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

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

[a] Values are given as mean ± SD.

[b] Values obtained from females successfully mated.

[c] Value obtained from 11 females, because corpora lutea were indistinguishable in one female.

[d] Values obtained from pregnant females surviving until scheduled sacrifice.

[e] [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.

[f] (No. of resorptions and dead fetuses/no. of implantations) × 100.

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

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

Death and/or moribund condition were observed after the administration of DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 of pregnancy and at 15.2 mg/kg bw/day and higher on days 4–7 of pregnancy, and significant increased incidence of females showing clinical signs of toxicity were found after the administration of DBTCl at 15.2 mg/kg bw/day and higher on days 0–3 of pregnancy and at 30.4 mg/kg bw/day on days 4–7 of pregnancy. These findings indicate that more severe general toxicity was induced by DBTCl on days 0–3 of pregnancy than that on days 4–7 of pregnancy. However, adverse effects on body weight gain were detected after the administration of DBTCl at 30.4 mg/kg bw/day on days 0–3 of pregnancy and at 7.6 mg/kg bw/day and higher on days 4–7 of pregnancy. Although the recovery of body weight gain was observed after the administration of DBTCl on days 0–3 of

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

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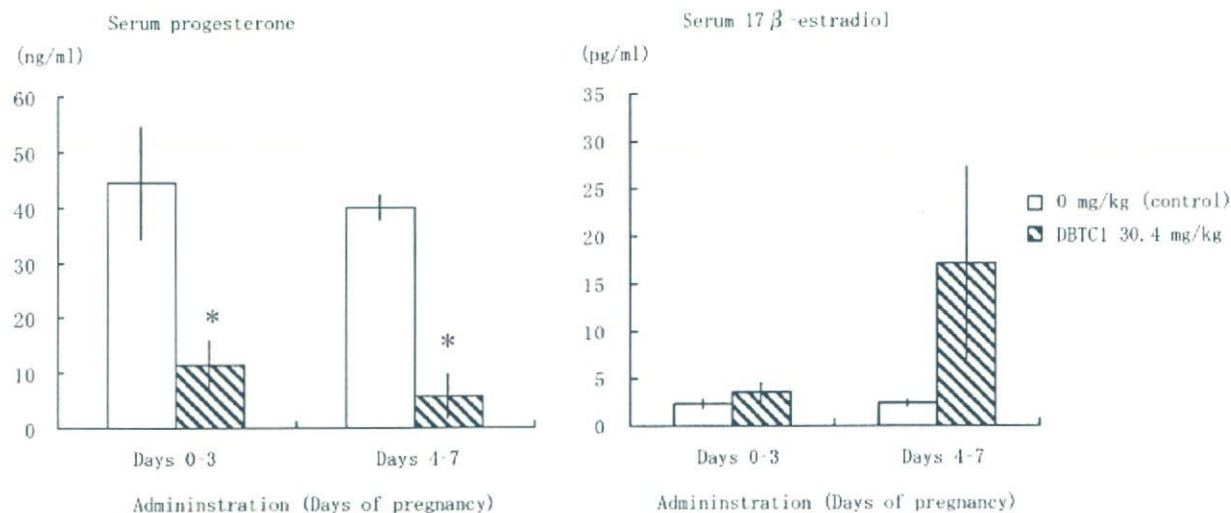
Serum progesterone
(ng/ml)

Serum 17 β -estradiol
(pg/ml)

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

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

The corpora lutea are essential up to the end of pregnancy in mice (Deansely, 1966). The embryo transport process in mice is triggered by progesterone and requires progesterone activity for its maintenance (Kendle and Lee, 1980). In mice, 24 h of progesterone priming is not only adequate for implantation, but this priming has a long-term effect on implantation

(Huet-Hudson and Dey, 1990). In our previous studies in rats, increases in the incidences of early embryonic loss were observed after the administration of DBTCl during early pregnancy (Ema and Harazono, 2000ab). The suppression of uterine decidualization and reduced levels of serum progesterone were found in female rats given DBTCl on days 0–3 or days 4–7 of pseudopregnancy (Harazono and Ema, 2003), and lowered reproductive parameters in female rats given DBTCl were recovered by the administration of progesterone (Ema et al., 2003). Based on these findings, we hypothesized that the decline in serum progesterone levels in pregnant animals was a primary mechanism for the implantation failure due to DBTCl in rats. In the present study in mice, a decline in serum progesterone levels was detected after the administration of DBTCl during early pregnancy. These findings are in good agreement with previous findings that DBTCl induced early embryonic loss and decreased serum progesterone levels in pregnant rats. There is a similarity in the effects of DBTCl on progesterone levels in early pregnancy in rats and mice, and these suggest that the decline in the serum progesterone levels is also the factor responsible for the DBTCl-induced pregnancy failure in mice. Early pregnancy failure was also caused by systemic activation of the CD-40 immune costimulatory pathway in mice (Erlebacher et al., 2004). They noted that pregnancy failure resulted from impaired progesterone synthesis by the corpus luteum of the ovary, an endocrine defect in turn associated with ovarian resistance to the gonadotropic effects of prolactin and that pregnancy failure also required the proinflammatory cytokine TNF-a and correlated with the luteal induction of the prolactin receptor signaling inhibitors suppressor of cytokine signaling 1

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

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

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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.
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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.).

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 $22 \pm 3\,°C$, with humidity of $50 \pm 20\%$, 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.

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
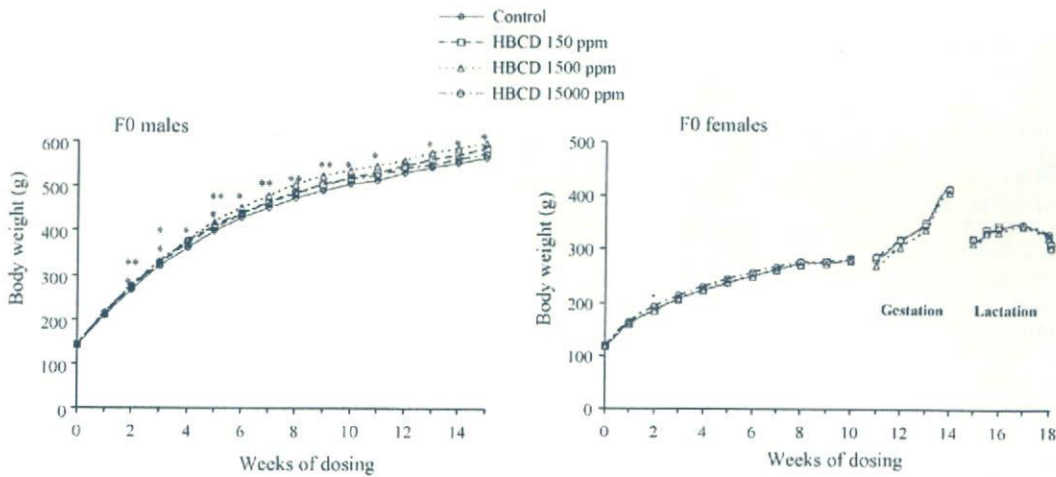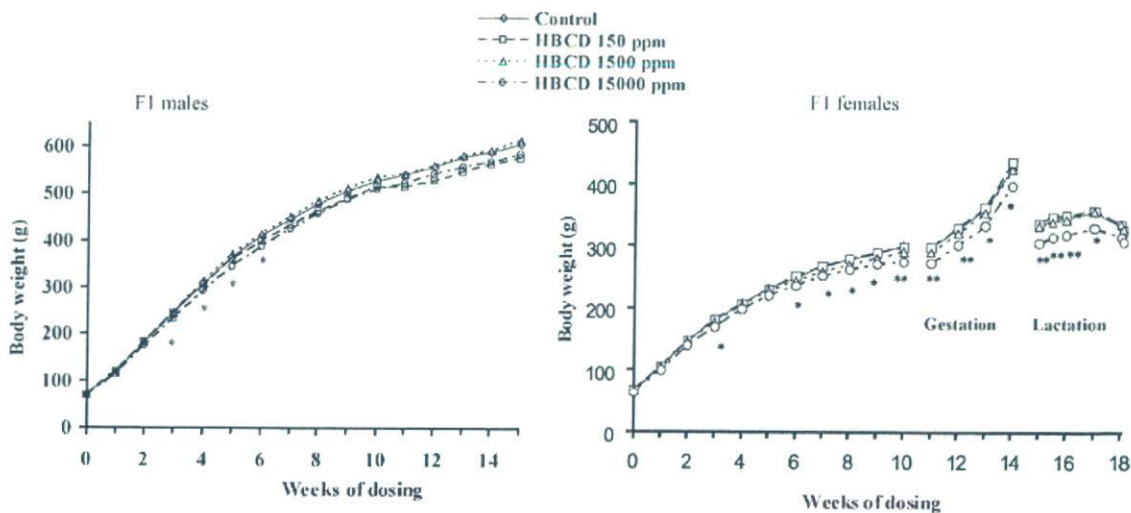

