.0	37.0 ± 2.2	38.2 ± 1.8	34.2 ± 3.2
Fibrinogen (mg/dL)	220 ± 10	221 ± 23	224 ± 16	207 ± 10	220 ± 10	221 ± 23	224 ± 16	207 ± 10
WBC ($10^2/\mu L$)	78 ± 22	61 ± 14	66 ± 23	55 ± 16	61 ± 16	54 ± 22	76 ± 20	49 ± 10
Differential leukocyte (%)								
Lymphocyte	94.7 ± 2.3	91.2 ± 2.9	91.7 ± 2.1	90.8 ± 5.9	90.2 ± 2.8	90.8 ± 4.2	92.2 ± 4.2	88.7 ± 7.2
Neutrophil	4.5 ± 1.8	7.8 ± 2.1	7.7 ± 1.5	8.5 ± 5.5	9.0 ± 3.0	8.2 ± 4.7	6.8 ± 3.9	10.0 ± 6.8
Eosinophil	0.5 ± 0.5	0.5 ± 0.8	0.3 ± 0.5	0.5 ± 0.5	0.3 ± 0.5	0.5 ± 0.8	0.5 ± 0.5	0.7 ± 0.5
Basophil	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Monocyte	0.3 ± 0.5	0.5 ± 0.5	0.3 ± 0.5	0.2 ± 0.4	0.5 ± 0.5	0.5 ± 0.5	0.5 ± 0.5	0.7 ± 0.5
No. of female rats	6	6	6	1	6	6	6	6
RBC ($10^4/\mu L$)	702 ± 13	749 ± 25*	735 ± 41	725	739 ± 21	751 ± 45	758 ± 30	753 ± 26
Hemoglobin (g/dL)	14.4 ± 0.4	14.7 ± 0.5	14.8 ± 0.7	15.2	14.6 ± 0.4	14.8 ± 0.8	14.8 ± 0.5	14.9 ± 0.4
Hematocrit (%)	41.8 ± 1.1	42.8 ± 1.1	42.8 ± 1.9	44.6	41.4 ± 1.1	42.1 ± 2.1	42.3 ± 1.6	42.4 ± 1.5
MCV (fL)	59.5 ± 2.1	57.1 ± 1.1*	58.2 ± 1.2	61.5	56.0 ± 0.9	56.1 ± 1.4	55.8 ± 1.6	56.3 ± 0.6
MCH (pg)	20.6 ± 0.7	19.7 ± 0.2*	20.1 ± 0.6	21.0	19.7 ± 0.5	19.7 ± 0.6	19.4 ± 0.7	19.8 ± 0.4
MCHC (g/dL)	34.6 ± 0.6	34.4 ± 0.4	34.5 ± 0.5	34.1	35.2 ± 0.6	35.2 ± 0.3	34.8 ± 0.3	35.1 ± 0.5
Platelet count ($10^4/\mu L$)	105.2 ± 8.4	115.9 ± 9.9	112.6 ± 11.9	109.5	96.1 ± 13.0	94.9 ± 5.4	94.2 ± 11.4	90.3 ± 2.7
Reticulocyte (‰)	77 ± 16	64± 15	71 ± 9	52	27 ± 3	25 ± 5	24 ± 5	23 ± 3
PT (s)	13.5 ± 0.4	13.7 ± 0.5	13.3 ± 0.3	13.3	13.5 ± 0.6	13.1 ± 0.2	13.4 ± 0.4	13.2 ± 0.3
APTT (s)	25.1 ± 2.0	23.9 ± 2.1	23.6 ± 2.6	25.8	23.9 ± 1.6	22.9 ± 1.4	23.2 ± 1.2	24.4 ± 1.4
Fibrinogen (mg/dL)	257 ± 21	255 ± 28	217 ± 19*	210	177 ± 20	179 ± 13	180 ± 7	188 ± 29
WBC ($10^2/\mu L$)	49 ± 14	57 ± 10	54 ± 16	74	32 ± 15	39 ± 11	38 ± 10	37 ± 14
Differential leukocyte (%)								
Lymphocyte	86.7 ± 8.0	87.2 ± 5.6	89.0 ± 5.2	75.0	90.3 ± 3.7	89.8 ± 4.3	87.5 ± 5.5	88.7 ± 4.4
Neutrophil	12.0 ± 7.6	11.8 ± 5.5	9.5 ± 4.4	23.0	9.0 ± 3.8	9.2 ± 3.9	11.2 ± 5.3	10.3 ± 4.7
Eosinophil	0.5 ± 0.5	0.2 ± 0.4	0.5 ± 0.8	0.0	0.3 ± 0.5	0.7 ± 0.8	0.7 ± 0.8	0.7 ± 0.5
Basophil	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Monocyte	0.8 ± 0.8	0.8 ± 0.8	1.0 ± 0.6	2.0	0.3 ± 0.5	0.3 ± 0.5	0.7 ± 0.8	0.3 ± 0.5

[a] Values are given as the mean ± SD.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

the recovery period, only dose independent changes were found as follows. Relative brain weight was significantly lower and absolute liver weight was significantly higher at 0.78 mg/kg bw/day in males. In females, relative heart weight was significantly lower at 0.78 mg/kg bw/day. In addition, relative brain weight was significantly lower than controls at 2.33 mg/kg bw/day, and absolute heart weight was significantly higher than controls at 7.0 mg/kg bw/day. Absolute and relative weights of the testes, epididymides, ovaries, and uterus showed no significant difference from controls in any dinoseb-treated groups.

Necropsy and Histopathological Findings

Extramedullary hematopoiesis in the spleen was observed in six animals in the control female group with severity ranging from slight to moderate. Reduction in extramedullary hematopoiesis in the spleen was significant at 2.33 mg/kg bw/day in the main group females with severity ranging from slight to mild. At 7.0 mg/kg bw/day, one surviving female showed slight extramedullary hematopoiesis in the spleen, but the other surviving female did not show splenic extramedullary hematopoiesis. No histopathological

