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-

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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*,c	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

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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.

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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).

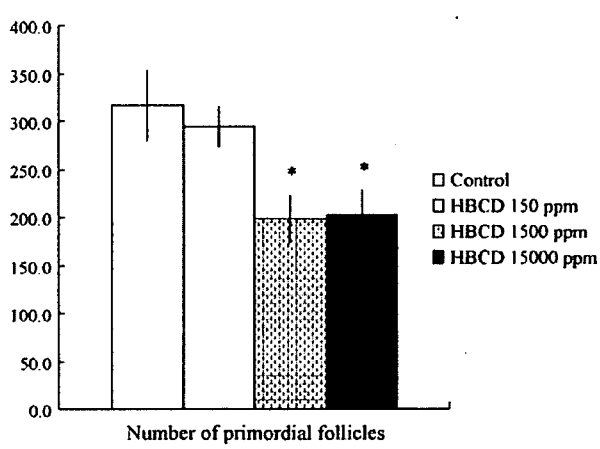

Fig. 3. Number of primordial follicles in the ovary of F1 female rats. Values are given as the mean ± S.E.M. (*) Significantly different from the control, $P < 0.05$.

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

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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] 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).

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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

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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] 2.08 ± 0.58[c]	1.65 ± 0.08 1.96 ± 0.16	1.60 ± 0.10 2.01 ± 0.29	1.46 ± 0.09** 2.31 ± 0.33**
Thymus (mg)[a]	343 ± 92[b] 414 ± 97[c]	336 ± 57 397 ± 54	360 ± 88 441 ± 69	282 ± 71 434 ± 81
Liver (g)[a]	3.87 ± 0.90[b] 4.72 ± 0.59[c]	4.02 ± 0.55 4.74 ± 0.35	4.12 ± 0.83 5.04 ± 0.40**	3.88 ± 0.68 6.00 ± 0.25**
Kidney (mg)[a,d]	965 ± 167[b] 1201 ± 173[c]	958 ± 99 1134 ± 56**	933 ± 135 1155 ± 85	749 ± 100** 1170 ± 96
Spleen (mg)[a]	360 ± 83[b] 443 ± 77[c]	361 ± 54 429 ± 64	346 ± 78 426 ± 69	263 ± 50** 411 ± 66
Adrenal (mg)[a,d]	23.4 ± 5.1[b] 28.7 ± 4.4[c]	25.1 ± 3.6 29.7 ± 3.2	24.3 ± 5.2 29.9 ± 4.0	19.6 ± 3.2* 30.4 ± 2.0
Testis (mg)[a,d]	476 ± 138[b] 574 ± 123[c]	510 ± 81 600 ± 55	475 ± 136 572 ± 93	385 ± 92 589 ± 54
Epididymis (mg)[a,d]	73.7 ± 16.8[b] 90.7 ± 14.1[c]	73.6 ± 10.7 87.2 ± 10.6	71.8 ± 17.5 87.3 ± 9.6	61.7 ± 9.5* 96.2 ± 10.5
Ventral prostate (mg)[a]	40.6 ± 9.7[b] 50.2 ± 9.3[c]	42.3 ± 9.5 50.2 ± 10.7	41.7 ± 12.1 50.8 ± 9.6	29.5 ± 6.8** 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

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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