Fig. 2. Body weights of F1 male and female rats. (*) Significantly different from the control, $P < 0.05$. (**) Significantly different from the control, $P < 0.01$.

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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	$22.6 \pm 0.5^{**}$	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	$15.6 \pm 2.0 (17)^{k}$
Day 14	$36.1 \pm 4.8 (23)^{k}$	36.3 ± 3.6	36.1 ± 3.9	$33.5 \pm 2.6 (17)^{k}$
Day 21	$61.1 \pm 7.1 (23)^{k}$	62.3 ± 6.5	61.9 ± 6.5	$55.4 \pm 4.0 (17)^{k,*}$
Female pup weight during lactation (g)[d]				
Day 0	$6.3 \pm 0.5 (23)^{k}$	6.6 ± 0.7	$6.8 \pm 0.6^{*}$	6.5 ± 0.7
Day 4	$9.6 \pm 1.4 (23)^{k}$	10.3 ± 1.8	10.4 ± 1.5	9.2 ± 1.6
Day 7	$15.4 \pm 2.8 (23)^{k}$	17.0 ± 2.5	16.9 ± 2.3	$15.1 \pm 1.6 (17)^{k}$
Day 14	$33.5 \pm 5.3 (23)^{k}$	35.5 ± 3.6	35.7 ± 3.6	$32.6 \pm 3.0 (17)^{k}$
Day 21	$56.5 \pm 8.0 (23)^{k}$	59.9 ± 6.4	60.5 ± 5.9	$53.2 \pm 4.7 (17)^{k}$
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	$6.7 \pm 0.7 (22)^{k}$	7.1 ± 0.6	6.6 ± 0.6
Day 4	$9.1 \pm 2.3 (22)^{k}$	$9.3 \pm 1.3 (22)^{k}$	9.0 ± 1.8	$8.0 \pm 1.3 (19)^{k}$
Day 7	$14.7 \pm 3.9 (22)^{k}$	$15.4 \pm 2.8 (22)^{k}$	$14.3 \pm 3.6 (19)^{k}$	$11.5 \pm 2.9 (17)^{k,*}$
Day 14	$31.4 \pm 8.0 (22)^{k}$	$33.8 \pm 5.0 (22)^{k}$	$31.0 \pm 7.2 (18)^{k}$	$24.2 \pm 6.6 (14)^{k,**}$
Day 21	$53.0 \pm 12.6 (22)^{k}$	$56.2 \pm 6.7 (22)^{k}$	$54.1 \pm 10.1 (18)^{k}$	$42.6 \pm 8.3 (13)^{k,**}$
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

Table 1 (Continued)

HBCD (ppm)	0 (control)	150	1500	15,000
Day 4	$8.9 \pm 2.3 \ (22)^k$	$8.5 \pm 1.3 \ (22)^k$	8.8 ± 1.8	$7.3 \pm 1.3 \ (20)^{k,**}$
Day 7	$14.3 \pm 3.5 \ (21)^k$	$14.2 \pm 2.8 \ (22)^k$	13.5 ± 3.9	$10.7 \pm 2.6 \ (17)^{k,**}$
Day 14	$31.2 \pm 6.5 \ (21)^k$	$31.3 \pm 5.1 \ (22)^k$	29.3 ± 7.3	$23.9 \pm 5.9 \ (13)^{k,**}$
Day 21	$52.0 \pm 10.0 \ (21)^k$	$52.8 \pm 6.6 \ (22)^k$	51.2 ± 10.8	$41.6 \pm 8.4 \ (13)^{k,**}$

[a] Incidence of females with normal estrous cycles (%) = (no. of females with normal estrous cycles/no. of females examined) × 100.
[b] Copulation index (%) = (no. of animals with successful copulation/no. of animals paired) × 100.
[c] Fertility index (%) = (no. of animals that impregnated a female or were pregnant/no. of animals with successful copulation) × 100.
[d] Values are given as the mean ± S.D.
[e] Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.
[f] Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.
[g] Sex ratio = total no. of male pups/total no. of pups.
[h] Viability index on postnatal day 0 (%) = (no. of live pups on postnatal day 0/no. of pups delivered) × 100.
[i] Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of live pups on postnatal day 0) × 100.
[j] Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live pups on postnatal day 4 after cull) × 100.
[k] Data were obtained from the numbers of litters in parentheses because females that had no male and/or female pups and/or experienced total male and/or female pup loss during lactation were excluded.
[*] Significantly different from the control, $P < 0.05$.
[**] Significantly different from the control, $P < 0.01$.

to controls. One dam experienced total litter loss by day 5 of lactation at 15,000 ppm; however, there were no significant differences in the copulation index, fertility index, gestation index, pre-coital interval, number of implantations, delivery index, number of F1 pups delivered, or viability of F1 pups during lactation between the control and HBCD-treated groups. Mean body weight of female F1 pups on PND 0 was significantly higher at 1500 ppm, and that of male F1 pups on PND 21 was significantly lowered at 15,000 ppm, compared to controls.

Table 1 also shows the reproductive and developmental parameters for F1 parent/F2 offspring. In F1 females, there were extended diestrus vaginal smears in a few control and HBCD-treated rats, but no significant effect of HBCD was found on the incidence of females with normal estrous cycles. All pairs in all groups copulated. One female each in the control and 150 ppm groups, and three females each at 1500 and 15,000 ppm were not impregnated. One pregnant female did not deliver live pups at 1500 ppm. One dam experienced total litter loss by day 4 of lactation in the control group and by day 2 of lactation at 150 ppm. At 15,000 ppm, eight dams experienced total litter loss by days 4, 5, 7, 9, 11, 13 or 18 of lactation, and a significantly increased incidence of dams with total litter loss was noted. No clear clinical signs of toxicity were noted in these dams with total litter loss. No significant changes were observed in the copulation index, fertility index, gestation index, pre-coital interval, gestation length, number of implantations, delivery index, number of F2 pups delivered or the sex ratio of F2 pups. A significantly decreased viability index was noted in F2 pups on PNDs 4 and 21 at 15,000 ppm. Mean body weights were significantly lowered compared to controls in male F2 pups on PNDs 7, 14 and 21 and in female F2 pups on PNDs 4, 7, 14 and 21 at 15,000 ppm.

3.3. Developmental landmarks (F1 and F2)

Table 2 presents physical development of F1 and F2 pups. There was no significant difference in the incidence of male and female F1 and F2 pups that displayed pinna unfolding, or incisor eruption between the control and HBCD-treated groups. The incidence of male and female F1 pups showing completion of eye opening was increased compared to controls at 1500 ppm. In F2 pups, the incidence of pups showing eye opening was lowered compared to controls in males at 15,000 ppm and in females at 1500 and 15,000 ppm. The AGD and AGD per cube root of body weight ratio were not significantly different between control and HBCD-treated groups in male and female F1 and F2 pups.