TABLE IV. Blood biochemical findings in male and female rats given dinoseb

	Main Group				Recovery Group			
Dose (mg/kg bw/day)	0 (control)	0.78	2.33	7.0	0 (control)	0.78	2.33	7.0
No. of male rats	6	6	6	6	6	6	6	6
AST (IU/L)[a]	85.3 ± 19.0	85.9 ± 17.7	93.5 ± 20.1	91.3 ± 7.3	120.7 ± 44.1	115.1 ± 29.7	103.2 ± 24.4	102.7 ± 16.8
ALT (IU/L)	37.6 ± 6.7	34.3 ± 3.3	37.8 ± 5.5	40.1 ± 4.6	39.0 ± 13.8	35.9 ± 9.8	34.9 ± 12.1	29.9 ± 4.9
ALP (IU/L)	584.0 ± 91.4	641.4 ± 106.4	651.0 ± 166.2	950.3 ± 338.4	373.8 ± 56.4	310.4 ± 53.7	304.9 ± 77.7	256.2 ± 56.9**
γ-GTP (IU/L)	0.34 ± 0.15	0.46 ± 0.19	0.53 ± 0.20	0.53 ± 0.11	0.41 ± 0.22	0.29 ± 0.21	0.27 ± 0.12	0.39 ± 0.08
Total protein (g/dL)	5.8 ± 0.2	5.9 ± 0.2	5.8 ± 0.2	5.6 ± 0.2	5.8 ± 0.2	5.8 ± 0.2	5.8 ± 0.4	5.4 ± 0.1
Albumin (g/dL)	2.95 ± 0.16	2.92 ± 0.12	2.91 ± 0.07	2.98 ± 0.15	2.93 ± 0.19	2.75 ± 0.12	2.84 ± 0.16	2.71 ± 0.07*
A/G ratio	1.04 ± 0.09	0.99 ± 0.03	1.00 ± 0.05	1.14 ± 0.10	1.04 ± 0.07	0.91 ± 0.05**	0.96 ± 0.05*	1.00 ± 0.04
Total bilirubin (mg/dL)	0.07 ± 0.02	0.06 ± 0.03	0.07 ± 0.04	0.05 ± 0.03	0.13 ± 0.03	0.12 ± 0.04	0.14 ± 0.02	0.11 ± 0.02
BUN (mg/dL)	17.7 ± 3.7	16.6 ± 1.3	16.4 ± 1.8	20.2 ± 1.3	15.3 ± 1.6	16.9 ± 2.2	16.7 ± 2.3	18.5 ± 2.1*
Creatinine (mg/dL)	0.27 ± 0.04	0.28 ± 0.02	0.28 ± 0.04	0.34 ± 0.04*	0.26 ± 0.03	0.30 ± 0.06	0.29 ± 0.04	0.29 ± 0.01
Glucose (mg/dL)	118.4 ± 7.3	119.6 ± 10.3	120.1 ± 9.2	112.7 ± 9.1	115.6 ± 15.2	121.4 ± 17.2	114.0 ± 22.5	102.7 ± 7.1
Total cholesterol (mg/dL)	68.0 ± 5.4	61.0 ± 4.4	56.7 ± 9.0*	60.6 ± 6.4	45.9 ± 10.0	60.6 ± 8.8	61.3 ± 10.6*	58.8 ± 11.2
Triglyceride (mg/dL)	63.4 ± 9.7	55.9 ± 21.3	62.8 ± 13.3	56.8 ± 15.4	37.5 ± 25.6	35.7 ± 17.7	36.9 ± 13.5	29.2 ± 10.7
Na (mEq/L)	144.5 ± 1.1	144.0 ± 1.3	144.3 ± 1.2	145.0 ± 0.7	144.8 ± 0.7	144.4 ± 0.7	145.2 ± 1.3	145.5 ± 0.9
K (mEq/L)	4.34 ± 0.27	4.35 ± 0.33	4.46 ± 0.29	4.12 ± 0.18	4.02 ± 0.34	4.23 ± 0.25	4.28 ± 0.22	4.16 ± 0.19
Cl (mEq/L)	105.8 ± 1.6	105.4 ± 1.0	105.1 ± 1.6	026.1 ± 1.1	106.8 ± 0.7	105.1 ± 1.4*	105.8 ± 0.9	106.9 ± 1.1
Ca (mg/dL)	9.9 ± 0.3	9.7 ± 0.3	9.6 ± 0.3	9.5 ± 0.3	9.4 ± 0.3	9.4 ± 0.3	9.5 ± 0.1	9.3 ± 0.2
Inorganic phosphate (mg/dL)	6.2 ± 1.3	6.2 ± 0.9	7.0 ± 0.6	5.7 ± 0.9	7.0 ± 0.5	7.3 ± 0.9	7.3 ± 0.7	7.4 ± 0.6
No. of female rats	6	6	6	1	6	6	6	6
AST (IU/L)	90.0 ± 16.4	98.7 ± 29.3	94.4 ± 21.4	78.4	86.0 ± 17.0	91.7 ± 11.7	85.8 ± 11.1	92.5 ± 11.2
ALT (IU/L)	23.8 ± 2.2	25.3 ± 3.9	26.6 ± 5.1	32.4	23.2 ± 3.9	26.5 ± 6.0	22.8 ± 1.6	26.2 ± 5.9
ALP (IU/L)	192.6 ± 46.8	178.8 ± 48.0	202.2 ± 23.1	194.7	129.3 ± 39.4	122.4 ± 17.5	135.5 ± 27.6	169.8 ± 49.1
γ-GTP (IU/L)	0.45 ± 0.12	0.65 ± 0.23	0.49 ± 0.17	0.55	0.46 ± 0.25	0.58 ± 0.25	0.59 ± 0.21	0.66 ± 0.26
Total protein (g/dL)	6.5 ± 0.2	6.4 ± 0.3	6.7 ± 0.5	6.1	6.5 ± 0.5	6.5 ± 0.3	6.2 ± 0.2	6.2 ± 0.4
Albumin (g/dL)	3.19 ± 0.15	3.16 ± 0.20	3.48 ± 0.25*	3.29	3.61 ± 0.36	3.59 ± 0.26	3.40 ± 0.16	3.32 ± 0.30
A/G ratio	0.98 ± 0.05	0.98 ± 0.07	1.10 ± 0.02*	1.17	1.24 ± 0.08	1.25 ± 0.11	1.23 ± 0.08	1.18 ± 0.09
Total bilirubin (mg/dL)	0.11 ± 0.02	0.10 ± 0.02	0.09 ± 0.02	0.05	0.10 ± 0.03	0.11 ± 0.03	0.10 ± 0.03	0.12 ± 0.03
BUN (mg/dL)	20.5 ± 2.5	18.9 ± 4.4	23.5 ± 1.9	18.4	18.2 ± 1.4	16.1 ± 1.7	18.1 ± 2.5	18.0 ± 1.6
Creatinine (mg/dL)	0.39 ± 0.01	0.38 ± 0.05	0.40 ± 0.01	0.44	0.37 ± 0.02	0.34 ± 0.04	0.39 ± 0.02	0.36 ± 0.05
Glucose (mg/dL)	127.2 ± 13.2	129.0 ± 14.3	123.3 ± 13.7	130.7	120.7 ± 20.5	123.2 ± 9.8	126.3 ± 11.4	119.2 ± 15.1
Total cholesterol (mg/dL)	72.2 ± 5.5	81.9 ± 20.7	70.3 ± 9.3	86.4	81.5 ± 8.4	87.0 ± 10.8	75.7 ± 11.2	84.4 ± 17.4
Triglyceride (mg/dL)	41.8 ± 13.7	56.9 ± 24.5	54.3 ± 16.7	82.4	25.7 ± 5.4	38.9 ± 22.7	42.7 ± 29.2	22.7 ± 6.4
Na (mEq/L)	141.1 ± 1.0	140.7 ± 0.8	140.5 ± 0.9	142.2	143.6 ± 0.9	142.9 ± 1.4	142.7 ± 1.3	144.2 ± 0.5
K (mEq/L)	4.23 ± 0.32	4.14 ± 0.34	4.08 ± 0.31	3.38	4.04 ± 0.27	4.16 ± 0.17	3.98 ± 0.25	3.83 ± 0.26
Cl (mEq/L)	104.6 ± 1.8	104.3 ± 0.9	104.3 ± 2.1	104.9	108.0 ± 1.3	107.1 ± 1.5	107.0 ± 1.0	107.2 ± 1.7
Ca (mg/dL)	10.9 ± 0.3	10.8 ± 0.3	10.7 ± 0.5	10.4	9.6 ± 0.3	9.7 ± 0.2	9.5 ± 0.2	9.5 ± 0.3
Inorganic phosphate (mg/dL)	7.8 ± 1.2	7.6 ± 0.8	6.8 ± 1.1	5.6	4.3 ± 0.7	4.4 ± 0.7	4.3 ± 0.6	4.7 ± 0.4

[a] Values are given as the mean ± SD.

* Significantly different from the control group ($p < 0.05$).

** Significantly different from the control group ($p < 0.01$).

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TABLE V. Organ weight of male and female rats given dinoseb