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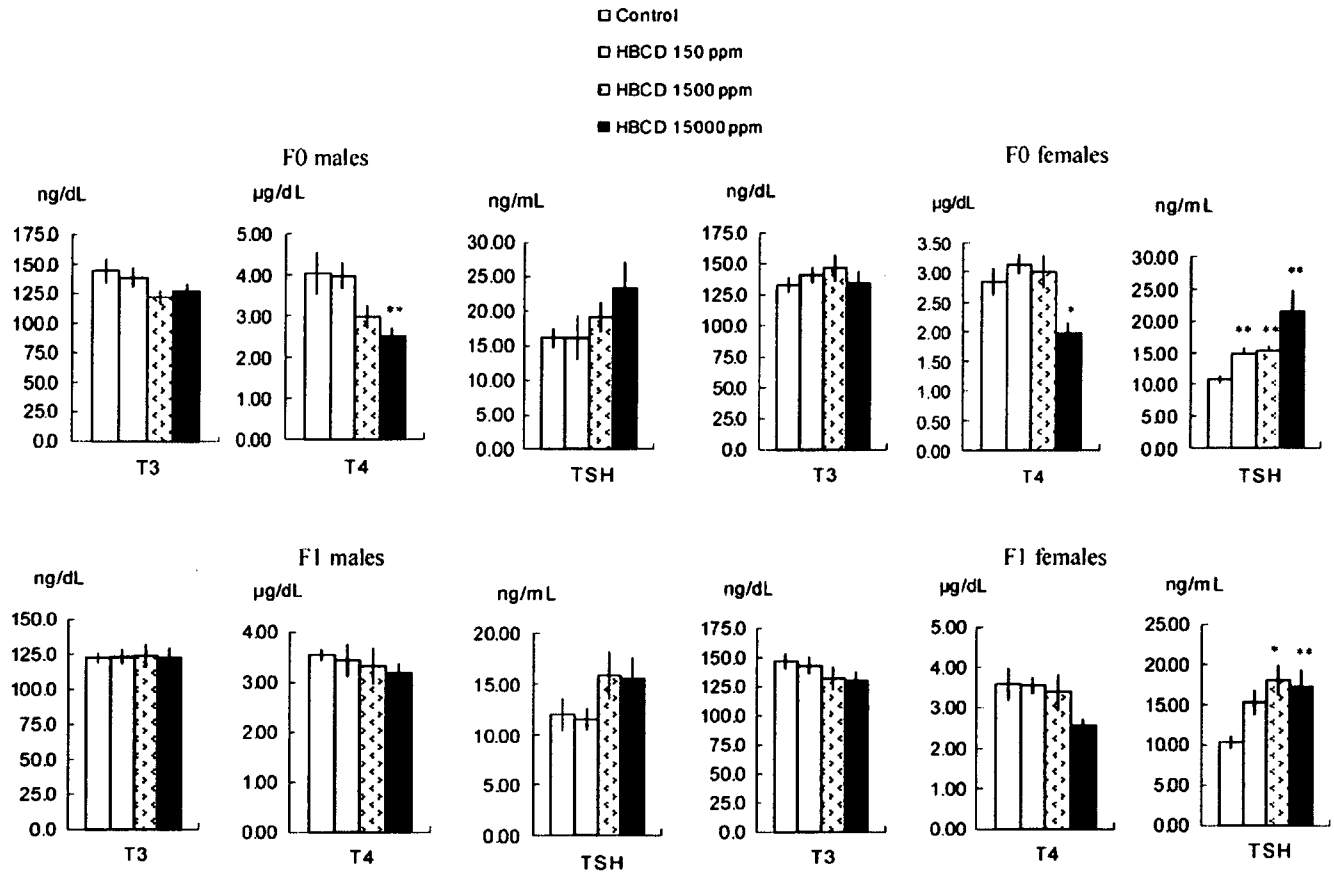

Fig. 4. Serum levels of T3, T4 and TSH in F0 and F1 rats. Values are given as the mean ± S.E.M. (*) Significantly different from the control, $P<0.05$. (**) Significantly different from the control, $P<0.01$.

