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).

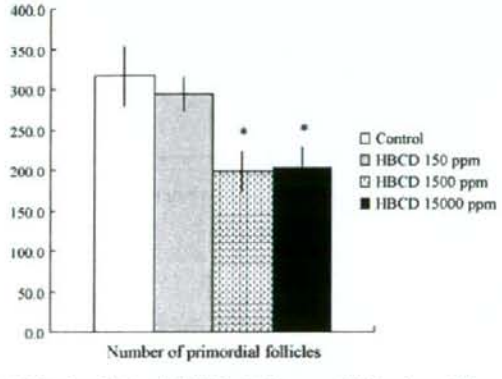

Fig. 3. Number of primordial follicles in the ovary of F1 female rats. Values are given as the mean ± S.E.M. (*) Significantly different from the control, $P < 0.05$.

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

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

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

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

3.10. Serum hormone levels (F0 and F1 adults)

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

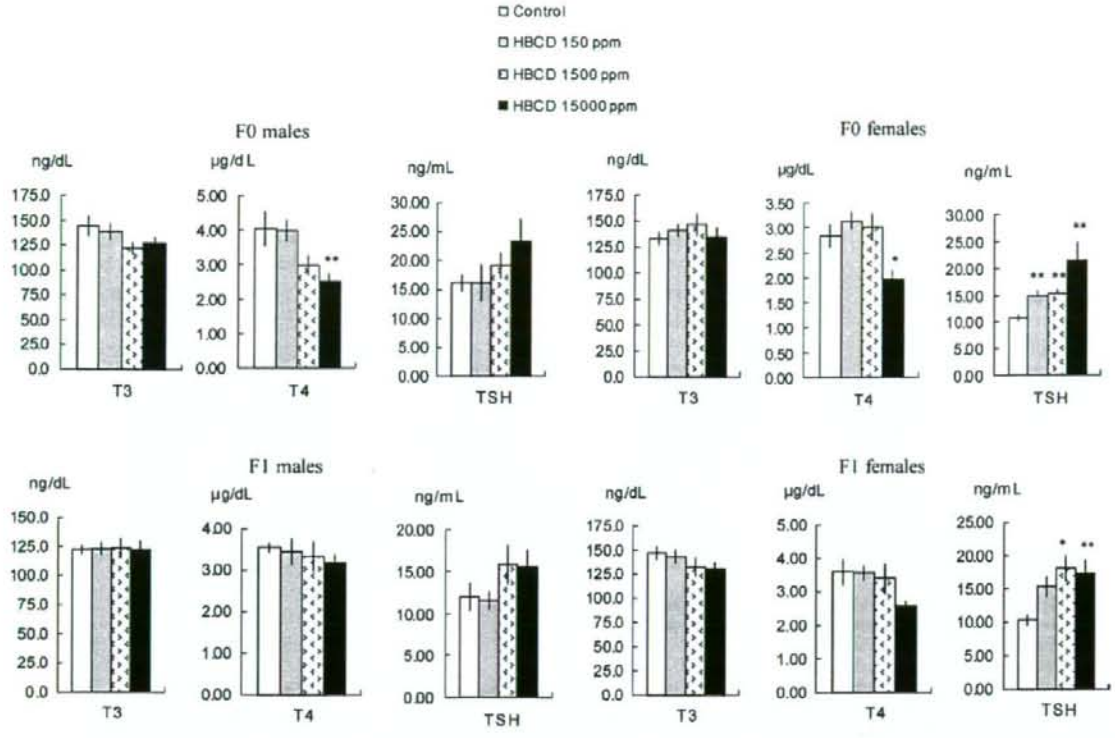

Fig. 4. Serum levels of T3, T4 and TSH in F0 and F1 rats. Values are given as the mean ± S.E.M. (*) Significantly different from the control, $P<0.05$. (**) Significantly different from the control, $P<0.01$.

[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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References

[1] Reistad T, Fonnum F, Mariussen E. Neurotoxicity of the pentabrominated diphenyl ether mixture, DE-71, and hexabromocyclododecane (HBCD) in rat cerebellar granule cells in vitro. Arch Toxicol 2006;80:785–96.

[2] BSEF (Bromine Science and Environmental Forum). Major brominated flame retardants volume estimates: total market demand by region. Brussels; 2003 [cited 2007, Aug 28]. Available from: www.bsef.com.