Dose (mg/kg bw/day)		Main Group				Recovery Group			
		0 (Control)	0.78	2.33	7.0	0 (Control)	0.78	2.33	7.0
No. of male rats		6	6	6	6	6	6	6	6
Body weight	(g)[a]	451 ± 19	449 ± 27	438 ± 42	409 ± 29	458 ± 22	474 ± 19	461 ± 29	461 ± 20
Brain	(g)[b]	2.04 ± 0.05	2.02 ± 0.04	2.07 ± 0.04	2.04 ± 0.06	2.12± 0.08	2.02 ± 0.10	2.09 ± 0.09	2.03 ± 0.13
	(g%)[c]	0.45 ± 0.02	0.45 ± 0.02	0.48 ± 0.05	0.50 ± 0.03*	0.46 ± 0.02	0.43 ± 0.02*	0.46 ± 0.03	0.44 ± 0.02
Pituitary	(mg)	12.7 ± 1.1	13.3 ± 1.9	13.2 ± 1.0	11.7 ± 1.1	14.3 ± 1.2	15.1 ± 1.5	13.6 ± 0.8	15.4 ± 1.0
	(mg%)	2.8 ± 0.2	3.0 ± 0.5	3.0 ± 0.3	2.9 ± 0.4	3.1 ± 0.2	3.2 ± 0.2	3.0 ± 0.2	3.4 ± 0.2
Thyroids	(mg)	20.7 ± 5.6	19.1 ± 6.4	23.0 ± 3.1	20.3 ± 3.5	18.5 ± 2.8	22.1 ± 4.2	20.6 ± 2.0	21.1 ± 4.5
	(mg%)	4.6 ± 1.3	4.3 ± 1.6	5.3 ± 0.6	5.0 ± 1.0	4.0 ± 0.6	4.7 ± 0.7	4.5 ± 0.4	4.6 ± 1.0
Thymus	(mg)	367 ± 126	260 ± 76	296 ± 120	245 ± 54	268 ± 41	316 ± 90	265 ± 86	296 ± 64
	(mg%)	82 ± 30	58 ± 16	67 ± 22	60 ± 14	59 ± 10	66 ± 17	58 ± 18	65 ± 15
Heart	(g)	1.41 ± 0.14	1.40 ± 0.07	1.37 ± 0.14	1.35 ± 0.11	1.52 ± 0.19	1.56 ± 0.09	1.62 ± 0.28	1.55 ± 0.25
	(g%)	0.31 ± 0.02	0.31 ± 0.01	0.31 ± 0.02	0.33 ± 0.02	0.33 ± 0.05	0.33 ± 0.03	0.35 ± 0.05	0.33 ± 0.04
Liver	(g)	15.98 ± 1.00	15.65 ± 1.04	15.49 ± 1.93	15.57 ± 1.02	10.44 ± 1.36	11.87 ± 0.72*	11.48 ± 1.09	11.17 ± 0.49
	(g%)	3.55 ± 0.21	3.49 ± 0.18	3.54 ± 0.28	3.81 ± 0.18	2.88 ± 0.24	2.51 ± 0.17	2.50 ± 0.27	2.42 ± 0.05
Spleen	(mg)	763 ± 110	791 ± 77	704 ± 61	682 ± 111	774 ± 70	696 ± 93	785 ± 208	780 ± 80
	(mg%)	170 ± 30	177 ± 17	162 ± 20	166 ± 16	170 ± 21	147 ± 20	173 ± 58	169 ± 13
Kidneys	(g)	2.91 ± 0.19	2.97 ± 0.15	2.96 ± 0.19	2.82 ± 0.19	3.10 ± 0.51	2.86± 0.07	2.87 ± 0.24	2.92 ± 0.15
	(g%)	0.65 ± 0.05	0.66 ± 0.05	0.68 ± 0.07	0.69 ± 0.05	0.68 ± 0.11	0.60 ± 0.03	0.62 ± 0.04	0.64 ± 0.02
Adrenals	(mg)	50.7 ± 8.3	57.4 ± 8.6	54.8 ± 6.3	58.8 ± 11.2	55.6 ± 7.8	52.4 ± 6.8	53.2 ± 9.9	61.4 ± 7.3
	(mg%)	11.3 ± 2.1	12.8 ± 2.1	12.5 ± 1.2	14.4 ± 2.8	12.2 ± 1.8	11.0 ± 1.2	11.5 ± 1.8	13.3 ± 1.5
Testes	(g)	3.11 ± 0.35	3.18 ± 0.42	3.36 ± 0.18	3.33 ± 0.30	3.29 ± 0.34	3.30 ± 0.40	3.25 ± 0.28	3.36 ± 0.27
	(g%)	0.69 ± 0.10	0.71 ± 0.11	0.78 ± 0.10	0.82 ± 0.06	0.72 ± 0.08	0.70 ± 0.09	0.71 ± 0.05	0.73 ± 0.06
Epididymides	(mg)	1120 ± 71	1170 ± 100	1158 ± 74	1150 ± 88	1250 ± 135	1255 ± 101	1222 ± 80	1224 ± 101
	(mg%)	249 ± 23	262 ± 28	267 ± 29	281 ± 14	274 ± 31	265 ± 25	265 ± 8	265 ± 17
No. of female rats		6	6	6	2	6	6	6	6
Body weight	(g)	301 ± 12	298 ± 14	301 ± 11	295	297 ± 26	306 ± 8	327 ± 13**	310 ± 9
Brain	(g)	1.97 ± 0.08	1.95 ± 0.05	2.01 ± 0.08	1.98	1.99 ± 0.09	1.91 ± 0.09	1.95 ± 0.08	1.99 ± 0.02
	(g%)	0.66 ± 0.03	0.66 ± 0.03	0.67 ± 0.03	0.68	0.67 ± 0.08	0.63 ± 0.03	0.60 ± 0.03*	0.64 ± 0.02
Pituitary	(mg)	16.7 ± 2.8	16.2 ± 2.1	16.7 ± 2.3	15.8	18.9 ± 3.18	20.1 ± 3.0	18.9 ± 3.7	18.3 ± 2.6
	(mg%)	5.5 ± 0.8	5.5 ± 0.7	5.6 ± 0.9	5.3	6.5 ± 1.5	6.6 ± 1.1	5.8 ± 1.3	5.9 ± 0.9
Thyroids	(mg)	16.8 ± 2.4	16.5 ± 3.6	17.7 ± 4.0	17.4	20.0 ± 3.9	20.2 ± 1.9	18.0 ± 3.0	22.7 ± 4.4
	(mg%)	5.6 ± 0.8	5.6 ± 1.2	5.9 ± 1.3	5.9	6.8 ± 1.6	6.6 ± 0.7	5.5 ± 1.1	7.4 ± 1.5
Thymus	(mg)	234 ± 48	266 ± 73	292 ± 77	225	284 ± 59	273 ± 29	282 ± 75	303 ± 65
	(mg%)	78 ± 17	90 ± 25	97 ± 26	76	96 ± 19	89 ± 8	86 ± 22	98 ± 21
Heart	(g)	1.03 ± 0.08	0.99 ± 0.08	1.00 ± 0.05	1.03	0.95 ± 0.06	0.90 ± 0.03	1.00 ± 0.05	1.04 ± 0.07**
	(g%)	0.34 ± 0.03	0.33 ± 0.02	0.33 ± 0.02	0.35	0.32 ± 0.03	0.29 ± 0.01*	0.31 ± 0.01	0.34 ± 0.02
Liver	(g)	9.92 ± 0.90	9.66 ± 0.92	9.84 ± 0.55	10.70	7.33 ± 0.67	7.62 ± 0.59	8.03 ± 0.66	7.95 ± 0.59
	(g%)	3.29 ± 0.26	3.24 ± 0.21	3.27 ± 0.15	3.64	2.47 ± 0.15	2.49 ± 0.14	2.46 ± 0.24	2.57 ± 0.13
Spleen	(mg)	690 ± 79	679 ± 145	719 ± 100	606	537 ± 86	522 ± 93	560 ± 69	593 ± 28
	(mg%)	229 ± 26	227 ± 42	239 ± 31	203	182 ± 31	170 ± 28	172 ± 28	192 ± 8
Kidneys	(g)	2.14 ± 0.15	2.05 ± 0.20	2.04 ± 0.11	1.99	1.94 ± 0.09	1.98 ± 0.07	1.95 ± 0.16	2.01 ± 0.19
	(g%)	0.71 ± 0.06	0.69 ± 0.05	0.68 ± 0.04	0.67	0.66 ± 0.06	0.65 ± 0.02	0.60 ± 0.05	0.65 ± 0.05
Adrenals	(mg)	74.6 ± 7.6	70.0 ± 10.4	69.9 ± 7.2	83.5	72.9 ± 6.1	62.1 ± 12.2	71.1 ± 6.2	70.5 ± 11.1
	(mg%)	24.9 ± 3.1	23.5 ± 2.9	23.2 ± 2.1	28.6	24.7 ± 3.1	20.3 ± 3.9	21.8 ± 2.5	22.7 ± 3.1
Ovaries	(mg)	110.0 ± 15.3	100.9 ± 8.3	102.2 ± 8.9	120.1	82.9 ± 20.3	79.9 ± 10.0	91.9 ± 14.9	96.3 ± 4.2
	(mg%)	36.6 ± 4.8	34.0 ± 3.2	34.0 ± 2.9	40.9	27.7 ± 4.8	26.1 ± 3.0	28.2 ± 4.7	31.1 ± 0.9
Uterus	(mg)	605 ± 165	570 ± 123	631 ± 135	2133	672 ± 139	622 ± 174	706 ± 168	576 ± 161
	(mg%)	202 ± 58	193 ± 46	210 ± 47	742	229 ± 59	203 ± 55	216 ± 49	186 ± 48

[a] Values are given as the mean ± SD.
[b] Absolute organ weight.
[c] Relative organ weight.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

changes were found in the epididymides, ovaries, and uterus in the dinoseb-treated groups (data not shown).

Sperm Analysis

T6 Table VI shows results of sperm analysis at the end of the administration period in male rats given dinoseb. Sperm analysis showed that there were no significant differences between the 0.78 and 2.33 mg/kg bw/day groups and controls in any of the sperm tests. The motile sperm rate, progressive sperm rate, straight line velocity, and viability rate in the 7.0 mg/kg bw/day dose group were significantly lower than controls, and the amplitude of lateral head displacement, abnormal sperm rate, and abnormal tail rate were significantly higher than controls.

T7 Table VII shows results of sperm analysis at the end of the recovery period in male rats given dinoseb. At completion of the recovery period, sperm analysis revealed no significant changes at 0.78 and 2.33 mg/kg bw/day. At 7.0 mg/kg bw/day, a significantly lower viability rate and survivability rate, and higher abnormal sperm rate and abnormal head rate were found.

Reproductive and Developmental Findings

T8 Table VIII shows the reproductive and developmental findings in rats given dinoseb. No change attributable to the chemical was noted in the number of estrous cases, copulation index, number of conceiving days, number of pregnant females, fertility index, gestation length, number of corpora lutea, number of implantation sites, implantation index or delivery, and nursing conditions. 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. Slight focal atrophy of the seminiferous tubule was observed in one male at 7.0 mg/kg bw/day. No changes attributable to the chemical were noted in the total number of births, number of stillbirths, number of live pups born, sex ratio, delivery index, birth index, live birth index, general condition, number of live pups on day 4 of lactation, viability index, body weight of offspring, appearance, and necropsy findings in the 0.78 and 2.33 mg/kg bw/day dose groups. Acaudate or short tail was observed in each one pup at 0.78 mg/kg bw/day.

DISCUSSION

The present study was conducted to obtain initial information on the possible repeated dose toxicity and reproductive and developmental toxicity of dinoseb in rats. The data show that dinoseb exerts general toxicity and reproductive and developmental toxicity, but is unlikely to possess teratogenic potential under this test condition.

The dosage used in the present study was sufficiently high such that it should be expected to induce general toxic effects such as body weight and food consumption changes. A significant decrease in body weight gain was found at

TABLE VI. Sperm analysis of male rats at completion of the administration period

Dose (mg/kg bw/day)	0 (Control)	0.78	2.33	7.0
No. of animals	6	66	6	
Sperm motility[a]				
Motile sperm rate (%)	71.3 ± 2.8	72.8 ± 5.3	75.0 ± 7.4	59.2 ± 6.0**
Progressive sperm rate (%)	34.4 ± 3.7	37.3 ± 7.5	38.2 ± 8.4	22.9 ± 4.5*
Path velocity (μm/s)	150.7 ± 4.8	152.3 ± 6.1	153.6 ± 6.9	143.8 ± 6.0
Straight line velocity (μm/s)	110.4 ± 4.2	112.6 ± 5.5	111.7 ± 6.4	97.7 ± 8.2**
Curvilinear velocity (μm/s)	339.4 ± 11.4	335.9 ± 17.0	344.9 ± 12.8	319.4 ± 15.9
Amplitude of lateral head displacement (μm)	19.9 ± 0.3	19.5 ± 0.7	19.9 ± 1.0	20.9 ± 0.4*
Beat cross frequency (Hz)	29.5 ± 0.8	28.3 ± 1.6	29.6 ± 1.4	28.5 ± 1.7
Viability rate (%)[b]	99.8 ± 0.2	99.4 ± 0.5	99.4 ± 0.9	97.9 ± 2.2*
Survivability rate (%)[c]	76.7 ± 8.2	83.4 ± 6.4	80.9 ± 6.8	66.9 ± 10.1
Sperm morphology[d]				
Abnormal sperm rate (%)	2.5 ± 1.8	1.9 ± 1.0	2.0 ± 1.0	6.9 ± 4.6*
Abnormal head rate (%)	2.5 ± 1.8	1.9 ± 0.9	1.8 ± 0.8	5.6 ± 4.3
Abnormal tail rate (%)	0.0 ± 0.0	0.1 ± 0.1	0.2 ± 0.3	1.3 ± 1.0**
No. of sperm in left cauda epididymis ($\times 10^6$)	268.8 ± 25.1	342.9 ± 50.1	334.8 ± 49.2	308.4 ± 114.2
No. of sperm/g weight of left cauda epididymis ($\times 10^6$)	1070.0 ± 94.9	281.1 ± 109.5	1238.2 ± 114.3	1227.4 ± 279.5

[a] Values are given as the mean ± SD.
[b] (Number of live sperm + number of sperm that died during incubation/number of sperm examined) × 100.
[c] (Number of live sperm/number of sperm examined) × 100.
[d] (Number of abnormal sperm/number of sperm examined) × 100.
* Significantly different from the control group ($p < 0.05$).
** Significantly different from the control group ($p < 0.01$).