[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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Title: **Repeated Dose and Reproductive Toxicity of the Ultraviolet Absorber 2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole in Rats**

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Running head: *Repeated Dose and Reproductive Toxicity of UV Absorber*

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ABSTRACT

2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB) is widely used as a UV absorber. In this study, the repeated dose and reproductive toxicity of DBHCB was evaluated in rats. Crj:CD(SD)IGS rats were given DBHCB by gavage at 0, 2.5, 25 or 250 mg/kg/day. Male and female rats were dosed beginning 28 days before mating and each female rat was mated with a male rat of the same dosage group. Males were dosed for a total of 56-57 days, and females were dosed for a total of 55-69 days up to day 3 of lactation throughout the mating and pregnancy periods. Ten males from each group were killed on the next day of the last administration, and ten females were killed on day 4-6 after parturition. Five rats/sex treated at 0 and 250 mg/kg/day for 56 days were then kept without treatment for 14 days (recovery period). No death was found in any group. No effects of DBHCB on general condition, body weight, food consumption or reproductive/developmental parameters were observed. Significant increases in serum albumin and A/G ratio at 25 mg/kg/day and higher and ALP levels at 250 mg/kg/day were noted in males. The absolute and relative weights of the liver were significantly increased in males at 25 mg/kg/day and higher. Significantly increased serum albumin and absolute and relative liver weight were also found in males at 250 mg/kg/day after the recovery period. No changes in these parameters were observed in females of any DBHCB-treated groups. No significant changes in organ histopathology were found in males or females. These findings indicated a sex difference in the toxicity of DBHCB in rats.

INTRODUCTION

Benzotriazole UV absorbers, which have a phenolic group attached to the benzotriazole structure, are known to have the most excellent absorption capacity within the full spectrum of UV absorption (Tenkazai.com, 2007), and are therefore used in a variety of polymers. 2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (CAS No. 3864-99-1; DBHCB), one of the benzotriazole UV absorbers, is a slightly yellowish powder that is stable under ordinary conditions and insoluble in water. The annual production and import from April 2005 to March 2006 was 532 tons in Japan (METI, 2006). This chemical provides effective light stabilization, and prevents the yellowing and degradation of polymers such as polypropylene, high density polyethylene, unsaturated polyesters, styrene-based thermoplastic elastomers, polyamides and impact polystyrenes (Chemical Land21, 2005). The finished polymers, which contain DBHCB less than 0.5% by weight of polyethylene phthalate polymers in compliance with 21 CFR 177.1630 (FDA, 2005a), may be used in contact with foods and used under certain conditions as described in 21 CFR 176.170 (FDA, 2000; 2005b). UV absorbers are used in food packaging to prevent polymer degradation and/or a change in the quality of the packed food due to UV light.

There is growing concern that humans have been exposed to these chemicals from environmental contamination and from contamination of packaged food. Exposure could lead to adverse effects due to the potential toxicity of the chemicals. Important information can be gained by studying the biological effects of environmental chemicals in laboratory animals.

Only limited information on the toxicity of DBHCB is available. DBHCB was not estrogenic in a recombinant yeast assay (Miller et al., 2001) or a yeast two-hybrid assay (Kawamura et al., 2003). It has been found that the oral LD50 for DBHCB is greater than 5000 mg/kg in rats, that DBHCB causes slight skin and eye irritation in rabbits, and that DBHCB treatment resulted in dose-dependent increases in the liver weight and signs of liver toxicity at 22-800 mg/kg/day, but not at 3.7 mg/kg/day, in rats (Everlight Chemical Industrial Corporation, 2002). We previously reported that maternal administration of DBHCB on days 5-19 of pregnancy caused no adverse effects in dams and fetuses at doses up to 1000 mg/kg/day (Ema et al., 2006).

Although testing for reproductive toxicity has become an important part of the overall toxicology profile for chemicals, no report is available for the reproductive toxicity of DBHCB. The present study was therefore conducted using a study design similar to the OECD Guideline 422 Combined Repeated Dose Toxicity Study with Reproduction/Developmental Toxicity Screening Study in rats (OECD, 1996).