Table 3 shows reflex ontogeny in F1 and F2 pups. All male and female F1 pups in all groups completed the surface righting reflex, negative geotaxis reflex and mid-air righting reflex. No significant changes were observed in reflex response time, except for faster response in the surface righting in males at 15,000 ppm, in F1 pups of both sexes in HBCD-treated groups. In F2 pups, a few pups failed to complete the reflex response in HBCD-treated groups, and a significantly low incidence of females completed mid-air righting was noted at 15,000 ppm; however, there was no significant difference in the incidence of male and female pups with completed response in other reflexes and in the reflex response time between control and HBCD-treated groups.

Table 4 presents data on sexual development in F1 rats. No significant differences between control and HBCD-treated groups were noted in the age at preputial separation in males or vaginal opening in females, or body weight at the age of preputial separation or vaginal opening.

3.4. Behavioral effects (F1)

Spontaneous locomotor activity for 10 min intervals and for a total of 60 min was not significantly different between control and HBCD-treated groups in male and females F1 rats (data not shown).

On the first day of the T-maze test, the pre-test swimming trials in the straight channel revealed that all male and female F1 rats in each group could swim satisfactorily, and no sig-

Table 2

Physical development in F1 and F2 pups

HBCD (ppm)	0 (control)	150	1500	15,000
F1 pups				
No. of litters examined	24	21	20	18
Pinna unfolding (%)[a,b]				
Male	86.0 ± 26.5	92.5 ± 16.5	93.6 ± 15.7	81.3 ± 27.9
Female	85.8 ± 29.5 (23)[c]	94.7 ± 14.7	97.3 ± 7.5	86.4 ± 23.8
Incisor eruption (%)[a,b]				
Male	91.6 ± 17.6 (23)[c]	96.4 ± 12.0	92.1 ± 17.0	89.7 ± 19.9 (17)[c]
Female	94.9 ± 11.4 (23)[c]	95.2 ± 10.1	92.5 ± 20.0	92.2 ± 15.4 (17)[c]
Eye opening (%)[a,b]				
Male	48.2 ± 41.5 (23)[c]	56.7 ± 37.9	$77.1 \pm 36.3^{*}$	45.8 ± 34.6 (17)[c]
Female	49.3 ± 37.8 (23)[c]	66.7 ± 41.3	$82.9 \pm 33.5^{**}$	54.9 ± 41.4 (17)[c]
AGD[a]				
Male pup AGD (mm)	5.37 ± 0.41	5.44 ± 0.36	5.38 ± 0.32	5.20 ± 0.51
Male pup AGD/(bw$^{1/3}$)	2.49 ± 0.11	2.48 ± 0.10	2.44 ± 0.12	2.46 ± 0.14
Female pup AGD (mm)	2.60 ± 0.23 (23)[c]	2.67 ± 0.16	2.62 ± 0.18	2.57 ± 0.23
Female pup AGD/(bw$^{1/3}$)	1.22 ± 0.09 (23)[c]	1.23 ± 0.06	1.20 ± 0.06	1.23 ± 0.06
F2 pups				
No. of litters examined	23	22	20	21
Pinna unfolding (%)[a,b]				
Male	79.9 ± 36.4 (22)[c]	90.5 ± 22.8	82.1 ± 29.8	70.1 ± 39.2 (20)[c]
Female	73.6 ± 39.6	90.6 ± 22.8	81.5 ± 31.1	66.8 ± 40.9
Incisor eruption (%)[a,b]				
Male	86.4 ± 25.3 (22)[c]	92.8 ± 19.6	97.2 ± 11.8 (18)[c]	86.3 ± 27.7 (14)[c]
Female	85.7 ± 26.9 (21)[c]	90.9 ± 26.2	97.5 ± 11.2	90.0 ± 28.0 (15)[c]
Eye opening (%)[a,b]				
Male	72.7 ± 40.0 (22)[c]	62.5 ± 40.6	47.2 ± 44.8 (18)[c]	33.9 ± 34.7 (14)[c,**]
Female	82.9 ± 26.8 (21)[c]	72.7 ± 37.7	$53.8 \pm 40.3^{*}$	48.1 ± 42.0 (13)[c,*]
AGD[a]				
Male pup AGD (mm)	5.12 ± 0.54 (22)[c]	5.12 ± 0.41	5.04 ± 0.42	4.84 ± 0.39 (19)[c]
Male pup AGD/(bw$^{1/3}$)	2.46 ± 0.12 (22)[c]	2.44 ± 0.13	2.43 ± 0.08	2.42 ± 0.12 (19)[c]
Female pup AGD (mm)	2.69 ± 0.30 (22)[c]	2.71 ± 0.24	2.71 ± 0.29	2.54 ± 0.21 (20)[c]
Female pup AGD/(bw$^{1/3}$)	1.30 ± 0.07 (22)[c]	1.33 ± 0.09	1.32 ± 0.09	1.32 ± 0.06 (20)[c]

[a] Values are given as the mean $\pm$ S.D.

[b] Incidence of animals that displayed pinna unfolding, incisor eruption or eye opening (%).

[c] Data were obtained from the numbers of litters in parentheses because females that had no male and/or female pups and/or experienced total male and/or female pup loss during lactation were excluded.

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

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

nificant changes were observed in the elapsed time to traverse the straight channel. In males, there were a significantly shorter elapsed time at 1500 and 15,000 ppm and fewer number of errors at 15,000 ppm on day 3 of the T-maze. In females, there was no significant difference in the elapsed time or number of errors of the T-maze between control and HBCD-treated groups (data not shown).

3.5. Necropsy and histopathology (F0, F1 and F2)

No compound-related gross lesions or microscopic alterations were observed in reproductive organs in male and female F0 and F1 adults showing reproductive difficulties, in male and female F0 and F1 adults of the highest dose group and in dead animals before scheduled sacrifice. There were no compound-related gross lesions or remarkable microscopic alterations in other tissues and organs, except for the thyroid, in male and female F0 and F1 adults.

Table 5 presents the histopathological findings in the thyroid of male and female F0 and F1 adults. Decreased size of follicles in the thyroid was found in F0 and F1 adults at 1500 ppm and higher, and in F1 females at 150 ppm as well. A significant increased incidence of rats with decreased follicle size was noted in F0 males (25%) and females (21%) and F1 females (21%) at 1500 ppm and F0 males (87%) and females (48%) and F1 males (46%) and females (54%) at 15,000 ppm, compared to controls (0%). Background incidence of decreased follicle size in the laboratory performed current study was 0% in a total of 56 males and 56 females in 6 studies (5–12/sex/study) form 1998 to 2004. Hypertrophy of the follicular cells in the thyroid was