TABLE VII. Sperm analysis of male rats at completion of the recovery period

Dose (mg/kg bw/day)	0 (control)	0.78	2.33	7.0
Number of animals	6	6	6	6
Sperm motility[a]				
Motile sperm rate (%)	78.5 ± 6.5	76.6 ± 10.3	72.7 ± 3.3	71.5 ± 4.4
Progressive sperm rate (%)	29.3 ± 9.5	30.4 ± 12.2	33.3 ± 11.2	26.5 ± 6.7
Path velocity (μm/s)	144.2 ± 8.0	147.9 ± 8.0	147.8 ± 8.6	141.3 ± 5.5
Straight line velocity (μm/s)	101.9 ± 6.2	104.1 ± 6.8	107.0 ± 10.3	97.7 ± 6.4
Curvilinear velocity (μm/s)	327.4 ± 22.2	335.4 ± 26.4	324.6 ± 22.6	324.2 ± 13.9
Amplitude of lateral head displacement (μm)	19.9 ± 0.3	19.5 ± 0.7	19.9 ± 1.0	20.9 ± 0.4*
Beat cross frequency (Hz)	20.2 ± 0.9	20.4 ± 0.8	19.8 ± 1.0	20.3 ± 0.8
Viability rate (%)[b]	99.9 ± 0.2	99.8 ± 0.3	99.7 ± 0.3	98.8 ± 1.1*
Survivability rate (%)[c]	84.1 ± 4.1	83.1 ± 3.3	81.0 ± 4.6	78.2 ± 3.1*
Sperm morphology[d]				
Abnormal sperm rate (%)	1.5 ± 1.0	2.4 ± 2.2	2.3 ± 1.4	4.6 ± 2.8*
Abnormal head rate (%)	1.5 ± 1.0	2.3 ± 2.2	2.2 ± 1.4	4.2 ± 2.5*
Abnormal tail rate (%)	0.0 ± 0.0	0.1 ± 0.2	0.1 ± 0.2	0.3 ± 0.4
No. of sperm in left cauda epididymis ($\times 10^6$)	358.4 ± 17.4	385.5 ± 70.5	347.8 ± 48.8	332.1 ± 28.8
No. of sperm/g weight of left cauda epididymis ($\times 10^6$)	1134.5 ± 72.6	1201.3 ± 1191.9	1118.9 ± 97.2	1087.3 ± 90.0

[a] Values are given as the mean ± SD.
[b] (Number of live sperm + number of sperm that died during incubation/number of sperm examined) × 100.
[c] (Number of live sperm/number of sperm examined) × 100.
[d] (Number of abnormal sperm/number of sperm examined) × 100.
* Significantly different from the control group ($p < 0.05$).

7.0 mg/kg bw/day in both sexes as expected. On the other hand, a significant increase in food consumption was observed from the mid stage of the administration period at 7.0 mg/kg bw/day in both sexes, and this effect was continued during the recovery period. In the 1930s, 2,4-dinitrophenol, a compound very similar to dinoseb, was prescribed as a weight-reducing agent. It is estimated that about 100 000 people in the United States used this drug while it was available. Because the energy is released as heat by uncoupling of electron transport from ATP synthesis, weight loss was remarkable (ATSDR, 1995; DOH, 2005). The decreased body weight gain with increased food consumption observed in this study is consistent with dinoseb's action as a metabolic activator. Because body weight gain was significantly increased during the recovery period at 7.0 mg/kg bw/day in males, the metabolic activation due to dinoseb seems temporary. At the end of the administration period, relative brain weight was significantly higher than controls with no significant difference in absolute weight in males at 7.0 mg/kg bw/day, suggesting that the difference in body weight in comparison with controls was responsible.

Transient salivation was observed immediately after administration at 0.78 mg/kg bw/day and higher, but it was considered that because of stimulation by dinoseb neither morphological changes in the salivary gland nor other neurological changes were observed. Females that died or were moribund showed a decrease in spontaneous motor activity, prone position, low body temperature, dyspnea, cyanosis, and bradypnea. In the acute toxicity, dinoseb can cause death because of respiratory and circulatory disorders (Rotterdam Convention, 2003). In this study, females that died or were moribund also showed adverse effects in the respiratory and circulatory systems with congestion in the lungs and livers.

Significant increases in RBC count, hematocrit, haemoglobin, or MCV found in the treated group animals indicate that dinoseb has a potential to induce polycythemia in rats. These changes were considered to be a result of regulation to low oxygen levels of cells. Dinoseb increases the rate of oxygen consumption in cells (Palmeira et al., 1994), and RBC production can be regulated by erythropoietin for need of oxygen (MedicineNet, 2001). Splenic extramedullary hematopoiesis, which was substantial in control pregnant rats, was significantly decreased in the 2.33 mg/kg bw/day females. This effect was also observed in one surviving female at 7.0 mg/kg bw/day. Reduction in splenic extramedullary hematopoiesis was considered to be a physiologic response to the decreased need for hematopoiesis because of polycythemia condition. In the blood chemistry, creatinine was significantly higher at 7.0 mg/kg bw/day in the main group males. However, no histopathological changes were found in the kidney, and other related parameters were not affected, suggesting that this change seems unlikely to be due to kidney failure but rather due to changes in muscle metabolism by dinoseb as an uncoupling agent. PT was significantly higher at 2.33 mg/kg bw/day (13.8 ± 0.3 s) and 7.0 mg/kg bw/day (14.1 ± 0.4 s), but

TABLE VIII. Reproductive and developmental findings in rats given dinoseb

Dose (mg/kg bw/day)	0 (control)	0.78	2.33	7.0
No. of females	12	12	12	12
No. of estrous cases before pairing (14 days)[a]	3.5 ± 0.5	3.3 ± 0.5	3.4 ± 0.5	3.3 ± 0.5
No. of pairs with successful copulation	12	12	12	12
Copulation index (%)[b]	100	100	100	100
No. of days till copulation after pairing[a]	2.3 ± 1.1	2.5 ± 1.2	2.4 ± 1.2	2.5 ± 1.3
No. of pregnant females	11	11	12	12
Fertility index (%)[c]	91.7	91.7	100.0	100.0
Length of gestation (days)[a]	22.2 ± 0.4	22.4 ± 0.9	22.2 ± 0.6	22.0
No. of corpora lutea[a]	16.6 ± 2.2	16.3 ± 2.1	16.1 ± 1.4	15.3 ± 1.2
No. of implantations[a]	14.6 ± 2.4	14.8 ± 1.8	14.6 ± 1.6	13.9 ± 1.1
Implantation index (%)[a,d]	87.6 ± 7.4	91.3 ± 5.5	90.6 ± 4.5	91.4 ± 6.6
No. of dead or moribund pregnant females during pregnancy	0	0	0	10
No. of dams delivered pups	11	11	12	2**
No. of dams without live pups	0	0	0	1
No. of dams with live pups	11	10	12	1**
No. of pups born[a]	13.9 ± 1.9	13.2 ± 3.4	13.2 ± 2.6	11.0
No. of stillbirths[a]	0.2 ± 0.4	1.2 ± 1.6	0.2 ± 0.4	6.0
No. of live pups born[a]	13.7 ± 1.8	12.0 ± 4.3	13.0 ± 2.5	5.0
Sex ratio at birth (male/female)[a,e]	1.09 ± 0.65 (74/77)	1.12 ± 0.44 (67/65)	1.28 ± 0.94 (80/76)	1.00 (5/5)
Delivery index (%)[a,f]	95.5 ± 5.5	88.2 ± 20.3	89.9 ± 14.1	84.5
Birth index (%)[a,g]	94.4 ± 5.7	79.4 ± 28.4	88.8 ± 13.4	41.5
Live birth index (%)[a,h]	98.8 ± 2.6	85.4 ± 29.7	98.8 ± 2.7	50
No. of live pups on day 4 of lactation[a]	13.6 ± 1.7	12.5 ± 1.6	12.8 ± 2.6	8.0
Viability index (%)[a,i]	99.4 ± 2.1	95.4 ± 10.3	98.1 ± 4.7	80
Body weight of pups[a]				
Male				
Day 0	6.8 ± 0.5	6.5 ± 0.4	6.7 ± 0.5	6.8
Day 4	11.1 ± 1.4	10.8 ± 1.1	10.6 ± 1.2	8.2
Female				
Day 0	6.4 ± 0.6	6.2 ± 0.5	6.3 ± 0.6	6.8
Day 4	10.2 ± 1.5	10.5 ± 1.3	9.9 ± 1.5	8.4
No. of fetuses (litters) with external malformation	0	2 (2)	0	0
Acaudate	0	1 (1)	0	0
Short tail	0	1 (1)	0	0

[a] Values are given as the mean ± SD.
[b] (Number of pairs with successful copulation/number of pairs) × 100.
[c] (Number of pregnant females/number of pairs with successful copulation) × 100.
[d] (Number of implantation scars/number of corpora lutea) × 100.
[e] Number of male pups/number of female pups.
[f] (Number of pups born/number of implantation scars) × 100.
[g] (Number of live pups born/number of implantation scars) × 100.
[h] (Number of live pups born/number of pups born) × 100.
[i] (Number of live pups on day 4/number of live pups born) × 100.
** Significantly different from the control group ($p < 0.01$).

these were within the range of historical background data for the laboratory that performed this study (16.2 ± 1.8 s). In the 7.0 mg/kg bw/day group, ALP and albumin were significantly lower and BUN was significantly higher. All of these differences were not observed at completion of the administration period and suggested not to be attributable to administration of the test substance. Other changes in hematology or blood chemistry were dose independent.

