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 $50 \pm 20\%$, 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

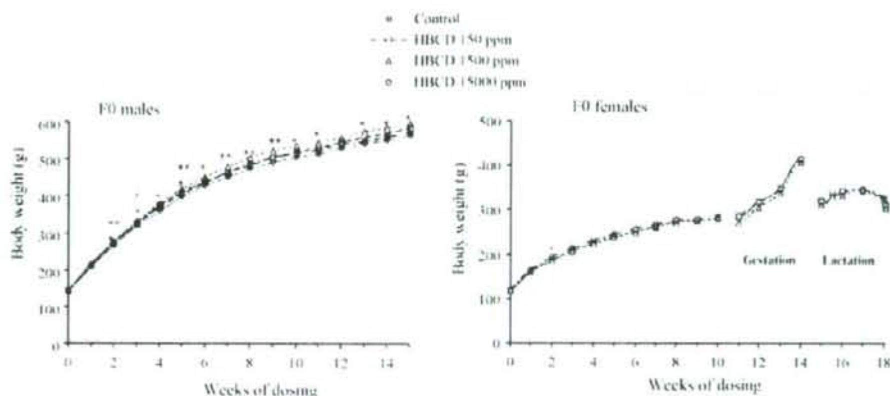

Fig. 1. Body weights of F0 male and female rats. (*) Significantly different from the control, $P < 0.05$. (**) Significantly different from the control, $P < 0.01$.

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

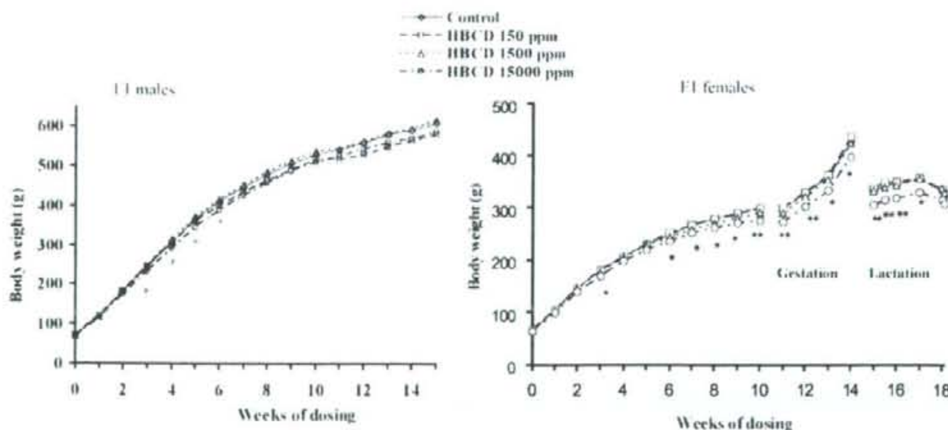

Fig. 2. Body weights of F1 male and female rats. (*) Significantly different from the control, $P < 0.05$. (**) Significantly different from the control, $P < 0.01$.

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

Table 1 (*Continued*)

HBCD (ppm)	0 (control)	150	1500	15,000
Day 4	8.9 ± 2.3 (22)[k]	8.5 ± 1.3 (22)[k]	8.8 ± 1.8	7.3 ± 1.3 (20)[k,**]
Day 7	14.3 ± 3.5 (21)[k]	14.2 ± 2.8 (22)[k]	13.5 ± 3.9	10.7 ± 2.6 (17)[k,**]
Day 14	31.2 ± 6.5 (21)[k]	31.3 ± 5.1 (22)[k]	29.3 ± 7.3	23.9 ± 5.9 (13)[k,**]
Day 21	52.0 ± 10.0 (21)[k]	52.8 ± 6.6 (22)[k]	51.2 ± 10.8	41.6 ± 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 ± 36.3*	45.8 ± 34.6 (17)[c]
Female	49.3 ± 37.8 (23)[c]	66.7 ± 41.3	82.9 ± 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 ± 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 ± 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 ± 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).

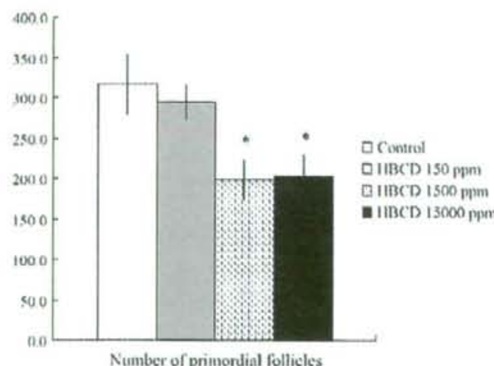

Fig. 3. Number of primordial follicles in the ovary of F1 female rats. Values are given as the mean ± S.E.M. (*) Significantly different from the control, $P < 0.05$.

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.