[3] American Chemical Council. HPV data summary and test plan for hexabromocyclododecane (HBCD); 2001.

[4] NRC (National Research Council). Hexabromocyclododecane, eds. Toxicological risks of selected flame-retardant chemicals. Washington, DC: National Academy Press; 2000. p. 53–71.

[5] Law RJ, Kohler M, Heeb NV, Gerecke AC, Schmid P, Voorspoels S, et al. Hexabromocyclododecane challenges scientists and regulators. Environ Sci Technol 2005;39:281A–7A.

[6] Sellstrom U, Kierkegaard A, de Wit C, Jansson B. Polybrominated diphenyl ethers and hexabromocyclododecane in sediment and fish from a Swedish river. Environ Toxicol Chem 1998;17:1065–72.

[7] Eljarrat E, de la Cal A, Raldua D, Duran C, Barcelo D. Occurrence and bioavailability of polybrominated diphenyl ethers and hexabromocyclododecane in sediment and fish from the Cinca River, a tributary of the Ebro River (Spain). Environ Sci Technol 2004;38:2603–8.

[8] Veith GD, De Feo DL, Bergstedt BV. Measuring and estimating the bioconcentration factor of chemicals in fish. J Fish Res Board Can 1979;36:1040–8.

[9] Morris S, Allchin CR, Zegers BN, Haftka JJ, Boon JP, Belpaire C, et al. Distribution and fate of HBCD and TBBPA brominated flame retardants in North Sea estuaries and aquatic food webs. Environ Sci Technol 2004;38:5497–504.
[10] Covaci A, Gerecke AC, Law RJ, Voorspoels S, Kohler M, Heeb NV, et al. Hexabromocyclododecanes (HBCDs) in the environment and humans: a review. Environ Sci Technol 2006;40:3679–88.
[11] Darnerud PO. Toxic effects of brominated flame retardants in man and in wildlife. Environ Int 2003;29:841–53.
[12] Thomsen C, Frøshaug M, Broadwell SL, Becher G, Eggesbø. Levels of brominated flame retardants in milk from the Norwegian human milk study: HUMIS. Organohalog Comp 2005;67:509–12.
[13] Fängström B, Athanassiadis I, Strid A, Odsjö T, Guvenius D, Norén K, et al. Temporal trends of PBDES and HBCDD in milk from Stockholm mothers, 1980–2004. Organohalog Comp 2006;68:774–7.
[14] Ryan JJ, Wainman BC, Schecter A, Moisey J, Sun WF. Trends of the brominated flame retardants, PBDES and HBCD, in human milks from North America. Organohalog Comp 2006;68:778–81.
[15] Clayson DB, Krewski DR. Objectives of toxicity testing. In: Arnold DL, Grice HC, Krewski DR, editors. Handbook of in vivo toxicity testing. San Diego: Academic Press; 1990. p. 3–18.
[16] de Wit CA. An overview of brominated flame retardants in the environment. Chemosphere 2002;46:583–624.
[17] Birnbaum LS, Staskal DF. Brominated flame retardants: cause for concern? Environ Health Perspect 2004;112:9–17.
[18] Chengelis CP. A 28-day oral (gavage) toxicity study of HBCD in rats. WIL-186004. Arlington, VA: Brominated Flame Retardant Industry Panel. Chemical Manufacturers Association; 1997.
[19] Chengelis CP. A 90-day oral (gavage) toxicity study of HBCD in rats. WIL-186012. Arlington, VA: Brominated Flame Retardant Industry Panel. Chemical Manufacturers Association; 2001.
[20] van der Ven LT, Verhoef A, van de Kuil T, Slob W, Leonards PE, Visser TJ, et al. A 28-day oral dose toxicity study enhanced to detect endocrine effects of hexabromocyclododecane in Wistar rats. Toxicol Sci 2006;94:281–92.
[21] Eriksson P, Fischer C, Wallin M, Jakobsson E, Fredriksson A. Impaired behaviour, learning and memory, in adult mice neonatally exposed to hexabromocyclododecane (HBCDD). Environ Toxicol Pharmacol 2006;21:317–22.
[22] Stump D. A prenatal developmental toxicity study of hexabromocyclododecane (HBCD) in rats. WIL-186009. Arlington, VA: Brominated Flame Retardant Industry Panel. Chemical Manufacturers Association; 1999.
[23] Murai T, Kawasaki H, Kanoh S. Studies on the toxicity of insecticides and food additives in pregnant rats: fetal toxicity of hexabromocyclododecane. Pharmacometrics (Japan) 1985;29:981–6 [Japanese].