It seems unlikely that dinoseb exerts reproductive toxicity to female rats when administered during the premating and early pregnancy period because no adverse effects on the estrous cyclicity, copulation, or fertility were caused by the administration of dinoseb in females. However, dinoseb is suggested to be reproductively toxic during mid and late pregnancy because death was found during late pregnancy in the main group females but not in the recovery group

females, more specifically in nonpregnant females, at 7.0 mg/kg bw/day.

In the 7.0 mg/kg bw/day dose group males, motile sperm rate, progressive sperm rate, straight line velocity, and viability rate were significantly lower than controls, and the amplitude of lateral head displacement, abnormal sperm rate, and abnormal tail rate were significantly higher than controls at the end of the administration period. The viability rate and survivability rate were also significantly lower than controls and the abnormal sperm rate and abnormal head rate were significantly higher than controls at the end of the recovery period. These effects are thought to be caused by the uncoupling effects of dinoseb rather than due to a body weight loss and a body temperature increase (Linder et al., 1982). On the histopathological findings of this study, focal atrophy of the seminiferous tubule in the testis was observed in one male at 7.0 mg/kg bw/day. However, this change was considered to be spontaneous because the degree of histopathological change was slight, and the incidence at this dose was low and not significantly different from the control group. There were no dose-related effects on spermatogenesis at stages of spermatogonia and spermatocyte in this study. However, in a feeding study of dinoseb (0–22.2 mg/kg bw/day) to Sherman rats, histopathological changes to spermatogonia, spermatocyte, spermatid, or sperm in the testes were observed after 20 or 30 days of administration at 22.2 mg/kg bw/day, and a critical effect to the spermatogonia was observed after 50 days of treatment at 22.2 mg/kg bw/day. After 11-week administration, sperm counts were significantly decreased at 9.1 mg/kg bw/day and above. In addition, spermatozoa were not found in sections of the epididymides at 22.2 mg/kg bw/day (Linder et al., 1982). Another spermatotoxicity study, in which males rats were treated with dinoseb for 5 days, showed no effect of treatment on sperm parameters in the cauda epididymis 3 days after the final dose but after 14 days, reduced sperm motility and increased incidence of tailless sperm were noted (Takahashi et al., 2004). These findings suggest that dinoseb affects sperm indirectly by disturbing spermatogenesis or maturation process of sperm in the epididymis and that prolonged exposure to dinoseb in feed affects the early stage of spermatogenesis.

As for the developmental parameters, no changes attributable to the chemical were noted in the 0.78 and 2.33 mg/kg bw/day dose groups. Developmental toxicity of dinoseb was not completely estimated in the present study, because only one dam with live pups was obtained at the highest dose. At 0.78 mg/kg bw/day, one pup was observed to be acaudate and another to have a short tail. However, incidences of fetuses with an external malformation at this dose were very low and not significantly different from those in the control group. The external malformations observed in the present study are of the types that occur spontaneously among control rat fetuses (Kameyama et al., 1980; Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000). Prenatal developmental toxicology studies by feeding administration of dinoseb showed teratogenic effects such as hypoplastic tails or microphthalmia in rats (Spencer and Sing, 1982; Giavini et al., 1986), but teratogenic effects were not induced by gavage dose (Giavini et al., 1986) or intraperitoneal administration (McCormack et al., 1980; Daston et al., 1988) in rats. Giavini et al. (1986) reported that gavage dose of dinoseb (0, 2.5, 5, 10, or 15 mg/kg bw/day) induced both maternal toxicity and developmental toxicity without teratogenic effects; however, administration of dinoseb in a diet induced microphthalmia at 200 ppm (~15 mg/kg bw/day in feed) with reduction in maternal body weight gain. Based on the results of the present study, dinoseb is considered to be unlikely to exert teratogenic effects by gavage dose, which is in agreement with the literature. There are no studies showing differences in toxicokinetics of dinoseb by gavage dose and feed administration, but there are some examples that gavage and feed administration show differences in toxicokinetics of chemicals (Yuan et al., 1994, 1995). Differences in absorption, distribution, metabolism, and/or elimination may explain the different teratogenic responses of dinoseb observed in these studies. Further studies are needed to clarify the differences in the toxicokinetics of dinoseb by gavage dose and feeding.

The present study was performed in compliance with the OECD guideline 422 (OECD, 1996), and this screening test does not provide complete information on all aspects of reproduction and development because of the relatively small numbers of animals in the dose groups. In the present study, only two females delivered their pups at the highest dose group; therefore, the developmental toxicity to pups was not fully assessed. The LOAEL for males and NOAEL for females were 0.78 mg/kg bw/day based on increases in hematocrit in males at 0.78 mg/kg bw/day and higher and decreased extramedullary hematopoiesis of the spleen in females at 2.33 mg/kg bw/day. The NOAEL for reproductive/developmental toxicity was considered to be 2.33 mg/kg bw/day based on sperm motility and morphology in males and decreased number of dams with live pups in females.

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Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats

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Abstract

Male and female rats were fed a diet containing flame retardant hexabromocyclododecane (HBCD) at 0, 150, 1500 or 15,000 ppm throughout the study beginning at the onset of a 10-week pre-mating period and continuing through the mating, gestation and lactation periods for two generations. The mean daily intakes of HBCD during the whole period of administration were 10.2, 101 and 1008 mg/kg bw in F0 males, 14.0, 141 and 1363 mg/kg bw in F0 females, 11.4, 115 and 1142 mg/kg bw in F1 males, and 14.3, 138 and 1363 mg/kg bw in F1 females for 150, 1500 and 15,000 ppm, respectively. The incidence of rats with decreased thyroid follicles size was increased in F0 and F1 males and females at 1500 ppm and higher. Serum TSH levels were increased in F0 and F1 females at 1500 ppm and higher, and serum T4 levels were decreased in F0 males and females at 15,000 ppm. The number of the primordial follicles in the ovary of F1 females was reduced at 1500 ppm and higher. There were increases in the absolute and relative weights of the liver in male adults and male and female weanlings at 1500 ppm and higher, and in female adults at 15,000 ppm, and of the thyroid in male and female adults at 15,000 ppm. Decreased body weight and body weight gain associated with reduced food consumption were found in F1 males and females at 15,000 ppm. Decreases were found in the viability index of F2 pups and the body weight of male F1 and F2 pups and female F2 pups at 15,000 ppm. In F2 pups, there were low incidences of the completion of eye opening in males at 15,000 ppm and in females at 1500 ppm and higher, and of completed mid-air righting in females at 15,000 ppm. The data indicate that the NOAEL of HBCD in this study was 150 ppm (10.2 mg/kg bw/day). The estimated human intake of HBCD is well below the NOAEL in the present study.

Keywords: Hexabromocyclododecane; Brominated flame retardant; Two-generation reproductive toxicity; Developmental toxicity; Rat

1. Introduction

Although about 80 different brominated organic flame retardants are registered, tetrabromobisphenol A, the polybrominated diphenyl ethers and hexabromocyclododecane (HBCD) account for most of the total volume [1]. HBCD is a nonaromatic, brominated cyclic alkane used as an additive flame retardant. Total market demand for HBCD in 2001 was estimated as 2800 tons in America, 9500 tons in Europe, 3900 tons in Asia and 500 tons in the rest of the world [2]. The commercial product is a mixture of three stereoisomers, alpha, beta and gamma, which are typically present at approximately 6, 8 and 80%, respectively [3]. Its primary application is in extruded (XPS) and expanded (EPS) polystyrene foam that is used as thermal insulation in the building industry. HBCD is the only suitable flame retardant for these applications. A secondary, although important, application of HBCD is as a flame retardant for upholstery textiles [3,4]. The partition coefficient (Log Kow) value of 5.6 suggests that this chemical is suspected to have high bioaccumulation potential [4]. HBCD has been used for about 20 years, and is detected in practically all environmental media [5]. HBCD was identified in sediment from several places along the River Viskan in Sweden [6] and the River Cinca in Spain [7]. HBCD was detected in fishes, pike (*Esox lucius*) [6] and barbel (*Barbus graellsi*) [7], indicating that it is bioavailable and bioaccumulates. The bioconcentration factor of this compound is reported to be 18,100 in fathead minnow (*Pimephales promelas*) [8]. HBCD was also detected from common whelk (*Buccinium undatum*), sea star (*Asterias rubens*), hermit crab (*Pagurus bernhardus*), gadoid fish species whiting (*Merlangius merlangus*), cod (*Gadus morhua*),

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harbor seal (*Phoca vitulina*) and harbor porpoise (*Phocoena phocoena*) from the North Sea [9]. These findings show evidence of HBCD bioaccumulation at the trophic level and biomagnification in the ascending aquatic food chain [9]. As a result of widespread use and the physical and chemical properties, HBCD is now considered to be a ubiquitous contaminant in the environment and humans [5,10]. It could be hypothesized that food intake is the largest single source of human exposure to HBCD [11].