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] 0.363 ± 0.028[c]	2.22 ± 0.08 0.388 ± 0.036*	2.18 ± 0.09 0.358 ± 0.034	2.11 ± 0.07** 0.363 ± 0.032
Pituitary gland (mg)[a]	13.1 ± 1.5[b] 2.16 ± 0.22[c]	13.6 ± 1.6 2.37 ± 0.23**	13.2 ± 1.4 2.17 ± 0.22	13.3 ± 1.2 2.28 ± 0.23
Thyroid (mg)[a,d]	24.3 ± 4.9[b] 4.03 ± 0.79[c]	24.2 ± 3.0 4.22 ± 0.63	25.4 ± 4.7 4.15 ± 0.72	29.0 ± 5.6** 4.96 ± 0.87**
Thymus (mg)[a]	344 ± 72[b] 56.7 ± 10.8[c]	305 ± 92 52.8 ± 14.3	368 ± 100 59.8 ± 14.4	341 ± 76 58.3 ± 11.1
Liver (g)[a]	19.83 ± 2.06[b] 3.27 ± 0.18[c]	19.36 ± 3.13 3.34 ± 0.26	20.73 ± 3.01 3.37 ± 0.25	22.61 ± 3.04** 3.86 ± 0.28**
Kidney (g)[a,d]	3.74 ± 0.34[b] 0.618 ± 0.037[c]	3.59 ± 0.36 0.625 ± 0.052	3.77 ± 0.33 0.619 ± 0.074	3.77 ± 0.58 0.645 ± 0.080
Spleen (mg)[a]	885 ± 168[b] 146 ± 26[c]	840 ± 147 146 ± 22	878 ± 163 143 ± 22	851 ± 113 146 ± 17
Adrenal (mg)[a,d]	59.7 ± 11.0[b] 9.9 ± 1.6[c]	63.1 ± 15.8 10.9 ± 2.3	60.3 ± 10.7 9.9 ± 1.8	59.4 ± 6.7 10.2 ± 1.1
Testis (g)[a,d]	3.63 ± 0.33[b] 0.602 ± 0.069[c]	3.52 ± 0.27 0.614 ± 0.049	3.51 ± 0.35 0.576 ± 0.062	3.45 ± 0.36 0.593 ± 0.065
Epididymis (mg)[a,d]	1346 ± 107[b] 223 ± 24[c]	1328 ± 104 232 ± 24	1282 ± 109 210 ± 19	1357 ± 104 234 ± 23
Seminal vesicle (g)[a]	2.36 ± 0.26[b] 0.391 ± 0.051[c]	2.28 ± 0.22 0.398 ± 0.050	2.33 ± 0.29 0.382 ± 0.051	2.38 ± 0.22 0.409 ± 0.045
Ventral prostate (mg)[a]	834 ± 195[b] 137 ± 28[c]	779 ± 217 135 ± 34	803 ± 175 131 ± 30	789 ± 159 135 ± 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. Significantly 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 ± 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 developmental 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