Table 3
Reflex ontogeny in F1 and F2 pups

HBCD (ppm)	0 (control)	150	1500	15,000
F1 pups				
No. of pups examined (male/female)	24/23	21/21	20/20	17/17
Surface righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
Surface righting reflex response time (s)[a]				
Male	2.3 ± 1.1	2.0 ± 0.6	1.8 ± 0.5	$1.6 \pm 0.3^{**}$
Female	3.1 ± 1.8	2.4 ± 1.5	2.9 ± 2.6	2.6 ± 2.6
Negative geotaxis reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/100
Negative geotaxis reflex response time (s)[a]				
Male	17.7 ± 7.1	16.8 ± 8.0	15.2 ± 7.8	19.4 ± 5.9
Female	13.9 ± 6.2	11.5 ± 6.2	12.7 ± 6.3	17.0 ± 6.9
Mid-air righting reflex completion rate (%)				
Male/female	100 (23)[b]/100	100/100	100/100	100/100
F2 pups				
No. of pups examined (male/female)	22/22	22/22	19/20	19/18
Surface righting reflex completion rate (%)				
Male/female	100/100	100/100	100/100	100/88.9
Surface righting reflex response time (s)[a]				
Male	2.1 ± 1.7	2.0 ± 1.5	2.8 ± 2.5	2.2 ± 2.3
Female	2.3 ± 0.9	2.4 ± 1.7	2.1 ± 0.9	3.7 ± 3.7 (16)[b]
Negative geotaxis reflex completion rate (%)				
Male/female	100/100 (21)[b]	95.5/100	100/100	81.3 (16)[b]/88.2 (17)[b]
Negative geotaxis reflex response time (s)[a]				
Male	17.3 ± 8.6	14.7 ± 6.8 (21)[b]	15.2 ± 6.4	14.1 ± 6.7 (13)[b]
Female	12.4 ± 5.3 (21)[b]	12.0 ± 5.2	16.7 ± 6.4	14.6 ± 6.6 (15)[b]
Mid-air righting reflex completion rate (%)				
Male/female	100/100 (21)[b]	100/100	94.4 (18)[b]/90.0	100 (13)[b]/76.9 (13)[b,*]

Surface righting reflex on postnatal day 5 (three trials), negative geotaxis reflex on postnatal day 8 (one trial) and mid-air righting reflex on postnatal day 18 (three trials) were examined. Completion rate (%) = (no. of animals showing all positive responses of the trials/no. of animals examined) × 100.

[a] Values are given as the mean ± S.D.
[b] Data were obtained from the numbers of pups in parentheses.
[*] Significantly different from the control, $P < 0.05$.
[**] Significantly different from the control, $P < 0.01$.

also observed in F0 males at 1500 ppm and higher, and in F0 females at 1500 ppm.

Fig. 3 shows the number of the primordial follicles in the ovary of F1 females. The number of primordial follicles (mean ± S.D.) was significantly decreased at 1500 (197.9 ± 76.9) and 15,000 ppm (203.4 ± 79.5), but not at 150 ppm (294.2 ± 66.3), compared to controls (316.3 ± 119.5). The range of the background control data in the laboratory performed current study was 189.5–353.4 (mean = 295.6) in 4 studies using 10 females per study in 2005–2006.

Table 4
Sexual development in F1 males and females

HBCD (ppm)	0 (control)	150	1500	15,000
F1 rats				
Male preputial separation				
No. of males examined	24	24	24	24
Age (days)[a]	42.8 ± 1.7	41.7 ± 1.8	42.8 ± 2.2	43.7 ± 1.5
Body weight (g)[a]	225.6 ± 17.1	219.6 ± 20.0	235.0 ± 20.8	226.5 ± 16.2
Female vaginal opening				
No. of females examined	24	24	24	24
Age (days)[a]	30.9 ± 2.0	30.3 ± 2.6	30.1 ± 1.8	30.8 ± 2.2
Body weight (g)[a]	106.0 ± 13.8	102.9 ± 13.8	106.0 ± 10.6	100.7 ± 13.0

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

Table 5
Histopathological findings in the thyroid of F0 and F1 rats

HBCD (ppm)	0 (control)	150	1500	15,000
F0 males				
No. of males examined	24	24	24	23[a]
Decreased size of thyroid follicle[b]	0	0	6*	20**
Hypertrophy of thyroid follicular cells[b]	0	0	3	1
F0 females				
No. of females examined	24	24	24	23[a]
Decreased size of thyroid follicle[b]	0	0	5*	11**
Hypertrophy of thyroid follicular cells[b]	0	0	2	0
F1 males				
No. of males examined	24	24	22[a]	24
Decreased size of thyroid follicle[b]	0	0	2	11**
Hypertrophy of thyroid follicular cells[b]	0	0	0	0
F1 females				
No. of females examined	24	24	24	24
Decreased size of thyroid follicle[b]	0	1	5*	13**
Hypertrophy of thyroid follicular cells[b]	0	0	0	0

[a] The number of animals examined was 23 or 22 due to autolysis.
[b] Values are given as the number of animals that showed abnormal findings.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

There were no compound-related gross lesions and histopathological changes in male and female F1 and F2 pups and weanlings including dead pups.

3.6. Organ weights (F0 adults)

The mean body weight at scheduled sacrifice was significantly heavier at 1500 ppm in males compared to controls. In F0 males, there were a significantly decreased relative weight of the brain at 1500 ppm and decreased relative weight of the seminal vesicle at 1500 ppm and higher. On the other hand, there were significantly increased absolute and relative weights of the liver at 1500 ppm and higher and of the thyroid at 15,000 ppm. In F0 females, significant increases were found in the absolute weight of the thyroid, liver and adrenal, and relative weight of the liver at 15,000 ppm when compared with controls (data not shown).
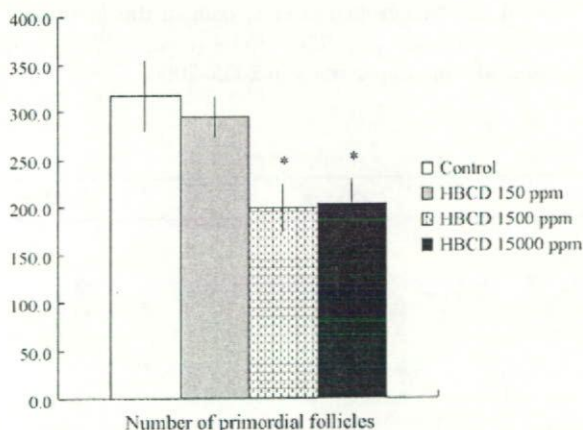

Fig. 3. Number of primordial follicles in the ovary of F1 female rats. Values are given as the mean ± S.E.M. (*) Significantly different from the control, $P < 0.05$.

3.7. Organ weights (F1 weanlings and adults)

Table 6 presents the organ weights of male and female F1 weanlings. The mean body weight at scheduled sacrifice was significantly lowered in males at 15,000 ppm compared to controls. In males, there were significant increases in the absolute and relative weights of the testis at 150 ppm, and relative weights of the testis and absolute and relative weight of the liver at 1500 ppm and higher. The absolute weights of the brain and kidney were significantly decreased at 15,000 ppm. In F1 females, significantly increased absolute and relative weights of the liver at 1500 ppm and higher, and decreased absolute weights of the brain and kidney at 15,000 ppm were observed.

Table 7 shows the organ weights of male F1 adult at scheduled sacrifice. The relative weights of the brain and pituitary were significantly higher at 150 ppm compared to controls. At 15,000 ppm, absolute weight of the brain was significantly decreased, and absolute and relative weights of the thyroid and liver were significantly increased compared to control.

The organ weights of female F1 adults at scheduled sacrifice are shown in Table 8. At 15,000 ppm, there were a significant decrease in the absolute weight of the brain and a significant increase in absolute and relative weights of the thyroid and liver.