HBCD was detected at ranging from 0.3 to 20 μg/g lipid in 49 samples of the 85 human breast milk samples collected from Norway between 1993 and 2001 [12]. The concentration of HBCD in the Stockholm human milk showed a fluctuating increase over time, and from 1980 the concentration increased from 0.13 pmol/g lipid to 0.60 pmol/g lipid in 2004 [13]. The HBCD concentration of human milks collected in 2002 to 2003 from North America was ranging from 0.3 to 10 μg/g lipid [14]. The presence of such a chemical compound in biological systems has aroused great concern about its toxicological potential. 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 [15]. The toxic effects of HBCD are briefly summarized by NRC [4], American Chemical Council [3], de Wit [16], Darnerud [11], Birnbaum and Staskal [17]. However, information on the effects of HBCD is insufficient to assess the overall toxicity of this compound. Following oral administration to male rats, HBCD was rapidly absorbed from the gastrointestinal tract, distributed primarily to the body fat, and eliminated rapidly, primarily in the feces [4]. In a 28-day repeated dose toxicity study, no toxic effects were noted in male and female SD rats at any dose of HBCD given by gavage at up to 1000 mg/kg bw/day [18]. In a 90-day repeated dose toxicity study in SD rats given HBCD at 0, 100, 300, or 1000 mg/kg bw/day by gavage, increased weights of the liver and prostate, and γ-glutamyltransferase, and decreased weight of the thyroid/parathyroid were found [19]. The author of this study concluded that these changes were probably of limited, if any, toxicological significance, because they were reversible, and not associated with specific target organ damage or diminished function. The dose-related effects of HBCD on the thyroid hormone axis were observed in a recent 28-day repeated dose study (OECD407) enhanced for endocrine and immune parameters using Wistar rats dosed by gavage at 0–200 mg/kg bw/day [20]. After a single dose of HBCD by gavage at 0.9 or 13.5 mg/kg bw by gavage on postnatal day (PND) 10, spontaneous activity and learning and memory in the water maze were altered when tested at the age of 3 months in NMRI mice [21]. As for the developmental toxicity of HBCD, two studies are available. There was no maternal or developmental toxicity in SD rats given HBCD by gavage on days 6–19 of pregnancy at any doses up to 1000 mg/kg bw/day [22]. No maternal or developmental toxicity was noted in Wistar rats given HBCD in diet at up to 1% (equivalent to 600 mg/kg bw/day) on days 0–20 of pregnancy [23]. No reproductive difficulties in dams or postnatal development in offspring were found even at the highest dose.

Although the testing for reproductive toxicity in an animal model is an important part of the overall toxicology, no information is available for the reproductive toxicity of HBCD at the present time; therefore, a two-generation reproductive toxicity study was conducted.

2. Materials and methods

This study was performed in 2005–2006 at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan) in compliance with the OECD guideline 416 Two-generation Reproduction Toxicity Study [24]. This study was conducted in accordance with the principles for Good Laboratory Practice [25], "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].

2.1. Chemical and dosing

Hexabromocyclododecane (HBCD; 1,2,5,6,9,10-hexabromocyclododecane; CAS No. 3194-55-6) was obtained from Wildlife International, Ltd. (Easton, MD). The test substance was a composite of HBCD commercial products from Albemarle Corporation (Baton Rouge, LA), Great Lakes Chemical Corporation (West Lafayette, IN) and Ameribrom Inc. (New York, NY), and Wildlife International, Ltd. prepared the composite. The preparation of HBCD was a mixture of three enantiomers. HBCD-α, HBCD-β and HBCD-γ, and their respective proportions in the used batch were 8.5, 7.9 and 83.7%. The HBCD (test substance number # 7086) used in this study was 99.7% pure, and was kept in a sealed container under cool (2–7 °C) and dark conditions. The purity and stability of the chemical were verified by analysis using liquid chromatography before and after the study.

Rats were given dietary HBCD at a concentration of 0 (control), 150, 1500 or 15,000 ppm. The dosage levels were determined based on the results of a previous 90-day oral repeated dose toxicity study [19] in male and female Crl:CD(SD)IGS BR rats given HBCD at 0, 100, 300 or 1000 mg/kg bw/day for 90 days. The author concluded that all test article-related changes, even at 1000 mg/kg bw/day, were reversible, not associated with specific target organ damage or diminished function (data not shown).

Dosed diet preparations were formulated by mixing HBCD into an appropriate amount of a powdered basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) for each dietary concentration. The control rats were fed a basal diet only. Analysis showed that the HBCD was homogeneous in the diet and stable for at least 21 days at room temperature, and was administered at the desired feed concentrations throughout the study.

2.2. Animals and housing conditions

Crl:CD(SD) rats were used throughout this study. Rats of this strain were chosen because they are the most commonly used in reproductive and developmental toxicity studies, and historical control data are available. Male and female rats at 4 weeks of age were purchased from Tsukuba Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The males and females were acclimated to the laboratory for 7 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. One hundred and ninety-two rats were randomly assigned 24/sex/group as F0 animals, and all animals were assigned a unique number and ear tattooed 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, Inc.).

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Animals were reared on a basal diet or diet containing HBCD and filtered tap water *ad libitum* and maintained in an air-conditioned room at 22 ± 3 °C, with humidity of 50 ± 20%, a 12-h light/dark (20:00–08:00) cycle and ventilation at 10–15 times/h.

2.3. Experimental design

Twenty-four F0 rats (5-week-old males and females)/sex/group were fed a diet containing HBCD at 0, 150, 1500 or 15,000 ppm for 10 weeks prior to the mating period. Administration of HBCD was continued throughout the mating, gestation and lactation periods. Twenty-four male and 24 female F1 weanlings (1 male and 1 female in each litter) in each group were selected as F1 parents on PNDs 21–25 to equalize the body weights among groups. The day on which F1 parental animals were selected was designated as 0 week of dosing for the F1 generation. The administration of HBCD in the diet was not suspended during PNDs 21–25. F1 selected rats were administered HBCD in the diet of their respective formulations in the same manner as described for F0 rats. Administration of HBCD in the diet was continued throughout the mating, gestation and lactation periods. On PND 26, unselected F1 weanlings and all F2 weanlings were necropsied.

2.4. Mating procedures

Each female was mated with a single male of the same dosage group until copulation occurred or the mating period had elapsed. The mating periods for F0 and F1 animals were 3 weeks. 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 as evidence of successful mating. The day of successful mating was designated as day 0 of pregnancy. F0 females that did not mate during the 3-week mating period were cohabited with another male from the same group who had been proven to copulate. For F1 matings, cohabitation of siblings was avoided.

2.5. Parental data

All adult rats were observed twice a day for clinical signs of toxicity, and body weights and food consumption were recorded weekly. For females exhibiting evidence of successful mating, body weight and food consumption of dams were recorded on days 0, 7, 14 and 20 of pregnancy and days 0, 4, 7, 14 and 21 of lactation. Daily vaginal lavage samples of each F0 and F1 female were evaluated for estrous cyclicity throughout the 2-week pre-cohabitation period and during cohabitation until evidence of copulation was detected. Females having repeated 4–6 day estrous cycles were judged to have normal estrous cycles. After weaning their pups, parental female rats were necropsied at the proestrous stage of the estrous cycle. For each female, the number of uterine implantation sites was recorded.

2.6. Litter data

Once insemination was confirmed, female rats were checked at least three times daily on days 21–25 of pregnancy to determine the time of delivery. The females were allowed to deliver spontaneously and nurse their pups until PND 21 (the day of weaning). 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, and live pups were counted, sexed, examined grossly, and individually weighed on PNDs 0, 4, 7, 14 and 21. On PND 4, litters were randomly adjusted to eight pups comprising of four males and four females. No adjustment was made for litters of fewer than eight pups. Pups were assigned a unique number and limb tattooed on PND 4.

2.7. Developmental landmarks

All F1 and F2 pups were observed for pinna unfolding on PND 3, incisor eruption on PND 11, and eye opening on PND 14. One male and one female F1 and F2 pup selected from each dam were evaluated for the surface righting reflex on PND 5, negative geotaxis reflex on PND 8, and mid-air righting reflex on PND 18 [26]. All F1 offspring selected as F1 parents were observed daily for male preputial separation beginning on PND 35 or female vaginal opening beginning on PND 25. Body weight of the respective F1 rats was recorded on the day of preputial separation or vaginal opening. The anogenital distance (AGD) was measured using calipers on PND 4 in all F1 and F2 pups, and the normalized value of AGD to body weight, AGD per cube root of body weight ratio, was calculated [27].

2.8. Behavioral tests

Spontaneous locomotor activity was measured with a multi-channel activity monitoring system (Supermex; Muromachi Kikai Co., Ltd., Tokyo, Japan) in 10 male and 10 female F1 rats selected from each group at 4 weeks of age. Rats were placed individually in transparent polycarbonate cages (27.6 W × 44.5 D × 20.4 H cm, CL-0108-1, CLEA Japan Inc., Tokyo, Japan), which were placed under an infrared sensor that detects thermal radiation from animals. Spontaneous motor activity was determined for 10 min intervals and for a total of 60 min.

A test in a water-filled multiple T-maze was conducted in 10 male and 10 female F1 rats selected from each group at 6 weeks of age. The apparatus was similar to that described by Biel [28]. The water temperature of the maze was kept 21–22 °C. As a preliminary swimming ability test, each rat was allowed to swim three times in a straight channel on the day before the maze trial, and then tested in the maze with three trials per day for the next three consecutive days. The elapsed time between entry into the water at the starting point and touching the goal ramp and number of errors were recorded. To prevent the exhaustion of the rats, no animal was allowed to remain in the water for more than 3 min in any trial.