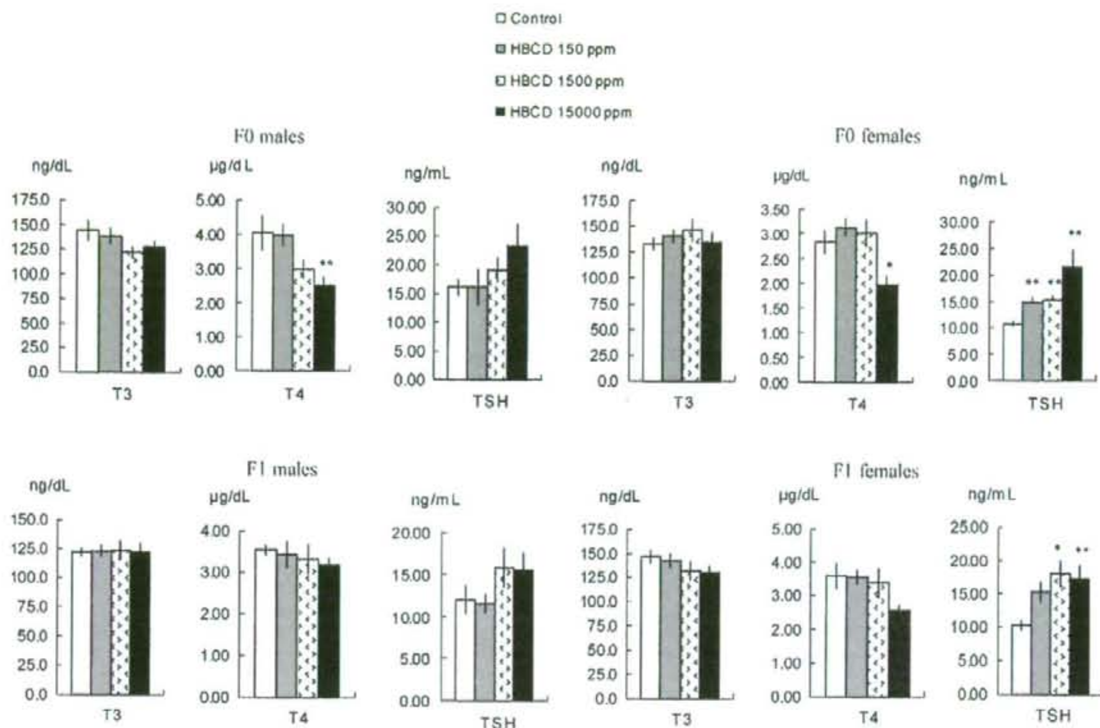

Fig. 4. Serum levels of T3, T4 and TSH in F0 and F1 rats. Values are given as the mean ± S.E.M. (*) Significantly different from the control, $P<0.05$. (**) Significantly different from the control, $P<0.01$.

[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.

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Review

Review of developmental toxicity of nitrophenolic herbicide dinoseb, 2-*sec*-butyl-4,6-dinitrophenol

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ABSTRACT

The present review paper summarizes the data available in the literature concerning prenatal exposure to dinoseb (2-*sec*-butyl-4,6-dinitrophenol; CAS No. 88-85-7), evaluating reported developmental toxicity in experimental animals. In particular, we have focused on the variable factors in the manifestation of the developmental toxicity of dinoseb. In this review, we showed that developmental toxicity of dinoseb was remarkably different between animal species used in experiments. Teratogenicity was detected in rats fed a diet containing dinoseb, in mice given dinoseb by gavage, intraperitoneally or subcutaneously, and in rabbits given dinoseb by gavage or dermally. Teratogenicity in rats given dinoseb by gavage was influenced by the dietary composition used in the experiments. We postulated that evaluation of the developmental toxicity after exposure by anticipated routes of human exposure would be important for risk assessment in humans.

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Contents

1. Introduction 328
2. Developmental toxicity of dinoseb 328
   2.1. Developmental toxicity in rats 328
      2.1.1. Gavage studies in rats 328
      2.1.2. Feeding studies in rats 329
      2.1.3. Intraperitoneal studies in rats 330
   2.2. Developmental toxicity of dinoseb in mice 330
      2.2.1. Gavage studies in mice 330
      2.2.2. Intraperitoneal studies in mice 330
      2.2.3. Subcutaneous studies in mice 332
   2.3. Developmental toxicity in rabbits 332
      2.3.1. Gavage studies in rabbits 332
      2.3.2. Dermal studies in rabbits 332
3. Discussion and conclusions 332
   Acknowledgement 333
   References 333

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1. Introduction

Dinoseb, 2-*sec*-butyl-4,6-dinitrophenol (CAS No. 88-85-7), is used as a nitrophenolic herbicide in soybeans, vegetables, fruits, nuts, citrus and other field crops for the selective control of grass and broadleaf weeds. It is also used as an insecticide in grapes and as a seed crop drying agent [1]. Dinoseb is a high volume chemical with production or importation exceeding 1000 tonnes per year in Organisation for Economic Co-operation and Development (OECD) member countries and widely used [2,3]. The volume of dinoseb imported into Japan is estimated to be 110 tonnes from April 2005 to March 2006 [4].

Dinoseb is a dark reddish-brown solid or dark orange viscous liquid, depending on the temperature (melting point 32–42 °C) [5]. Dinoseb is well absorbed from the gastrointestinal tract by the oral route in mice and can pass through the placenta into the fetus of mice [6]. A dermal study showed that in 6 h young and adult female rats absorbed about 44% of the dose, while at 120 h 75.9% was absorbed in young and 92.5% in adults [7]. Dinoseb shows relatively strong acute toxicity with the oral LD_{50} of 5–50 mg/kg in female rats [8], the intraperitoneal LD_{50} of 14.1–22.5 mg/kg in mice [9] and the dermal LD_{50} of 40 mg/kg in rabbits [9]. The inhalation LC_{50} is 33–290 mg/m^3 for 4-h exposure in rats [9]. The basic mechanism of toxicity is thought to be stimulation of oxidative metabolism in cell mitochondria by the uncoupling of oxidative phosphorylation [10]. Toxicity of dinoseb is enhanced by physical activity and high ambient temperature such as in an outdoor agricultural environment [10,11]. Early symptoms of dinoseb exposure include hyperthermia, sweating, headache and confusion. Severe exposure may result in restlessness, seizures, coma and death [10–12].

Exposure to dinoseb may occur by direct contact, ingestion and inhalation for users and producers, but potential indirect exposure to dinoseb via the environment 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 US [13]. 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 [13].