MATERIALS AND METHODS

Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in reproductive and developmental toxicity studies and historical control data are available. Males at 11 weeks of age and females at 10 weeks of age were purchased from Hino Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The rats were acclimatized to the laboratory for one week prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared on a basal diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water *ad libitum*. Rats were maintained in an air-conditioned room at 21.5-22.1°C, with a relative humidity of 47-67%, a 12-hour light/dark cycle, and ventilation

with 15 air changes/hour. Rats were housed individually, except during the acclimation, mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared using wooden chips as bedding (White Flake; Charles River Laboratories Japan, Inc.). This experiment was approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd. (SNBL; Kagoshima, Japan) and performed in accordance with the ethics criteria contained in the bylaws of the committee of SNBL.

Chemicals and Dosing

DBHCB was obtained from Musashino Chemical Laboratory, Ltd. (Kitaibaraki, Japan). The DBHCB (Lot no. 05004IX3) used in this study was 99.9% pure based on HPLC analysis, and it was kept in a dark and cool place at room temperature under airtight conditions. The purity and stability of the chemical were verified by analysis before the study.

DBHCB was suspended in 5% gum arabic solution. The volume of each dose was adjusted to 10 mL/kg body weight based on the latest body weight. The control rats were given only 5% gum arabic solution. Stability of the formulations kept in a dark and cool place under airtight conditions had been confirmed for up to 14 days. During use, the formulations were maintained under these conditions for no more than 7 days and were 97.3 to 100.1% of the target concentration.

The initial numbers of the rats were 15/sex at 0 (control) and 250 mg/kg/day, and 10/sex at 2.5 and 25 mg/kg/day. Male and female rats were dosed once daily beginning 28 days before mating and each female rat was mated with a male rat of the same dosage group. Males were dosed for a total of 56-57 days, and females were dosed for a total of 55-69 days to day 3 of lactation throughout the mating and pregnancy periods. Ten males from each group were killed after 56-57 days administration, and ten females were killed on day 4-6 after parturition. The remaining five rats/sex treated at 0 and 250 mg/kg/day for 56 days were kept without treatment for 14 days (recovery period). Dosage levels were determined based on the results of our dose-finding study in which significantly increased liver weight occurred in males at 250 mg/kg/day and higher, but not in females even at 1000 mg/kg/day, after administration of DBHCB for 14 days in rats.

Observations

All rats were observed twice a day for clinical signs of toxicity during the administration period and once a day during the non-administration period. The body weight was recorded twice a week in males, and twice a week during the pre-mating period, on days 0, 7, 14 and 20 of pregnancy and on days 0, 3 and 4 of lactation in females. Food consumption was recorded twice a week for males, and twice a week during the pre-mating period, on days 1, 4, 7, 11, 15, 17 and 20 of pregnancy and on days 1 and 3 of lactation for females.

Prior to scheduled terminal necropsy, blood samples for hematological and biochemical evaluation were collected from the abdominal aorta of five fasted male and female rats per group under anesthesia by intraperitoneal injection of sodium pentobarbital. Blood samples were analyzed for the following hematological parameters using K_2-EDTA as an anticoagulant: red blood cell count (RBC), white blood cell count (WBC), hematocrit value, hemoglobin concentration, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte ratio, and differential white blood cell ratio (Hematology System ADVIA 120, Bayer Diagnostics Manufacturing Ltd., Dublin, Ireland); using sodium citrate as

anticoagulant: prothrombin time (PT) and activated partial thromboplastin time (APTT) (Automated Blood Coagulation Measuring Apparatus CA-5000, Sysmex Corp., Kobe, Japan).

Serum samples obtained from centrifuged whole blood were analyzed for the following biochemistry parameters: aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase, total bilirubin, total protein, albumin, total cholesterol, triglyceride, glucose, blood urea nitrogen (BUN), creatinine, inorganic phosphorus, calcium, sodium, potassium, chloride (Automatic analyzer JCA-BM8, JOEL Ltd., Tokyo, Japan), total bile acid (Spectrophotometer U-3200, Hitachi Ltd., Tokyo, Japan), protein fraction (Automatic Electrophoresis Apparatus, AES-4000, Olympus Corp., Tokyo, Japan) and albumin/globulin (A/G) ratio.