2.9. Termination/necropsy adults

Parental rats were necropsied: males after the parturition of paired females, females after weaning of their pups. The proestrous stage of the estrous cycle was characterized by examination of the vaginal smears of female rats on the day of necropsy. A complete necropsy was performed on all rats found dead and those killed at the scheduled sacrifice. Live rats were euthanized by exsanguination under ether anesthesia. The external surfaces of the rats were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. Weights of the brain, pituitary, thyroid, thymus, liver, kidney, spleen, adrenal, testis, epididymis, seminal vesicle (with coagulating glands and their fluids), ventral prostate, uterus and ovary were recorded. Weights of the thyroid and seminal vesicle were measured after fixation. Major organs were stored in 10% neutral-buffered formalin. The testis and epididymis were fixed with Bouin's solution and preserved in 70% ethanol.

Histopathological evaluation of F0 and F1 adults was performed on the tissues specified below after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin: the pituitary, liver, thymus, kidney, spleen, adrenal, bone marrow, mesenteric lymph node, Peyer's patches, testis, epididymis, seminal vesicle, coagulating gland, ventral prostate, ovary, uterus, vagina and mammary gland of all males and females in the control and highest dose (15,000 ppm) groups and of females with abnormal estrous cycles, males and females without evidence of copulation or insemination and females with abnormal delivery or totally dead pups in all groups. Any organs or tissues of F0 and F1 adults showing gross alterations were evaluated histopathologically. The thyroid in all rats in all groups was examined histopathologically. In ten F1 females of each group, the number of primordial follicles was counted [29]. The right ovary was fixed in 10% neutral-buffered formalin and then dehydrated and embedded in paraffin in a longitudinal orientation by routine procedures. Sections were cut serially at 5 μm and every 20th section was serially mounted on a slide and stained with hematoxylin and eosin. About 40 sections per ovary were used to determine the primordial follicles.

2.10. Termination/necropsy pups

Following the adjustment of litter size on PND 4, culled pups were euthanized by inhalation of carbon dioxide and subjected to a gross external and internal necropsy. No tissues from these pups were collected.

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The weanlings not selected to become parents were euthanized and necropsied as described for the adults. Organ weights of one male and one female F1 and F2 weanling selected from each dam were measured as described above for adults. The weights of the pituitary, thyroid and seminal vesicle were not determined. All pups found dead before weaning were also necropsied.

In all male and female F1 and F2 weanlings whose organs were collected, histopathological evaluations of the liver, in the control and 15,000 ppm groups, and thyroid, in all groups, were performed after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin.

2.11. Hematological and blood biochemical parameters

On the day of the scheduled sacrifice, blood samples were collected from the abdominal aorta of adult rats under ether anesthesia.

Hematological examinations were performed for 10 males and 10 females of F0 and F1 rats randomly selected from each group. Blood samples were analyzed for the following hematological parameters, using 2K-EDTA as an anticoagulant: white blood cell (WBC) count and differential leukocyte count.

Blood biochemical evaluations were performed in 10 males and 10 females of F0 and F1 rats randomly selected from each group. Serum samples obtained from centrifuged whole blood were analyzed for biochemistry parameters such as total protein, albumin and globulin.

2.12. Serum hormone levels

On the day of the scheduled sacrifice, blood samples were collected from the abdominal aorta of adult rats. Eight males and eight proestrous females of F0 and F1 generations from each group were selected randomly for blood collection. Hormone levels were determined by Panapharm Laboratories Co., Ltd. (Uto, Japan). Serum levels of testosterone, 5α-dihydrotestosterone (DHT), luteinizing hormone (LH) and follicle stimulating hormone (FSH), thyroxine (T4), triiodothyronine (T3) and thyroid stimulating hormone (TSH) in males, and estradiol, progesterone, LH, FSH, T3, T4 and TSH in females were measured with a radioimmunoassay kit. Double antibody kits were used for measurement of testosterone, estradiol, progesterone, T3 and T4 concentration (Diagnostic Products Corp., Los Angeles, CA) and DHT concentration (Diagnostic Systems Laboratories Inc., Webster TX). Serum concentrations of LH, FSH and TSH were measured using (rat LH)[^{125}I], (rat FSH)[^{125}I] and (rat TSH)[^{125}I]assay systems (Amersham Biosciences Ltd., Little Chalfont, Buckinghamshire, UK), respectively.

2.13. Sperm parameters

Sperm parameters were determined for all F0 and F1 male adults on the day of the scheduled sacrifice. The right testis was used to count testicular homogenization-resistant spermatid heads. The right cauda epididymis was weighed and used for sperm analysis. Sperm motility was analyzed using a computer-assisted cell motion analyzer (TOX IVOS, Hamilton Thorne Biosciences, Beverly, MA). The percentage of motile sperm and progressively motile sperm, and the swimming speed and pattern were determined. After recording sperm motion, the cauda epididymal fluid was diluted and the sperm were enumerated using a hemacytometer under a light microscope. Sperm count per gram of epididymal tissue was obtained by dividing the total count by the gram weight of the cauda epididymis. Sperm were stained with eosin and mounted on a slide glass. Two hundred sperm in each sample were examined under a light microscope, and the percentage of morphologically abnormal sperm was calculated.

2.14. Statistical analysis

Statistical analysis was performed according to the methods of Gad [30]. Data on offspring before weaning were statistically analyzed using the litter as the experimental unit.

Body weight, body weight gain, food consumption, length of estrous cycle, pre-coital interval, gestation length, numbers of implantations and pups delivered, delivery index, sperm parameters, hematological and blood biochemical parameters, hormone levels, organ weight, organ/body weight ratio (relative organ weight), number of primordial follicles, reflex response time, age and body weight at sexual maturation, parameters of behavioral tests, AGD, AGD/cube root of body weight ratio, and viability of pups were analyzed for statistical significance using the following method. 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 the Mann–Whitney *U* test.

The incidence of pups with changes in clinical and gross internal observations, and completion rate of developmental landmarks and reflexes were analyzed by the Wilcoxon rank sum test.

The incidence of parent animals with changes in clinical, gross internal and histopathological findings, the incidence of weanlings with changes in histopathological findings, the incidence of females with normal estrous cycles, the copulation index, fertility index, gestation index, neonatal sex ratio and completion rate of the reflex response test were analyzed by Fisher's exact test.

The 0.05 level of probability was significant. The probability was designated as the cut-off for statistical significance.

3. Results

3.1. Clinical observations, body weight and food consumption during the pre-mating, mating, gestation and lactation periods (F0 and F1)

One F0 male at 15,000 ppm was euthanized at 13 weeks of dosing because of a moribund condition resulting from accidental injury in the home cage. One F1 male at 1500 ppm was dead from accidental injury in the home cage. One F0 male at 15,000 ppm and one F1 male at 1500 ppm died without any apparent clinical signs of toxicity at 5 and 7 weeks of dosing, respectively. In F0 females at 15,000 ppm, one was euthanized during the pre-mating period because of a moribund condition, and one died on day 22 of pregnancy due to dystocia. No significant difference was seen between control and HBCD-treated groups in the incidence of clinical signs of toxicity in either male or female F0 and F1 rats during the pre-mating, mating, gestation, or lactation period (data not shown).

Fig. 1 shows the body weights of F0 males and females during dosing. In F0 males, the mean body weight and/or body weight gain were significantly higher than those of controls almost throughout the dosing period at 1500 ppm and in the first 5 weeks of dosing at 15,000 ppm. In F0 females, the mean body weight gain was significantly increased on days 0–4 of lactation at 150 ppm and during weeks 0–3 of dosing at 15,000 ppm compared to controls, and the mean body weight was significantly increased on week 2 of dosing at 15,000 ppm. The body weight gain was significantly decreased on days 0–14 of pregnancy at 15,000 ppm compared to controls.

Fig. 2 presents the body weights of F1 males and females during dosing. Significant decreases compared to controls were observed in the body weight during weeks 3–6 of dosing and body weight gain during the first 6 weeks of dosing in F1 males at 15,000 ppm. Compared with control group, a significantly lowered mean body weight was observed during weeks 3 and 6–10 of dosing, the whole period of gestation and days 0–14

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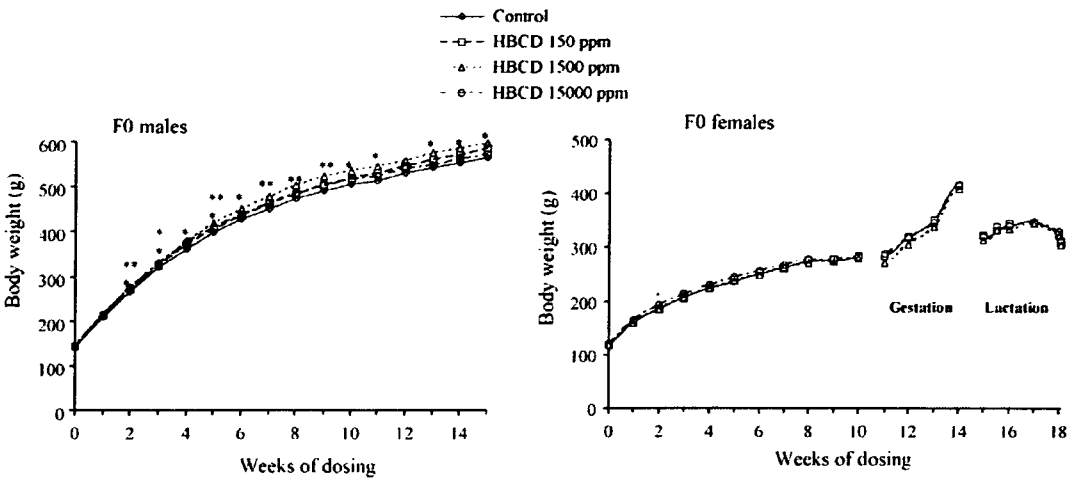

Fig. 1. Body weights of F0 male and female rats. (*) Significantly different from the control, $P < 0.05$. (**) Significantly different from the control, $P < 0.01$.

of lactation, and a significantly reduced mean body weight gain was observed during weeks 0–10 of dosing at 15,000 ppm in F1 females.