Dinoseb has an interesting history as a developmental toxicant. Dinoseb was approved for sale in the US in 1948 [1] and was one of the chemicals permitted on the market on the basis of safety tests conducted by Industrial Bio-Test Laboratory, a concern later found to have submitted many flawed and even fraudulent reports on its procedures and results [14]. In a later study, dinoseb showed teratogenicity in mice when administrated intraperitoneally, but not by gavage administration [15]. In a subsequent study, gavage dosing of dinoseb induced both maternal toxicity and developmental toxicity without teratogenic effects, but dietary administered dinoseb was reported to be teratogenic in rats [16]. In an unpublished study conducted in rabbits, neural malformations without maternal toxicity were observed after dermal application of dinoseb [13,17]. Dinoseb as a pesticide was banned in the US in 1986 and the EU in 1991, based on the potential risk of birth defects and other adverse health effects in humans [1,18].

We previously reported the results of a combined repeated dose toxicity study with reproduction/developmental toxicity screening test, in which Crj:CD(SD)IGS rats were dosed with dinoseb by gavage at 0, 0.78, 2.33 or 7.0 mg/kg bw/day. At the highest dose, the numbers of dams that delivered their pups and of dams with live pups at delivery were reduced, with only one dam delivering live pups at this dose. Developmental toxicity of dinoseb was not completely elucidated in our previous study because this screening test used a relatively small number of animals and investigated a limited number of endpoints. Only an external examination was performed in live newborns. No increased incidences of pups with malformations were noted [19]. Although our study provided limited information on the teratogenicity of dinoseb, our findings supported the results of a study of Giavini et al. [16] that gavage dosing of dinoseb was not teratogenic in rats. However, the same authors also showed that dietary administered dinoseb produced fetal malformations in rats [16]. These results indicate that the manifestation of developmental toxicity by dinoseb depends on the mode of administration in rats. However, these relationships are much more complicated than originally thought, and the composition of the diet has been shown to influence the ability of dinoseb to induce microphthalmia in pups [20].

Developmentally toxic effects of chemicals are influenced by the susceptibility of animal species and strains, the developmental stages of offspring and administration doses [21,22]. Teratogenicity is governed by dose–effect relations, but there are many variable factors such as the duration of chemical treatment [23], frequency of dosing [24], routes or modes of administration [25–28], the vehicle/suspending agent [29] or a combination of chemicals [30]. Dinoseb is one of the chemicals which show different developmental toxicity according to these variable factors. The present review paper summarizes the data available in the literature concerning prenatal exposure to dinoseb, evaluating reported developmental toxicity in experimental animals with particular focus on the variable factors in the manifestation of the developmental toxicity of dinoseb.

2. Developmental toxicity of dinoseb

The relationship between maternal toxicity and developmental toxicity has been expressed in several ways in an attempt to clarify the toxicity [31,32], however the relevance of these expressions has not been established. This paper focuses on the developmental toxicity of dinoseb, but both maternal and fetal toxicities are summarized to show their relationship with respect to dinoseb. It should be noted that the term dinoseb has been used in the literature to refer to several related chemicals based on 2-*sec*-butyl-4,6-dinitrophenol (CAS: 88-85-7). In this paper, dinoseb refers to the parent molecule only.

2.1. Developmental toxicity in rats

Table 1 shows the results of developmental toxicity studies of dinoseb in rats. There are oral (gavage and diet) and i.p. administration studies. The data were reviewed by routes of administration, in order of the most likely route of human intake. Only statistically significant effects are summarized unless noted otherwise.

2.1.1. Gavage studies in rats

In our previous study, male Crj:CD(SD)IGS rats were dosed dinoseb by gavage for a total of 42 days beginning 14 days before mating and females were dosed for a total of 44–48 days beginning 14 days before mating to day 6 of lactation at 0 (vehicle corn oil), 0.78, 2.33 or 7.0 mg/kg bw/day [19]. 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. Eight of twelve females died and two animals were moribund during late pregnancy at 7.0 mg/kg bw/day. Developmental toxicity of dinoseb was not completely estimated because only one dam with live pups was obtained at the highest dose and newborn rats were examined only externally. No increased incidence of pups with an external malformation was noted in the dinoseb-treated groups.

In teratology studies in rats, skeletal variation, delayed ossification and/or decreased fetal body weight was commonly observed