At the scheduled terminal necropsy, all rats were euthanized by exsanguination under anesthesia. All rats were subjected to gross necropsy, which included an external examination of all body orifices and surfaces, and examinations of all cranial, thoracic and abdominal organs. The brain, heart, liver, kidney, spleen, thymus, and adrenal gland in males and females, the testis, epididymis, seminal vesicle, and prostate in males, and the ovary in females were removed and weighed. Relative organ weights (mg or g/100 g body weight) were calculated on the basis of the terminal body weight. In females, the numbers of corpora lutea and implantation sites were recorded. Samples of tissues and organs were preserved in neural phosphate-buffered 10% formaldehyde solution. The testis and epididymis were fixed in Bouin's solution. Histopathological evaluations for five rats/sex /group were performed on the tissues specified below after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin; the brain, heart, thymus, kidney, spleen, adrenal gland, small and large intestine, lung, trachea, thyroid, submandibular and mesenteric lymph node, femur bone marrow, spinal cord, sciatic nerve, tibial nerve, urinary blabber, testis, epididymis, seminal vesicle, prostate, ovary and uterus in the control and highest dose groups, and the liver in all groups.

Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered evidence of successful mating. The day of successful mating was designated as day 0 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal day (PND) 4-6. The day on which parturition was completed was designated as PND 0. Litter size and numbers of live and dead pups were recorded, and the live pups were sexed and individually weighed on PNDs 0 and 4. Dead pups were examined grossly. On PND 4, the pups were euthanized by exsanguination under anesthesia, and gross external and internal examinations were performed.

Data Analysis

The statistical analysis of pups was carried out using the litter as the experimental unit. The body weight, body weight gain and food consumption, precoital interval, length of gestation, numbers of implantations and live pups per litter and pup weight, delivery index, viability index, hematological and blood biochemical parameters, and organ weight were analyzed with Bartlett's test for homogeneity of variance at the 5% level of significance. When the variance was homogeneous, Dunnett's test was performed to compare the mean value in the control group with that in each DBHCB group. When the variance was heterogeneous, a Dunnett-type test was performed to compare the mean value in the control

group with that in each DBHCB group after rank conversion. Recovery in the control and highest dose groups was analyzed in the following way. Variance ratio was analyzed by *F* test. If the variance ratio was equivalent, the groups were compared by Student's *t*-test. If the variance was not equivalent, the Wilcoxon test was performed.

RESULTS

No deaths or DBHCB-related clinical signs of toxicity were found in male or female rats of any groups. There was no significant difference in the body weight and body weight gain between control and DBHCB-treated groups in males and females including during pregnancy and lactation. No significant changes in the food consumption were found, except for a significant decrease on days 28-29 in males and an increase on days 31-32 in females at 250 mg/kg.

The reproductive and developmental findings in rats given DBHCB are presented in Table 1. Although one pair did not copulate in the control group, all pairs copulated and all copulated females were impregnated and delivered their pups in all DBHCB-treated groups. There was no significant difference in the copulation index, fertility index, gestation index, precoital interval or gestation length between control and DBHCB-treated groups. No effects of DBHCB were observed on the numbers of corpora lutea or implantations, preimplantation loss, numbers of pups delivered, live pups or stillborn or sex ratio of live pups. There was no significant difference in the viability or body weight of pups on PND 0 or 4 between control and DBHCB-treated groups. External and internal examinations revealed no morphological anomalies in pups of any group.