3.8. Organ weights (F2 weanlings)

Table 9 presents the organ weights of male F2 weanlings. The body weight at sacrifice was significantly reduced at 15,000 ppm compared to controls. A significant decrease was observed in the relative weight of the kidney at 150 ppm, and a significant increase was observed in the relative weight of the liver at 1500

Table 6

Organ weights of male and female F1 weanlings

HBCD (ppm)	0 (control)	150	1500	15,000
No. of male F1 weanlings examined	23	21	20	17
Body weight (g)[a]	85.7 ± 10.9	89.6 ± 8.1	87.7 ± 9.2	78.3 ± 5.8[*]
Brain (g)[a]	1.64 ± 0.09[b]	1.66 ± 0.05	1.62 ± 0.07	1.55 ± 0.06[**]
	1.94 ± 0.19[c]	1.87 ± 0.17	1.86 ± 0.18	1.99 ± 0.13
Thymus (mg)[a]	342 ± 68[b]	339 ± 50	369 ± 59	317 ± 57
	398 ± 55[c]	379 ± 45	421 ± 55	405 ± 70
Liver (g)[a]	3.94 ± 0.63[b]	4.12 ± 0.48	4.43 ± 0.59[*]	4.71 ± 0.58[**]
	4.60 ± 0.37[c]	4.60 ± 0.32	5.05 ± 0.32[**]	6.00 ± 0.44[**]
Kidney (mg)[a,d]	996 ± 125[b]	1035 ± 131	1004 ± 109	894 ± 99[*]
	1165 ± 74[c]	1155 ± 92	1146 ± 70	1140 ± 78
Spleen (mg)[a]	336 ± 62[b]	327 ± 41	334 ± 43	309 ± 69
	394 ± 64[c]	366 ± 42	383 ± 46	395 ± 81
Adrenal (mg)[a,d]	23.9 ± 3.0[b]	25.0 ± 3.3	26.1 ± 3.7	22.8 ± 3.6
	28.0 ± 2.6[c]	28.0 ± 3.9	29.9 ± 4.3	29.2 ± 4.8
Testis (mg)[a,d]	488 ± 100[b]	550 ± 70[*]	541 ± 92	494 ± 70
	565 ± 65[c]	614 ± 56[*]	615 ± 61[*]	631 ± 73[**]
Epididymis (mg)[a,d]	73.2 ± 9.5[b]	77.4 ± 9.8	78.3 ± 9.9	70.1 ± 11.6
	85.9 ± 9.8[c]	86.7 ± 10.3	89.3 ± 7.5	89.9 ± 15.3
Ventral prostate (mg)[a]	40.0 ± 12.0[b]	42.0 ± 7.7	42.1 ± 7.1	34.8 ± 9.4
	46.4 ± 10.3[c]	47.1 ± 8.8	48.2 ± 7.3	44.5 ± 11.1
No. of female F1 weanlings examined	23	21	20	14
Body weight (g)[a]	78.9 ± 10.6	83.2 ± 9.7	83.9 ± 8.3	72.1 ± 5.3
Brain (g)[a]	1.58 ± 0.09[b]	1.61 ± 0.07	1.59 ± 0.08	1.51 ± 0.06[*]
	2.04 ± 0.23[c]	1.96 ± 0.19	1.91 ± 0.14	2.10 ± 0.16
Thymus (mg)[a]	335 ± 64[b]	330 ± 58	370 ± 58	305 ± 31
	423 ± 58[c]	397 ± 63	441 ± 53	422 ± 33
Liver (g)[a]	3.61 ± 0.55[b]	3.83 ± 0.55	4.22 ± 0.56[**]	4.37 ± 0.41[**]
	4.57 ± 0.35[c]	4.59 ± 0.28	5.02 ± 0.32[**]	6.07 ± 0.36[**]
Kidney (mg)[a,d]	932 ± 102[b]	945 ± 112	958 ± 115	815 ± 85[**]
	1189 ± 85[c]	1136 ± 63	1143 ± 81	1129 ± 72
Spleen (mg)[a]	311 ± 53[b]	306 ± 44	304 ± 59	280 ± 40
	399 ± 75[c]	370 ± 51	363 ± 67	388 ± 48
Adrenal (mg)[a,d]	21.9 ± 3.5[b]	23.7 ± 2.8	24.2 ± 3.8	20.9 ± 3.4
	27.8 ± 3.8[c]	28.7 ± 4.0	28.9 ± 4.0	28.9 ± 4.1
Ovary (mg)[a,d]	20.8 ± 3.7[b]	22.8 ± 3.6	21.0 ± 4.0	20.9 ± 3.4
	26.5 ± 4.5[c]	27.5 ± 4.1	25.0 ± 3.8	28.9 ± 3.7
Uterus (mg)[a]	57.0 ± 10.9[b]	62.0 ± 14.1	64.1 ± 18.6	51.9 ± 12.4
	73.6 ± 17.5[c]	74.9 ± 17.7	76.0 ± 18.4	71.9 ± 16.2

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

[b] Absolute organ weight.

[c] Relative organ weight = organ weight (g or mg)/100 g body weight.

[d] Values are given as the total weights of the organs on both sides.

[*] Significantly different from the control, $P < 0.05$.

[**] Significantly different from the control, $P < 0.01$.

and 15,000 ppm. There were significantly decreased absolute weight of the brain, kidney, spleen, adrenal, epididymis and ventral prostate and increased relative weight of the brain at 15,000 ppm.

Table 10 also presents the organ weights of female F2 weanlings. At 15,000 ppm, a significant decrease compared to controls was found in the body weight at sacrifice. The absolute and relative weights of the ovary were significantly higher at 150 ppm. At 15,000 ppm, there were significantly reduced absolute weight of the brain, thymus, kidney, spleen, adrenal and uterus and increased relative weight of the brain, liver and ovary.

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Table 7
Organ weights of male F1 adults

HBCD (ppm)	0 (control)	150	1500	15,000
No. of male F1 adults examined	24	24	22	24
Body weight (g)[a]	605.6 ± 41.9	576.7 ± 59.0	613.3 ± 59.2	584.4 ± 54.9
Brain (g)[a]	2.19 ± 0.08[b]	2.22 ± 0.08	2.18 ± 0.09	2.11 ± 0.07**
	0.363 ± 0.028[c]	0.388 ± 0.036*	0.358 ± 0.034	0.363 ± 0.032
Pituitary gland (mg)[a]	13.1 ± 1.5[b]	13.6 ± 1.6	13.2 ± 1.4	13.3 ± 1.2
	2.16 ± 0.22[c]	2.37 ± 0.23**	2.17 ± 0.22	2.28 ± 0.23
Thyroid (mg)[a,d]	24.3 ± 4.9[b]	24.2 ± 3.0	25.4 ± 4.7	29.0 ± 5.6**
	4.03 ± 0.79[c]	4.22 ± 0.63	4.15 ± 0.72	4.96 ± 0.87**
Thymus (mg)[a]	344 ± 72[b]	305 ± 92	368 ± 100	341 ± 76
	56.7 ± 10.8[c]	52.8 ± 14.3	59.8 ± 14.4	58.3 ± 11.1
Liver (g)[a]	19.83 ± 2.06[b]	19.36 ± 3.13	20.73 ± 3.01	22.61 ± 3.04**
	3.27 ± 0.18[c]	3.34 ± 0.26	3.37 ± 0.25	3.86 ± 0.28**
Kidney (g)[a,d]	3.74 ± 0.34[b]	3.59 ± 0.36	3.77 ± 0.33	3.77 ± 0.58
	0.618 ± 0.037[c]	0.625 ± 0.052	0.619 ± 0.074	0.645 ± 0.080
Spleen (mg)[a]	885 ± 168[b]	840 ± 147	878 ± 163	851 ± 113
	146 ± 26[c]	146 ± 22	143 ± 22	146 ± 17
Adrenal (mg)[a,d]	59.7 ± 11.0[b]	63.1 ± 15.8	60.3 ± 10.7	59.4 ± 6.7
	9.9 ± 1.6[c]	10.9 ± 2.3	9.9 ± 1.8	10.2 ± 1.1
Testis (g)[a,d]	3.63 ± 0.33[b]	3.52 ± 0.27	3.51 ± 0.35	3.45 ± 0.36
	0.602 ± 0.069[c]	0.614 ± 0.049	0.576 ± 0.062	0.593 ± 0.065
Epididymis (mg)[a,d]	1346 ± 107[b]	1328 ± 104	1282 ± 109	1357 ± 104
	223 ± 24[c]	232 ± 24	210 ± 19	234 ± 23
Seminal vesicle (g)[a]	2.36 ± 0.26[b]	2.28 ± 0.22	2.33 ± 0.29	2.38 ± 0.22
	0.391 ± 0.051[c]	0.398 ± 0.050	0.382 ± 0.051	0.409 ± 0.045
Ventral prostate (mg)[a]	834 ± 195[b]	779 ± 217	803 ± 175	789 ± 159
	137 ± 28[c]	135 ± 34	131 ± 30	135 ± 22