Table 1
Developmental toxicity of dinoseb in rats

Strain	Dose	Exposure time	Developmental effect observed	Reference
Gavage				
Crj:CD(SD)IGS rat	7 mg/kg	44–48 days	↓Number of dams delivered and number of dams with live pups at delivery	[19]
CD rat	10 mg/kg (in corn oil)	GDs 5–14	Skeletal variations	[16]
	15 mg/kg (in corn oil)		Delayed ossification, ↓fetal weight	
	7.5, 10 mg/kg (twice/day)	GDs 5–12	Skeletal variations, ↓fetal weight	
	15 mg/kg (in NaOH)	GDs 5–12	Skeletal variations, delayed ossification ↓fetal weight	
Crl:CD rat	15 mg/kg (with diet B)	GDs 5–13	↓Fetal weight, microphthalmia	[20]
	15 mg/kg (with diet A)		↓Fetal weight	
Wistar/Han rat[a]	3 mg/kg	GDs 6–15	Absence of thoracic vertebrae	[13]
	10 mg/kg		Skeletal variations	
Feeding				
CD rat	200 ppm (15 mg/kg)	GDs 5–14	↓Fetal weight, microphthalmia, skeletal variations	[16]
Crl:CD rat	200 ppm (diet B)	GDs 5–13	↓Fetal weight, microphthalmia	[20]
	200 ppm (diet A)	GDs 5–13	No effects	
SD rat	150 ppm (9.23 mg/kg)	GDs 5–14	↑Total intra-uterine loss	[9,33]
	200 ppm (10.86 mg/kg)		↑Early embryonic loss, ↑resorptions, ↓fetal weight, hypoplastic tail	
Sharman rat[a]	Up to 200 ppm	153 days	↓Fertility, fecundity, neonate survival, weight gain, viability and lactation	[34]
CD(SD) rat[a]	1, 3, 10 mg/kg	Three-generation	↓Body weight gain in pups (F1, F2, F3)	[13,17]
		Next two-generation	↓Body weight gain in pups (F4, F5), absolute/relative gonadal weight (F4) Low viability index (F5)	
Intraperitoneal				
SD rat	8.0 mg/kg	GDs 9–11	↓Fetal body weight, dilated renal pelvis and ureters in fetuses Pathological changes in liver and kidney in fetuses and neonates	[35]
	9.0 mg/kg		↓Fetal crown-rump length, neonatal body weight	
SD rat	7.5 mg/kg	GDs 9–11, 10–12	↓Fetal body weight	[36]
	10.5 mg/kg		Functional defect of kidney (PND 6), ↓body weight in pups (PND 30), ↑Relative weight of kidney (PND 30)	

[a] Only secondary literature or abstract is available.

in fetuses of dams treated with dinoseb. Giavini et al. [16] administered dinoseb to pregnant CD rats by gavage in corn oil either once a day on gestation days (GDs) 5–14 at 0, 2.5, 5, 10 or 15 mg/kg bw/day or twice a day on GDs 5–12 at 15 (7.5 × 2) or 20 (10 × 2) mg/kg bw/day. Dinoseb was also dosed to pregnant rats on GDs 5–12 at 15 mg/kg bw/day in NaOH. This vehicle was selected to conform to a vehicle used in a study of Gibson [15] in which dinoseb in NaOH showed teratogenicity in mice when administered i.p. but not by gavage. An increased incidence of supernumerary ribs was observed at 10 mg/kg bw/day and higher, and fetal weight was decreased at 15 and 20 mg/kg bw/day regardless of frequency of dosing or vehicle. Delayed ossification of caudal vertebrae, metacarpals or sternebrae was observed at a single dose of 15 mg/kg bw/day (in both of corn oil and NaOH). These doses also caused maternal toxicities such as mortality and decreases in body weight gain. No malformations were observed in fetuses of dams treated with dinoseb under the test condition regardless of the dosing regime or vehicle used in the experiment.

Fetal body weight was decreased when pregnant Crl:CD rats were given dinoseb at 15 mg/kg bw/day with diet A (protein 21%, fat 3.5%, fiber 6.5%, ash 7.5% and N-free extractives 61.5%: Italiana Mangimi, Settimo Milanese, Italy) and diet B (protein 21%, fat 4.8%, fiber 4.2%, ash 8.5% and N-free extractives 61.5%: Mangimi Piccioni, Gessate, Italy) on GDs 5–13 [20]. Dinoseb induced microphthalmia in fetuses of animals fed diet B but did not induce maternal toxicity. Maternal mortality and decreased maternal body weight gain were observed when dinoseb was given with diet A. Although developmental toxicity was different based on the type of diet, there were no differences in dinoseb concentrations in maternal plasma and in embryo between the two dietary groups.

Wistar/Han rats were administrated dinoseb by gavage on GDs 6–15 at 0, 1, 3 or 10 mg/kg bw/day [13]. No information on vehicle was presented in this study. Only slight depressions were observed in food consumption and body weight gain of dams at 10 mg/kg bw/day. Fetuses at the highest dose showed a slight decrease in body weight, and increases in the incidence of skeletal ossification and the number of supernumerary ribs. At 3 mg/kg bw/day and higher, absence of thoracic vertebrae was observed. No further information is available for this study, but the result indicates that dosing of dinoseb by gavage is hazardous in Wistar/Han rats.

These findings in rats suggest that the composition of the diet used in experiments may influence teratogenic response. It is interesting to note that dinoseb-induced malformations were observed without maternal toxicity by gavage dosing of dinoseb in rats.