Table 2 shows the hematological findings in rats given DBHCB at the end of the administration period. A significantly decreased RBC at 250 mg/kg/day and shorter APTT at 25 and 250 mg/kg/day were observed in males. The number of neutrophils was significantly increased at 250 mg/kg/day in males. In females, the only significant change was a lowered number of eosinophils at 25 and 250 mg/kg/day. At the end of the recovery period, significantly increased numbers of platelets and neutrophils as well as increased neutrophil ratio, were observed in males at 250 mg/kg/day, in addition to a decreased lymphocyte ratio.

Table 3 presents the blood biochemical findings in rats given DBHCB at the end of the administration period. In males, significantly increased levels of ALAT at 25 mg/kg/day, decreased levels of creatinine at 25 mg/kg/day and higher were observed. Additionally, males presented decreased levels of total bilirubin and increased levels of ALP at 250 mg/kg/day were observed. The levels of total protein were significantly increased at 25 mg/kg/day. A significantly increased albumin percentage and A/G ratio and decreased α2-globulin percentage were found in males at 25 and 250 mg/kg/day, as well as a decreased percentage of β-globulin at 2.5 mg/kg/day and higher. In females, the levels of total cholesterol were significantly decreased at 2.5 and 25 mg/kg/day. No significant changes in other blood biochemical parameters were noted in males and females in the DBHCB-treated groups. At the end of the recovery period, significantly increased levels of total protein, albumin and total cholesterol and decreased creatinine levels and α2-globulin ratio were observed at 250 mg/kg/day in males. In females, parameters remained unchanged in all DBHCB-treated groups.

The organ weights of male rats given DBHCB at the end of the administration period are presented in Table 4. The absolute and relative weights of the liver were significantly higher at 25 mg/kg/day and higher. No significant changes in the weight of the reproductive organs

were found. At the end of recovery period, the absolute and relative weights of the liver at 250 mg/kg/day were still significantly increased.

Table 5 shows the organ weight of female rats given DBHCB at the end of the administration period. There were no significant changes in the absolute and relative weights of organs including the reproductive organs. At the end of the recovery period, no significant changes in the absolute or relative weight of organs were observed at 250 mg/kg/day.

No changes related to the administration of DBHCB were found in the necropsy findings. Histopathological examinations revealed no test compound-related toxicological changes in the liver of males and females in all the DBHCB-treated groups. There were also no changes in the other organs, including the male and female reproductive organs, in the 250 mg/kg/day group.

DISCUSSION

The present study was conducted to determine the repeated dose and reproductive toxicity of DBHCB. The data show that the repeated oral dosing of DBHCB caused changes in the liver in males, but not in females, and no changes in the reproductive function of male and female rats.

In the present study, there were no changes in the reproductive parameters regarding copulation, fertility, parturition and nursing of their pups in rats given DBHCB beginning 28 days before mating, during pregnancy and shortly after parturition. No changes in weight or histopathology were found in male and female reproductive organs. Moreover, the prenatal and postnatal developmental parameters regarding embryonic/fetal/neonatal survival and growth and morphological development of offspring were not affected by administration of DBHCB. These results are consistent with the results of our previous study in which no maternal or prenatal developmental toxicity was noted in rats given DBHCB by gavage on days 5-19 of pregnancy at 1000 mg/kg/day (Ema et al., 2006). These findings indicate that DBHCB has no potential for reproductive or developmental toxicity in rats.

On the hematological examination, changes in some parameters were noted in both male and female rats at higher doses. However, these changes are not considered to indicate toxicological significance because they were relatively small and were dose-independent. The lowered RBC, for example, in males at 250 mg/kg/day is unlikely to represent anemia because the degree of decrease is slight and other anemic parameters such as hematocrit, hemoglobin, MCV, MCH, MCHC and reticulocyte count, were not affected by the administration of DBHCB. Anemia is defined clinically as the condition characterized by a hemoglobin concentration below the lower reference limit (Hall, 2007). Regarding renal function, it has been described that serum creatinine levels parallel changes in BUN caused by alterations in renal blood flow, renal function or urinary outflow (Hall, 2007). The changes in creatinine levels in male rats at 25 mg/kg/day and higher are not thought to have toxicological significance because there were no changes in BUN or histopathological alterations of the kidney in the DBHCB-treated groups. In male rats, changes in some blood biochemical parameters suggestive of liver toxicity were observed at higher doses. The increased levels of total protein and albumin suggest an acceleration of protein synthesis in the liver, and these phenomena are supported by the increased weight of the liver at higher doses. These changes were only noted only in males, indicating a sex difference in the toxicity of DBHCB.