[a] Values are given as the mean ± S.D.
[b] Absolute organ weight.
[c] Relative organ weight = organ weight (g or mg)/100 g body weight.
[d] Values are given as the total weights of the organs on both sides.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

3.9. Hematological and blood biochemical parameters (F0 and F1 adults)

In male F0 and F1 and female F1 adults, no significant difference was noted in the total WBC or differential leukocyte count between control and HBCD-treated groups. In female F0 adults, there was a significantly lower percent of stabform and segmented neutrophils, and a higher percent of lymphocytes at 150 ppm compared to controls. Total protein and globulin were significantly higher in F0 males at 1500 and 15,000 ppm, in F0 females at 150 and 15,000 ppm and in F1 males at 15,000 ppm than those in controls (data not shown).

3.10. Serum hormone levels (F0 and F1 adults)

Fig. 4 shows serum hormone levels of T3, T4 and TSH in male and female F0 and F1 adult rats. There were no significant changes in T3 levels in F0 and F1 rats of both sexes. Lower levels of T4 compared to controls were observed at 15,000 ppm in F0 males and females. Signifi-

cantly increased levels of TSH were found in F0 females at 150 ppm and higher, and F1 females at 1500 ppm and higher.

In F0 adults, serum FSH levels were significantly decreased in males at 1500 ppm and increased in females at 15,000 ppm compared to controls. In F1 adults, significantly higher levels of DHT were observed in males at 1500 ppm. No significant differences in serum testosterone, estradiol, progesterone and LH levels were noted in F0 and F1 adults of both sexes between control and HBCD-treated groups (data not shown).

3.11. Sperm parameters (F0 and F1 adults)

A significantly lower number of epididymal sperm at 150 ppm and higher mean amplitude of lateral head displacement at 15,000 ppm was found in F0 males compared to controls. There were no significant changes in the sperm counts, the percentage of motile sperm and progressively motile sperm, swimming speed and pattern, and the percentage of morphologically abnormal sperm in F1 adults between control and HBCD-treated groups (data not shown).

Table 8
Organ weights of female F1 adults

HBCD (ppm)	0 (control)	150	1500	15,000
No. of female F1 adults examined	22	22	20	13
Body weight (g)[a]	322.9 ± 25.9	327.0 ± 24.8	328.6 ± 20.2	307.8 ± 30.5
Brain (g)[a]	2.07 ± 0.09[b]	2.06 ± 0.07	2.06 ± 0.08	1.97 ± 0.06**
	0.645 ± 0.045[c]	0.634 ± 0.053	0.630 ± 0.045	0.646 ± 0.056
Pituitary gland (mg)[a]	14.7 ± 1.5[b]	15.8 ± 2.7	15.5 ± 1.8	14.3 ± 3.0
	4.56 ± 0.43[c]	4.83 ± 0.81	4.72 ± 0.59	4.62 ± 0.68
Thyroid (mg)[a,d]	19.3 ± 3.3[b]	19.8 ± 3.5	21.5 ± 4.6	23.9 ± 4.5**
	6.01 ± 1.01[c]	6.08 ± 1.05	6.54 ± 1.36	7.76 ± 1.36**
Thymus (mg)[a]	250 ± 62[b]	233 ± 62	276 ± 80	259 ± 76
	77.4 ± 17.4[c]	71.6 ± 19.9	83.8 ± 21.8	83.9 ± 22.2
Liver (g)[a]	13.49 ± 1.59[b]	14.30 ± 1.29	14.35 ± 1.41	15.58 ± 2.38**
	4.18 ± 0.42[c]	4.39 ± 0.44	4.38 ± 0.47	5.05 ± 0.50**
Kidney (g)[a,d]	2.36 ± 0.23[b]	2.31 ± 0.19	2.39 ± 0.18	2.23 ± 0.26
	0.732 ± 0.054[c]	0.710 ± 0.068	0.729 ± 0.070	0.726 ± 0.051
Spleen (mg)[a]	632 ± 124[b]	595 ± 68	624 ± 93	578 ± 70
	195 ± 33[c]	183 ± 24	190 ± 27	188 ± 16
Adrenal (mg)[a,d]	70.8 ± 10.4[b]	73.9 ± 10.5	74.8 ± 9.6	71.7 ± 13.4
	22.0 ± 3.1[c]	22.6 ± 3.1	22.8 ± 2.8	23.3 ± 3.5
Ovary (mg)[a,d]	102.4 ± 12.9[b]	106.4 ± 13.2	108.6 ± 18.0	104.9 ± 16.9
	31.8 ± 4.2[c]	32.6 ± 3.9	33.1 ± 5.3	34.1 ± 4.2
Uterus (mg)[a]	966 ± 216[b]	913 ± 188	955 ± 204	949 ± 156
	299 ± 64[c]	282 ± 65	291 ± 64	313 ± 69

[a] Values are given as the mean $\pm$ S.D.
[b] Absolute organ weight.
[c] Relative organ weight = organ weight (g or mg)/100 g body weight.
[d] Values are given as the total weights of the organs on both sides.
** Significantly different from the control, $P < 0.01$.

4. Discussion

In the present study, unscheduled deaths and euthanasia due to moribund condition were noted in a few animals. The deaths, euthanasia and clinical signs observed in the present study were not thought to be attributable to the administration of HBCD, because these incidences were very low and inconsistent across generations and sexes and these occurrences are not uncommon in toxicological studies. Lowered body weight and body weight gain accompanied by decreased food consumption were observed at 15,000 ppm in F1 males and females. These findings suggest that a dietary level of 15,000 ppm is generally toxic to rats.

Although a few F0 and F1 adults showed reproductive difficulties, necropsy and the histopathology of the reproductive organs revealed no compound-related changes in these rats. No adverse effects on spermatogenic endpoints observed in the present study are consistent with the previous results of sperm analysis [19].

Lowered body weight of pre-weaning pups was found at 15,000 ppm. More pronounced effects were noted on viability and body weight in F2 pups at this dose. These findings indicate that the dose levels of 15,000 ppm used in this study were potent enough to have adverse effects on the survival and growth of pups. Lochry [31] noted strong correlations between develop-

mental landmark parameters and pup body weight data, which were consistently the more sensitive indicator of the developmental status of offspring. A higher completion rate of eye opening was noted in male and female F1 pups at 1500 ppm, but this rate was not dose-dependent and was not accompanied by changes in body weight. A lower completion rate of eye opening was found in female F2 pups at 1500 ppm and higher, and in male F2 pups at 15,000 ppm, and was associated with lowered body weight. This decreased rate in F2 pups seems to be due to lowered body weight. The lowered completion rate of mid-air righting reflex in female F2 at 15,000 ppm seemed to be due to decreased body weight, because reflex responses are also dependent on physical development [32]. These findings of pre-weaning developmental parameters suggest that high doses (>1500 ppm) of HBCD affect the growth of offspring and the resulting decreased body weight is associated with delays of pre-weaning developmental landmarks and reflex ontogeny.

In the present study, HBCD-related effects were not found on sex hormone-dependent events, such as estrous cyclicity, AGD [33], male preputial separation [34], female vaginal opening [35] or the weight of reproductive organs, or on sex hormone levels at scheduled necropsy. These findings suggest that HBCD has no effects on androgenic/estrogenic events or sexual differentiation.