2.1.2. Feeding studies in rats

Feeding of dinoseb to CD rats on GDs 5–14 produced a specific teratogenic effect, increased incidence of fetuses with microphthalmia, reduced fetal weight and increased incidence of fetuses with supernumerary ribs at 200 ppm (15 mg/kg bw/day) accompanied by decreased maternal body weight gain [16]. An increased incidence of fetuses with microphthalmia and reduced fetal weight were also observed when pregnant Crl:CD rats were given dinoseb in diet B at 200 ppm on GDs 5–13. At this dose, maternal food consumption and body weight gain were decreased compared to the control groups [20]. When dinoseb was fed with diet A, maternal food consumption and body weight gain were reduced, but no effects were found in fetuses [20]. These findings indicate that the developmental toxicity, including teratogenicity, of dinoseb in rats was influenced by diet compositions.

Following feeding of dinoseb on GDs 5–14 at 0, 50, 100, 150, 200, 250 300 and 350 ppm (0, 3.26, 6.9, 9.23, 10.86, 9.38, 9.49 and 8.6 mg/kg bw/day) in SD rats, the number of resorp-

tions at 200–350 ppm, early embryo loss at 200–350 ppm, and total intra-uterine loss at 150–350 ppm were increased in a dose-related manner [9,33]. Body weight gain in dams was decreased at 150–350 ppm. At 200 ppm, hypoplastic tail was observed in 8 out of 62 fetuses and fetal weight was decreased. In decidualized females given dinoseb on days 7–10 of pseudopregnancy, uterine protein and glycogen concentrations were decreased at 200 ppm and higher in a dose-related manner. The authors suggest a toxic role of dinoseb in the uterine environment.

Hall et al. [34] provide a brief summary of a subchronic feeding study in which Sherman male and female rats were fed a diet containing dinoseb at 0, 50, 100, 150, 200, 300, 400 and 500 ppm for 153 days. The 300, 400 and 500 ppm groups were terminated at day 21 of administration due to mortality of 14, 100 and 100%, respectively, and only animals fed dinoseb up to 200 ppm were evaluated. Fertility, fecundity, neonate survival, weight gain, viability and lactation were depressed. No further details are available.

In an unpublished five-generation study, decreased body weight gains were observed in parents during the pre-mating period (F0, F1 and F2) at 10 mg/kg bw/day dinoseb in diet and in pups on postnatal day (PND) 21 (F1, F2 and F3) at 1, 3 and 10 mg/kg bw/day, but weights at birth were similar to controls. Body weight gain in F4 and F5 pups was increased and absolute and relative gonadal weights in F4 pups were decreased at all dose levels. A low viability index was obtained (from F4 to F5) at all dose levels. No detailed information is available for this study [13,17].

Taken together, 150–200 ppm dinoseb by feeding dose represents a threshold dose at which maternal toxicity and developmental toxicity including teratogenicity begin to appear, but adverse effects on embryos/fetuses were reduced when dinoseb was given with diet A.

2.1.3. Intraperitoneal studies in rats

Two i.p. studies in rats showed similar results on developmental toxicity. When dinoseb was given to SD rats on GDs 9–11 at doses up to 15.8 mg/kg bw/day in NaOH, all pregnant rats given dinoseb at 11.2 mg/kg bw/day and higher, and 3 of the 16 pregnant rats at 9.0 mg/kg bw/day died. There were dilated renal pelvis and ureters in fetuses, decreased body weight in fetuses, and pathological changes in the liver and kidney in both fetal and neonatal rats at 8.0 mg/kg bw/day without maternal toxicity. At 9.0 mg/kg bw/day, fetal crown-rump length (CRL) was decreased, and neonatal body weight was decreased on PNDs 1 and 7 but not on PND 42. In surviving dams, dinoseb did not affect the number of live fetuses or the resorption rate in surviving dams [35].

When dinoseb was administrated i.p. to pregnant SD rats on GDs 9–11 or 10–12 at 0–18.0 mg/kg bw/day in NaOH, fetal body weight was decreased at 7.5 mg/kg bw/day and higher, but weights at birth and on PND 6 were not affected. Maternal death was observed at 8.0 mg/kg bw/day and higher, and 10.5 mg/kg bw/day was an approximate LD_{50} in pregnant rats. Postnatal observation on PND 30 revealed that there was a body weight reduction and an increase in relative kidney weight at 10.5 mg/kg bw/day. On PND 6, there was a deficit in urinary concentrating ability in pups of dams given dinoseb on GDs 9–11 at 10.5 mg/kg bw/day [36].