Food consumption was generally paralleled to the body weights/body weight gains during most of the study (data not shown).

The mean daily intakes of HBCD were 12.5, 125 and 1238 mg/kg bw during the pre-mating period, 9.6, 96 and 941 mg/kg bw during the gestation period, and 23.4, 240 and 2200 mg/kg bw during the lactation period in F0 females for 150, 1500 and 15,000 ppm, respectively. The mean daily intakes of HBCD were 14.0, 138 and 1365 mg/kg bw during the pre-mating period, 9.7, 100 and 995 mg/kg bw during the gestation period, and 19.6, 179 and 1724 mg/kg bw during the lactation period in F1 females for 150, 1500 and 15,000 ppm, respectively. The mean daily intakes of HBCD during the whole period were 10.2, 101 and 1008 mg/kg bw in F0 males, 14.0, 141 and 1363 mg/kg bw in F0 females, 11.4, 115 and 1142 mg/kg bw in F1 males, and 14.3, 138 and 1363 mg/kg bw in F1 females for 150, 1500 and 15,000 ppm, respectively.

3.2. Reproductive effects (F0 parents/F1 offspring and F1 parents/F2 offspring)

Table 1

presents the reproductive and developmental parameters for F0 parent/F1 offspring. HBCD produced no significant deviations in estrous cycles, although a few control and HBCD-treated rats had extended estrus or diestrus. Copulation was not observed in two males and two females at 1500 ppm and two males and one female at 15,000 ppm. Two females each at 150 and 1500 ppm did not become pregnant and three females at 15,000 ppm neither. One pregnant female each at 150 and 15,000 ppm did not deliver live pups. There were significantly longer gestation length and lower sex ratio of live pups at 1500 ppm compared

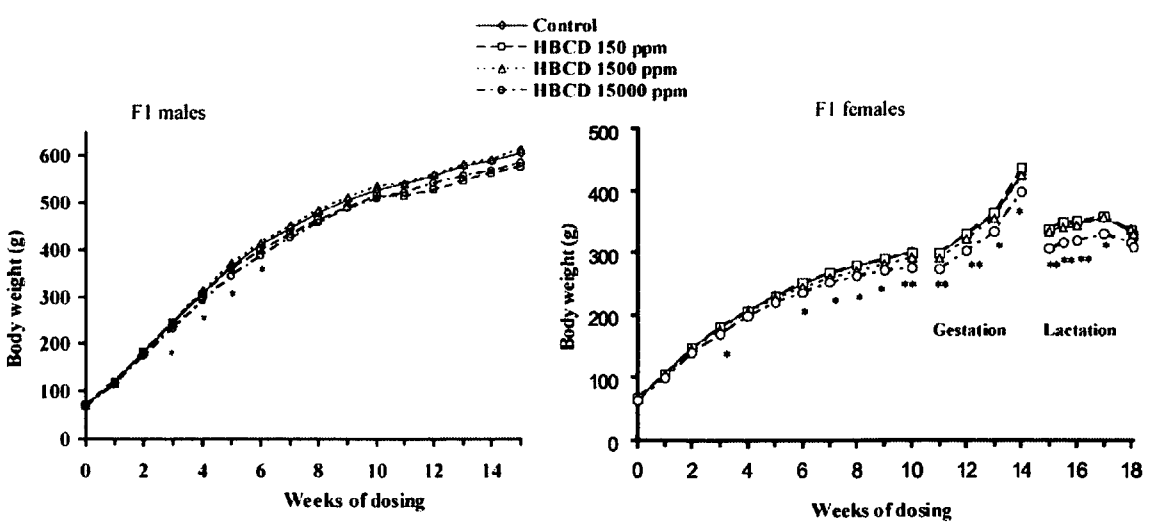

Fig. 2. Body weights of F1 male and female rats. (*) Significantly different from the control, $P < 0.05$. (**) Significantly different from the control, $P < 0.01$.

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Table 1
Reproductive and developmental findings in F0 parents/F1 offspring and F1 parents/F2 offspring

HBCD (ppm)	0 (control)	150	1500	15,000
F0 parents/F1 offspring				
No. of rats (male/female)	24/24	24/24	24/24	23/23
Females with normal estrous cycles (%)[a]	91.7	95.8	87.5	87.0
Copulation index (male/female) (%)[b]	100/100	100/100	91.7/91.7	91.3/95.7
Fertility index (male/female) (%)[c]	100/100	91.7/91.7	90.9/90.9	85.7/86.4
No. of pregnant females	24	22	20	19
Pre-coital interval (days)[d]	3.4 ± 3.9	3.1 ± 3.3	2.7 ± 1.4	3.5 ± 4.3
No. of implantations[d]	14.2 ± 2.1	13.7 ± 3.3	14.5 ± 1.4	14.5 ± 2.7
Gestation index (%)[e]	100	95.5	100	94.7
Delivery index (%)[f]	92.0	89.3	90.7	93.6
Gestation length (days)[d]	22.1 ± 0.3	22.3 ± 0.5	22.6 ± 0.5*	22.2 ± 0.4
No. of pups delivered[d]	13.0 ± 2.3	13.3 ± 1.7	13.3 ± 2.6	13.5 ± 2.8
No. of litters	24	21	20	18
Sex ratio of F1 pups[g]	0.524	0.471	0.426*	0.572
No. of litters totally lost	0	0	0	1
Viability index during lactation (%)[h,i,j]				
Day 0	99.6	97.5	98.8	99.2
Day 4	95.6	98.7	98.7	95.8
Day 21	93.2	99.4	98.1	93.8
Male pup weight during lactation (g)[d]				
Day 0	6.8 ± 0.5	6.9 ± 0.6	7.2 ± 0.7	6.8 ± 0.6
Day 4	10.2 ± 1.7	10.7 ± 1.8	10.8 ± 1.6	9.5 ± 1.8
Day 7	16.4 ± 3.1	17.5 ± 2.4	16.9 ± 2.2	15.6 ± 2.0 (17)[k]
Day 14	36.1 ± 4.8 (23)[k]	36.3 ± 3.6	36.1 ± 3.9	33.5 ± 2.6 (17)[k]
Day 21	61.1 ± 7.1 (23)[k]	62.3 ± 6.5	61.9 ± 6.5	55.4 ± 4.0 (17)[k,*]
Female pup weight during lactation (g)[d]				
Day 0	6.3 ± 0.5 (23)[k]	6.6 ± 0.7	6.8 ± 0.6*	6.5 ± 0.7
Day 4	9.6 ± 1.4 (23)[k]	10.3 ± 1.8	10.4 ± 1.5	9.2 ± 1.6
Day 7	15.4 ± 2.8 (23)[k]	17.0 ± 2.5	16.9 ± 2.3	15.1 ± 1.6 (17)[k]
Day 14	33.5 ± 5.3 (23)[k]	35.5 ± 3.6	35.7 ± 3.6	32.6 ± 3.0 (17)[k]
Day 21	56.5 ± 8.0 (23)[k]	59.9 ± 6.4	60.5 ± 5.9	53.2 ± 4.7 (17)[k]
F1 parents/F2 offspring				
No. of rats (male/female)	24/24	24/24	23/24	24/24
Females with normal estrous cycles (%)[a]	95.8	91.7	91.7	91.7
Copulation index (male/female) (%)[b]	100/100	100/100	100/100	100/100
Fertility index (male/female) (%)[c]	95.8/95.8	95.8/95.8	87.0/87.5	87.5/87.5
No. of pregnant females	23	23	21	21
Pre-coital interval (days)[d]	2.6 ± 1.6	3.4 ± 4.1	3.3 ± 3.7	2.3 ± 1.3
No. of implantations[d]	14.3 ± 2.5	14.7 ± 3.4	14.0 ± 3.2	14.3 ± 2.8
Gestation index (%)[e]	100	100	95.2	100
Delivery index (%)[f]	91.4	94.8	88.1	92.6
Gestation length (days)[d]	22.5 ± 0.5	22.4 ± 0.6	22.4 ± 0.5	22.4 ± 0.5
No. of pups delivered[d]	13.2 ± 3.4	13.9 ± 3.3	13.4 ± 2.4	13.1 ± 2.4
No. of litters	23	23	20	21
Sex ratio of F2 pups[g]	0.523	0.492	0.517	0.486
No. of litters totally lost	1	1	0	8**
Viability index during lactation (%)[h,i,j]				
Day 0	98.6	97.7	96.0	97.8
Day 4	86.9	87.3	92.1	68.4*
Day 21	85.0 (22)[k]	89.6 (22)[k]	71.3	49.7 (20)[k,*]
Male pup weight during lactation (g)[d]				
Day 0	6.8 ± 0.8	6.7 ± 0.7 (22)[k]	7.1 ± 0.6	6.6 ± 0.6
Day 4	9.1 ± 2.3 (22)[k]	9.3 ± 1.3 (22)[k]	9.0 ± 1.8	8.0 ± 1.3 (19)[k]
Day 7	14.7 ± 3.9 (22)[k]	15.4 ± 2.8 (22)[k]	14.3 ± 3.6 (19)[k]	11.5 ± 2.9 (17)[k,*]
Day 14	31.4 ± 8.0 (22)[k]	33.8 ± 5.0 (22)[k]	31.0 ± 7.2 (18)[k]	24.2 ± 6.6 (14)[k,*]
Day 21	53.0 ± 12.6 (22)[k]	56.2 ± 6.7 (22)[k]	54.1 ± 10.1 (18)[k]	42.6 ± 8.3 (13)[k,*]
Female pup weight during lactation (g)[d]				
Day 0	6.5 ± 0.8	6.3 ± 0.6	6.7 ± 0.6	6.2 ± 0.6

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