Transient changes were noted in performance in the water-filled T-maze in F1 males at 1500 ppm and higher, but HBCD

Table 9
Organ weights of male F2 weanlings

HBCD (ppm)	0 (control)	150	1500	15,000
No. of male F2 weanlings examined	22	22	18	13
Body weight (g)[a]	82.2 ± 17.1	84.6 ± 8.7	81.3 ± 13.4	64.7 ± 11.2**
Brain (g)[a]	1.62 ± 0.13[b]	1.65 ± 0.08	1.60 ± 0.10	1.46 ± 0.09**
	2.08 ± 0.58[c]	1.96 ± 0.16	2.01 ± 0.29	2.31 ± 0.33**
Thymus (mg)[a]	343 ± 92[b]	336 ± 57	360 ± 88	282 ± 71
	414 ± 97[c]	397 ± 54	441 ± 69	434 ± 81
Liver (g)[a]	3.87 ± 0.90[b]	4.02 ± 0.55	4.12 ± 0.83	3.88 ± 0.68
	4.72 ± 0.59[c]	4.74 ± 0.35	5.04 ± 0.40*	6.00 ± 0.25**
Kidney (mg)[a,d]	965 ± 167[b]	958 ± 99	933 ± 135	749 ± 100**
	1201 ± 173[c]	1134 ± 56**	1155 ± 85	1170 ± 96
Spleen (mg)[a]	360 ± 83[b]	361 ± 54	346 ± 78	263 ± 50**
	443 ± 77[c]	429 ± 64	426 ± 69	411 ± 66
Adrenal (mg)[a,d]	23.4 ± 5.1[b]	25.1 ± 3.6	24.3 ± 5.2	19.6 ± 3.2*
	28.7 ± 4.4[c]	29.7 ± 3.2	29.9 ± 4.0	30.4 ± 2.0
Testis (mg)[a,d]	476 ± 138[b]	510 ± 81	475 ± 136	385 ± 92
	574 ± 123[c]	600 ± 55	572 ± 93	589 ± 54
Epididymis (mg)[a,d]	73.7 ± 16.8[b]	73.6 ± 10.7	71.8 ± 17.5	61.7 ± 9.5*
	90.7 ± 14.1[c]	87.2 ± 10.6	87.3 ± 9.6	96.2 ± 10.5
Ventral prostate (mg)[a]	40.6 ± 9.7[b]	42.3 ± 9.5	41.7 ± 12.1	29.5 ± 6.8**
	50.2 ± 9.3[c]	50.2 ± 10.7	50.8 ± 9.6	47.3 ± 15.8

[a] Values are given as the mean ± S.D.
[b] Absolute organ weight.
[c] Relative organ weight = organ weight (g or mg)/100 g body weight.
[d] Values are given as the total weights of the organs on both sides.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

did not cause any toxicological changes in spontaneous locomotor activity in F1 rats of both sexes. Previously, decreased locomotion at low and high doses and worse performance in the Morris water maze at high doses were reported in male mice given a single gavage dose with HBCD at 0.9 and 13.5 mg/kg bw on PND 10 [21]. The discrepancy in the behavior of offspring between the present and previous studies could be explained by the difference in the actual intake of HBCD in pups between the direct exposure of pups and maternal exposure, indirectly to pups via maternal milk, and by differences in the animal species used in these studies. Further studies are needed to clarify the transfer of HBCD to the nervous system in pre-weaning animals and species difference.

The changes in absolute and/or relative weight of the brain, pituitary, thymus, kidney, spleen, adrenal, testis, epididymis, seminal vesicle, ventral prostate, ovary and uterus observed in adults and/or weanlings of either sexes or generation are not thought to have toxicological significance, because these changes were not dose-dependent or were inconsistent across age, sex and generation. Increased absolute and/or relative weights of the liver were noted regardless of sex, age and generation in the present study. Previously, an increase in absolute and relative liver weight was reported in rat dams given dietary HBCD at 1.0% [23]. A dose-dependent weight increase of the liver was noted only in females given HBCD by gavage for 28 days [20]. Gavage dose of HBCD for 28 days caused increased absolute and relative weights of the liver, but

not test article-related histopathological lesions, in male rats at 1000 mg/kg bw/day and in female rats at 350 mg/kg bw/day and higher [18]. In a rat 90-day repeated dose toxicity study of HBCD by gavage, increased absolute and relative weights of the liver were detected at 100 mg/kg bw/day and higher in males and females [19]. The liver change in males was characterized as minimal hepatocellular vacuolation, and a slight increase in the severity of this change was found in females at 300 mg/kg bw/day and higher. In females, minimal and mild centrilobular hepatocellular hypertrophy were also observed at 1000 mg/kg bw/day; however, the author concluded that these increases in liver weight were an adaptive, rather than a toxic response, and are not uncommon in rats, and are most likely the results of microsomal induction because of the absence of test article-related histopathological and serum chemistry changes [18,19]. It is known that hepatic enzyme induction produces increased liver weight without accompanied histopathological changes in rats [36]. In the present study, neither histopathological change in the liver in any sex, generation or age, nor gender difference in the effects of HBCD on the liver were noted; however, the increased levels of total protein and globulin, in F0 males and females and F1 males, observed in the present study were considered to result from the increased liver weight. The induction of CYP2B1 mRNA, CYP2B1/2B2 protein and 7-pentoxyresorufin O-depentylase activity, suggesting phenobarbital-type induction, was caused in juvenile/young rats given HBCD in feed for 28 days [37]. These findings suggest

Table 10
Organ weights of female F2 weanlings

HBCD (ppm)	0 (control)	150	1500	15,000
No. of female F2 weanlings examined	21	22	20	13
Body weight (g)[a]	75.3 ± 12.5	75.8 ± 8.5	73.1 ± 12.8	57.9 ± 11.6**
Brain (g)[a]	1.57 ± 0.11[b]	1.58 ± 0.07	1.55 ± 0.12	1.41 ± 0.15**
	2.14 ± 0.37[c]	2.11 ± 0.20	2.17 ± 0.35	2.48 ± 0.34**
Thymus (mg)[a]	338 ± 85[b]	324 ± 50	331 ± 69	260 ± 80**
	447 ± 81[c]	429 ± 57	451 ± 51	445 ± 83
Liver (g)[a]	3.55 ± 0.64[b]	3.57 ± 0.48	3.63 ± 0.74	3.42 ± 0.77
	4.70 ± 0.27[c]	4.70 ± 0.28	4.94 ± 0.32	5.89 ± 0.44**
Kidney (mg)[a,d]	916 ± 131[b]	885 ± 98	868 ± 144	679 ± 138**
	1226 ± 93[c]	1169 ± 65	1194 ± 84	1177 ± 103
Spleen (mg)[a]	325 ± 59[b]	302 ± 42	299 ± 62	225 ± 45**
	436 ± 61[c]	399 ± 43	412 ± 61	392 ± 53
Adrenal (mg)[a,d]	22.1 ± 4.2[b]	21.5 ± 2.6	21.5 ± 4.3	17.6 ± 3.1**
	29.5 ± 4.1[c]	28.4 ± 3.4	29.4 ± 3.1	30.7 ± 2.6
Ovary (mg)[a,d]	20.0 ± 3.9[b]	22.9 ± 2.6*	20.9 ± 3.9	18.2 ± 4.0
	26.9 ± 5.1[c]	30.5 ± 3.9*	28.8 ± 4.2	32.1 ± 7.5*
Uterus (mg)[a]	60.8 ± 16.1[b]	63.6 ± 15.1	57.0 ± 15.7	47.6 ± 11.4*
	80.9 ± 16.3[c]	84.4 ± 21.0	78.7 ± 21.7	83.7 ± 20.3

[a] Value are given as the mean ± S.D.
[b] Absolute organ weight.
[c] Relative organ weight = organ weight (g or mg)/100 g body weight.
[d] Values are given as the total weights of the organs of both sides.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

that the increased liver weight and blood biochemistry changes observed in the present study may be attributable to enzyme induction.