As described above, dinoseb produced suggestive renal damage in rat offspring following maternal administration. However, pathological changes in the kidney observed in prenatal rats were reduced in incidence or not detected at 42-day postpartum in the study of McCormack et al. [35]. In the study of Daston et al. [36], a deficit in urinary concentrating ability observed during postnatal development also disappeared after functional maturation (PND 30). Prenatal incidence of dilated renal pelvis was not dose-dependent. Moreover, Woo and Hoar [37] noted that the renal parenchyma increased in weight rapidly, but that the renal papilla increased in length solely during late pregnancy, and they suggested that this discrepancy in growth rate frequently resulted in the kidney with an enlarged renal pelvis. Taken all together, these renal effects appear to be a developmental delay, but not a permanent functional impairment.

2.2. Developmental toxicity of dinoseb in mice

Table 2 shows the results of developmental toxicity studies of dinoseb in mice. There are gavage, i.p. and s.c. administration studies. The data were reviewed by routes of administration, in order of the most likely route of human intake. Only statistically significant effects are summarized unless noted otherwise.

2.2.1. Gavage studies in mice

Pregnant CD-1 mice were administered dinoseb in corn oil on GDs 8–12 at 15 mg/kg bw/day, the expected maximum tolerated dose level of dinoseb. No effects were observed in reproductive and developmental parameters [38]. Pregnant CD-1 mice were given dinoseb in corn oil by gavage at 26 or 33 mg/kg bw on GD 7. Two out of 40 pregnant animals died at 33 mg/kg bw, but percent mortality and body weight of pregnant mice were not changed. An increased incidence of supernumerary ribs was observed in both dinoseb-treated groups. The authors noted that increased incidence of supernumerary ribs may be a response to a non-specific disruption in maternal status [39].

Administration of dinoseb to pregnant CD-1 mice by gavage on GDs 7–8 at 50 mg/kg bw/day in NaOH produced reduced fetal weight and increased incidence of fetuses with supernumerary ribs (71% in litters) without maternal death. The authors suggest that supernumerary ribs are indicative of basic alterations in the development of the axial skeleton [40]. A similar study conducted by Rogers et al. [41] observed a dose-related increased incidence of mouse fetuses with supernumerary ribs following maternal administration of dinoseb in NaOH at 50 mg/kg bw/day on GDs 7–8 and suggested that increased incidence of supernumerary ribs in fetuses is toxicologically significant. Skeletal anomalies such as sternum or vertebral centrum defects and fused ribs were also detected in fetuses of mice given dinoseb on GDs 7–8 at 50 mg/kg bw/day in NaOH. Although the treatment regimes of Branch et al. [40] and Rogers et al. [41] were essentially same, they obtained different developmental effects in fetuses of mice given dinoseb at 50 mg/kg bw/day. Rogers et al. [41] used 25 pregnant mice. On the other hand, Branch et al. [40] used only two pregnant mice, which is too small to evaluate the developmental toxicity. Therefore, it appears that a gavage dosing of dinoseb on GDs 7–8 at 50 mg/kg bw/day can induce teratogenic effects without maternal toxicity in CD-1 mice.

Dinoseb was administered to pregnant Swiss-Webster mice during GDs 7–15, 9–11 or 13–15 by gavage up to 50 mg/kg bw/day in NaOH. Gavage dosing of dinoseb produced no increased incidence of gross or soft-tissue anomalies. When dinoseb was given by gavage on GDs 9–11, six out of eight pregnant animals died at 50 mg/kg bw/day, but no effects were observed on developmental parameters. Skeletal variations such as supernumerary ribs and vertebrae were observed after doses of 20 and/or 32 mg/kg bw/day during GDs 7–15. The fetal CRL was also reduced at 32 mg/kg bw/day after dosing with dinoseb on GDs 7–15. A dose of 32 mg/kg bw/day dinoseb during GDs 13–15 induced absent or not ossified sternebrae. The dose levels that caused these adverse effects in fetuses were also lethal to some dams [15].

2.2.2. Intraperitoneal studies in mice

No adverse effects were observed in reproductive and developmental parameters after an i.p. dosing of dinoseb on GDs 7–15