The NOAEL for repeated dose toxicity of DBHCB is considered to be 2.5 mg/kg/day in

male rats based on the increased levels of albumin and weight of the liver, and 250 mg/kg/day, the highest dose used in the present study, in female rats. Our findings indicate that male rats have more than 100-fold greater susceptibility to DBHCB toxicity than female rats. Previously, we showed a sex differences in toxicity in the 28-day and 52-week repeated dose toxicity studies of a structurally similar compound, 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole (HDBB), which is also used as an ultraviolet absorber (Hirata-Koizumi et al., 2008ab). In the 28-day repeated dose toxicity study using rats given HDBB by gavage at 0, 0.5, 2.5, 12.5 or 62.5 mg/kg/day, adverse effects on the liver and heart were noted at all doses in males and at 12.5 mg/kg and higher in females (Hirata-Koizumi et al., 2008a). In the 52-week repeated dose toxicity study with rats given HDBB by gavage at 0, 0.1, 0.5 or 2.5 mg/kg/day in males and 0, 0.5, 2.5 or 12.5 mg/kg/day in females, toxic effects were observed in the liver at 0.5 mg/kg/day and higher in males and 12.5 mg/kg/day in females (Hirata-Koizumi et al., 2008b).

It has been recognized that there are sex differences in the toxicity of chemical compounds in rats. A recent subchronic toxicity study showed that fluoranthene, a polycyclic aromatic hydrocarbon, had greater effects on males than females, especially in the kidney, in F344 rats (Knuckles et al., 2004). On the other hand, female rats exhibited a higher susceptibility to hypothermic effects and inhibition of hypothalamic cholinesterase by the carbamate cholinesterase inhibitor, rivastigmine (Wang et al., 2001). These findings suggest that sexual hormones may play an important role in sex differences in toxicity. It has already been shown that orchidectomy resulted in the complete ablation of the above-mentioned sex differences in hypothalamic cholinesterase inhibition induced by rivastigmine (Wang et al., 2001). Testosterone is likely to interfere with the effects of rivastigmine, because testosterone decreases cholinesterase inhibition in gonadectomised males and females. More recently, we showed that castration markedly reduced sex differences in the toxicity of HDBB in male and female rats (Hirata-Koizumi et al., 2008c). We also reported no sex differences in susceptibility to the toxic effects of HDBB in preweaning rats (Hirata-Koizumi et al., 2008d). It is important to investigate the role of sex steroids in the mediation of sex differences in susceptibility to DBHCB toxicity and to determine the toxic effects of DBHCB in preweaning rats . A repeated-dose toxicity study of DBHCB is currently in progress using castrated and pre-weaning male and female rats.

To date, there has been no available data for human exposure to this chemical. Actual human exposure to DBHCB may be very low because it was not detected in polyethylenterephthalate bottles in Brazil (Monteiro et al., 1998) or polyethylene products in Japan (Kawamura et al., 1997). Consideration of these findings and the results of the present study together suggest that the human risk of adverse effects from DBHCB exposure is very low.

In conclusion, administration of DBHCB during pre-mating, mating, and pregnancy as well as shortly after parturition caused no changes in the reproductive function of male and female rats. DBHCB produced increases in the liver weight, albumin levels, and A/G ratio at 25 mg/kg/day and higher as well as ALP levels at 250 mg/kg/day in males and but no change in females. These findings indicate a sex difference in the toxicity of DBHCB in rats.

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