In the previous 90-day repeated dose toxicity study, HBCD caused increases in the absolute and relative weights of the thyroid/parathyroid in females and thyroid follicular cell hypertrophy in males and females at 300 mg/kg bw/day and higher, and depressed serum T4 levels in males at 100 mg/kg bw/day and higher and in females at 300 mg/kg bw/day and higher [19]. van der Ven et al. [20] described that the most striking effect of HBCD was on the thyroid hormone axis, including lowered T4 levels, increased immunostaining for TSH in the pituitary, increased weight/activation of the pituitary and thyroid, induction of hepatic T4-glucuronyl transferase, and decreased thyroid follicles size, and these effects were restricted to females. They also noted that higher sensitivity in females may be due to higher liver concentrations of HBCD than in males [20]. In the present study, reduced levels of serum T4 in males and females at 15,000 ppm and increased levels of serum TSH at 1500 ppm and higher in females were observed. It seems likely that the lowered T4 levels may be related to enhanced elimination of T4 due to the induction of hepatic drug metabolizing enzymes and that increased TSH levels may be due to feedback resulting from decreased T4 levels. The increased TSH levels in F0 females at 150 ppm were not considered to have toxicological meaning, because these changes were not accompanied by histopathological changes in the thyroid or decreased T4 levels, or were inconsistent across generations at this dose. Increased thyroid

weight at 15,000 ppm and decreased thyroid follicle size and hypertrophy of thyroid follicular cells at 1500 ppm and higher were also noted in male and female F0 and F1 generations. These present findings are essentially consistent with the previous findings [19,20].

Primordial follicles preserve oocytes during the reproductive life span and constitute a stockpile of nongrowing follicles in mammalian ovaries. The primordial follicle population represents a female's total reproductive potential, because primordial follicles do not proliferate or grow [38]. It is reported that busulfan destroyed primordial germ cells, rendering the individual deficient in primordial follicles [39,40]. A reduced primordial stockpile was observed in female offspring of SD rats given busulfan on day 13–15 of pregnancy [41]. In a continuous breeding study in which female Long-Evans hooded rat offspring, after maternal intraperitoneal injection of busulfan on day 14 of pregnancy, were bred with control males for eight breeding cycles, the number of pups delivered was reduced at 2.5 and 5.0 mg/kg bw and no pups were delivered at 10 mg/kg bw [42]. Gray et al. [43] mentioned that continuous breeding of females exposed to reproductive toxicants during critical developmental periods is more useful than a single breeding trial in the detection of subfertility. In the present study, histopathological examinations of the ovary of F1 females revealed a decreased number of primordial follicles at 1500 and 15,000 ppm. Variation exists in primordial follicle counts dependent upon the methodology used [44], but follicle counts provide a more sensitive indicator of potential toxicity than did measures of fertility [45]. Parker
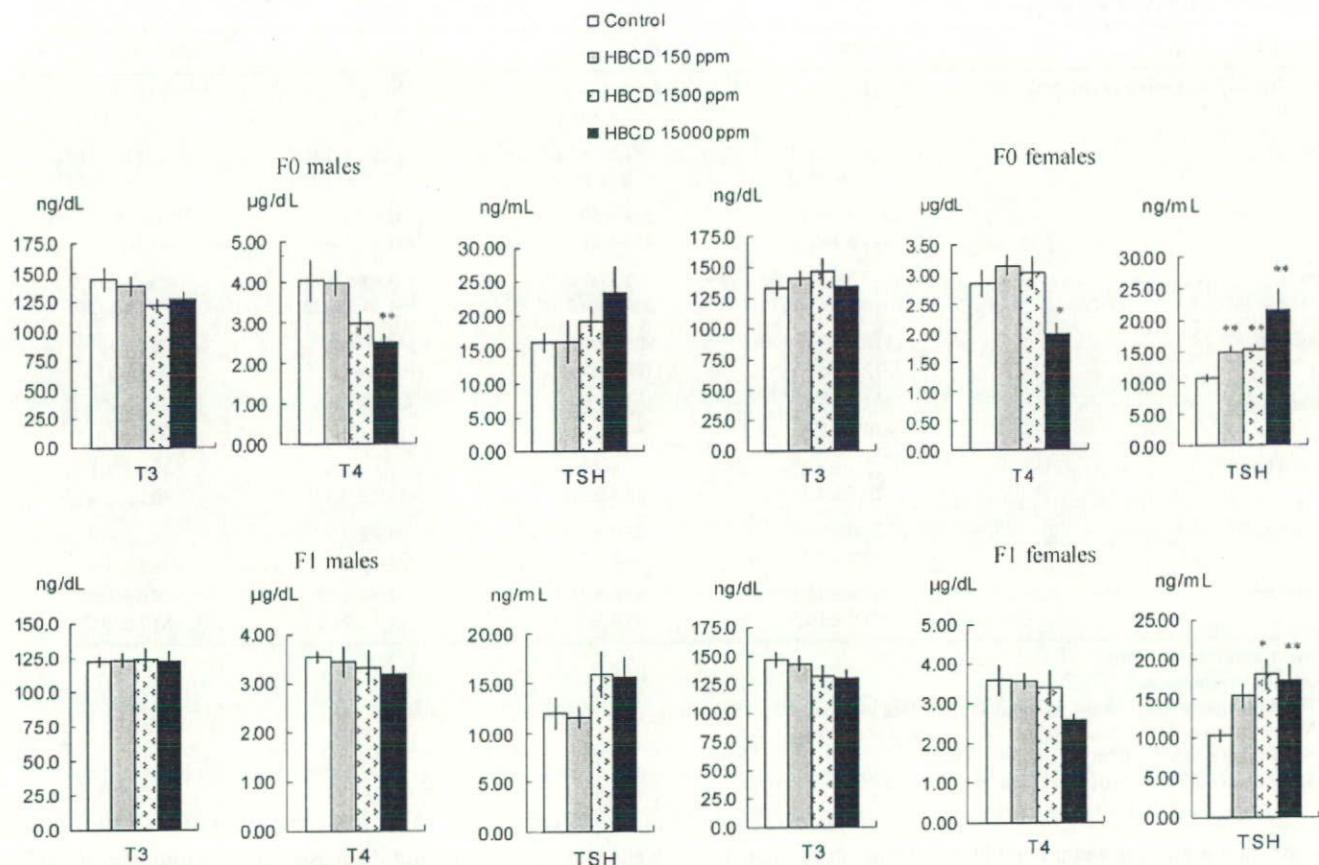
Fig. 4. Serum levels of T3, T4 and TSH in F0 and F1 rats. Values are given as the mean ± S.E.M. (*) Significantly different from the control, $P < 0.05$. (**) Significantly different from the control, $P < 0.01$.

[46] noted that a decrease in primordial follicle count is usually considered a biomarker of an adverse reproductive effect because no recovery is possible. Although these findings suggest that HBCD is potentially reproductively toxic, no adverse effects on reproductive parameters in F1 dams, or on the numbers of implantations or F2 pups delivered were noted in the present study. In the present study, F1 parent rats were subjected to a single breeding trial. A continuous breeding study of HBCD may be needed to clarify the reproductive toxicity of HBCD, especially the adverse effects of HBCD on the reproductive life span.

In conclusion, the results of the two-generation reproductive toxicity study described here provide a more comprehensive toxicity profile of HBCD than has been previously reported, and the NOAEL of HBCD in this study was considered to be 150 ppm (10.2 mg/kg bw/day) in rats. NCR [4] estimated that the average oral dose rate was 0.026 mg/kg bw/day. The estimated human intake of HBCD is well below the NOAEL in the present study.

Acknowledgement

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