Table 2
Developmental toxicity of dinoseb in mice

Strain	Dose (mg/kg)	Exposure time	Developmental effect observed	Reference
Gavage				
CD-1 mouse	15	GDs 8–12	No effects	[38]
CD-1 mouse	26, 33	GD 7	Supernumerary ribs	[39]
CD-1 mouse	50	GDs 7–8	Supernumerary ribs, ↓fetal weight	[40]
CD-1 mouse	50	GDs 7–8	Supernumerary ribs, sternum and vertebral centrum defects, fused ribs	[41]
SW mouse	50	GDs 9–11	No effects (6/8 dams died)	[6]
	20	GDs 7–15	Supernumerary ribs and vertebrae	
	32		↓Fetal crown-rump length	
	32	GDs 13–15	Absent or not ossified sternebrae	
Intraperitoneal				
SW mouse	10	GDs 9–11	Soft-tissue malformation	[6]
	17.7		Gross and skeletal malformations	
	18.8		↓Fetal body weight, number of fetuses, ↑resorption	
	20		↓Fetal crown-rump length	
	12.5–17.7	GDs 13–15	↓Fetal body weight, ↑resorption	
	5	GDs 7–15	No effects	
	15.8	GDs 9–11	↓PAH uptake by renal cortical slices	[42]
SW mouse	15.8 (combination with SKF-525A)	GDs 9–11	Hydronephrosis, ectrodactyly, ↑resorption	[43]
	17.7, 18.8 (combination with phenobarbital)	GDs 9–11	Hydronephrosis	
	14.1	GDs 9–11	Delayed ossification	
	15.8		External malformations	
	17.7		Hydronephrosis	
	18.8		↓Fetal body weight, ↑resorption	
	14.1	GDs 9–11 (24 h deprivation)	Delayed ossification, ↓fetal body weight	
	15.8		Hydronephrosis, ectopic kidney, internal hydrocephalus External and skeletal malformations	
	14.1	GDs 9–11 (48 h deprivation)	↓Fetal body weight	
	15.8		External and skeletal malformations	
SW mouse	7.5	GDs 9–11 (32 °C)	↓Fetal body weight, external, soft tissue and skeletal malformations, delayed ossification	[44]
	15.8	GDs 9–11 (room temperature; wet)	↓Fetal body weight, external and soft tissue malformations, ↑resorption	
	15.8	GDs 9–11 (6 °C; wet)	↓Fetal body weight, external and soft-tissue malformations	
	15.8	GDs 9–11 (room temperature; dry)	↓Fetal body weight	
	17.7		External and soft-tissue malformations, skeletal retardation, variation and malformation	
	17.7	GDs 9–11 (6 °C; dry)	↓Fetal body weight, external and soft-tissue malformations, skeletal retardation, variation and malformation	
Subcutaneous				
SW mouse	17.7	GDs 10–12	Fused ribs and vertebrae, absent or not ossified sternebrae	[6]
	17.7	GDs 14–16	Cleft palate, ↑resorption, ↓number of fetuses, fetal body weight, fetal crown-rump length	
	17.7	GDs 8–16	Skeletal variations, ↓fetal body weight, fetal crown-rump length, absent or not ossified sternebrae	

at 5 mg/kg bw/day in Swiss–Webster mice; however, teratogenicity was obtained after i.p. dosing of dinoseb on GDs 13–15 and 9–11 [15]. An increased incidence of soft-tissue malformation such as internal hydrocephalus was observed at 10–15.8 mg/kg bw/day in NaOH after i.p. treatment of dinoseb on GDs 9–11. At these doses, no maternal toxicity was observed. Increased incidences of defects in the limbs, tail, ribs, sternebrae and vertebrae, internal hydrocephaly and hydronephrosis were also induced at 17.7 mg/kg bw/day. Fetal body weight and number of fetuses were decreased at 18.8 mg/kg bw/day, and fetal CRL was decreased at 20.0 mg/kg bw/day. At 17.7–20.0 mg/kg bw/day, dinoseb produced hyperthermia and death in dams. Dinoseb at 12.5 and 17.7 mg/kg bw/day on GDs 13–15 caused increased resorptions and decreased fetal body weight, but not maternal toxicity. Unlike dosing with dinoseb on GDs 9–11, teratogenicity was not observed after administration of dinoseb on GDs 13–15 up to 17.7 mg/kg bw/day.

In a later review study for perinatal nephropathies, Gibson [42] stated that an incidence of 30–40% fetuses with hydronephrosis was observed at caesarean section due to i.p. administration of dinoseb on GDs 9–11; however, no grossly observable hydronephrosis was evident in pups at 1 or 2 weeks of age. Renal alteration observed in offspring of mice given dinoseb seems to be a transient dilatation of the renal pelvis, which is also suggested by studies in rats [35,36]. On the other hand, i.p. treatment of dinoseb on GDs 9–11 at 15.8 mg/kg bw/day caused a impairment in *p*-aminophippuric acid (PAH) uptake into renal cortical slices of offspring at 1 and 2 weeks of age, and this effect was also evident in 7 weeks of age [42].

Effects of food deprivation, Phenobarbital, an inducer of chemical metabolism, and 2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride (SKF-525A), an inhibitor of chemical metabolism, on the developmental toxicity of dinoseb were evaluated in Swiss–Webster mice [43]. Pregnant mice were treated i.p. with dinoseb at doses of 0–18.8 mg/kg bw/day on GDs 9–11. These treatments were preceded by 24 or 48 h food deprivation or by pretreatment with phenobarbital or SKF-525A. Dinoseb-induced external and skeletal anomalies were increased by 24 h food deprivation. Effects of phenobarbital pretreatments on dinoseb-induced developmental toxicity were inconsistent at 17.7 and