[24] OECD. OECD Test Guideline for Testing of Chemicals. Proposal for Updating Guideline 416, Two-generation Reproduction Toxicity Study (Organization for Economic Co-operation and Development); 2001.
[25] ME, MHLW and METI (Ministry of the Environment, Ministry of Health, Labour and Welfare, and Ministry of Economy, Trade and Industry, Japan). On standard of Testing Facility Conducting Studies Concerning New Chemical Substances, November 21, 2003, revised April 1, 2005.
[26] Altman J, Sudarshan K. Postnatal development of locomotion in the laboratory rat. Anim Behav 1975;23:896–920.
[27] Gallavan Jr RH, Holson JF, Stump DG, Knapp JF, Reynolds VL. Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Reprod Toxicol 1999;13:383–90.
[28] Biel W. Early age differences in maze performance in the albino rats. J Genet Psychol 1940;56:439–45.
[29] Heindel JJ. Oocyte quantitation and ovarian histology. In: Daston G, Kimmel C, editors. An evaluation and interpretation of reproductive endpoints for human health risk assessment. Washington DC: International Life Sciences Institute Press; 1999. p. 57–74.
[30] Gad SC. Statistics for toxicologist. In: Hayes AW, editor. Principles and methods of toxicology. 4th ed. Philadelphia: Taylor and Francis Group; 2001. p. 285–364.
[31] Lochry EA. Concurrent use of behavioral/functional testing in existing reproductive and developmental toxicity screens: practical considerations. J Am Coll Toxicol 1987:433–9.
[32] ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use). ICH Harmonized Tripartite Guideline: Detection of Toxicity to Reproduction for Medicinal Products and Toxicity to Male Fertility, S5 (R2), Current Step 4 version; 2005.
[33] Heinrichs WL. Current laboratory approaches for assessing female reproductive toxicity. In: Dixon RL, editor. Reproductive toxicology. New York: Raven Press; 1985. p. 95–108.
[34] Korenbrot CC, Huhtaniemi IT, Weiner RI. Preputial separation as an external sign of pubertal development in the male rat. Biol Reprod 1977;17:298–303.
[35] Parker Jr CR, Mahesh VB. Hormonal events surrounding the natural onset of puberty in female rats. Biol Reprod 1976;14:347–53.
[36] Amacher DE, Schomaker SJ, Burkhardt JE. The relationship among microsomal enzyme induction, liver weight and histological change in rat toxicology studies. Food Chem Toxicol 1998;36:831–9.
[37] Germer S, Piersma AH, van der Ven L, Kamyschnikow A, Fery Y, Schmitz HJ, et al. Subacute effects of the brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A on hepatic cytochrome P450 levels in rats. Toxicology 2006;218:229–36.
[38] Kezele P, Skinner MK. Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. Endocrinology 2003;144:3329–37.
[39] Forsberg JG, Olivecrona H. The effect of prenatally administered Busulphan on rat gonads. Biol Neonat 1966;10:180–92.
[40] Merchant H. Rat gonadal and ovarioan organogenesis with and without germ cells. An ultrastructural study. Dev Biol 1975;44:1–21.
[41] Hirshfield AN. Relationship between the supply of primordial follicles and the onset of follicular growth in rats. Biol Reprod 1994;50:421–8.
[42] Gray Jr LE. Chemically induced alterations of reproductive development in female mammals. In: Boeklheide K, Chapin RE, Hoyer PB, editors. Comprehensive toxicology (reproductive and endocrine toxicology). Oxford: Elsevier Science; 1997. p. 329–38.
[43] Gray Jr LE, Ostby JS, Kavlock RJ, Marshall R. Gonadal effects of fetal exposure to the azo dye congo red in mice: infertility in female but not male offspring. Fundam Appl Toxicol 1992;19:411–22.
[44] Christian MS, Brown RW. Control observations regarding primordial follicle counts in EPA multigeneration studies in Crl Sprague-Dawley ("Gold Standard") rats. Reprod Toxicol 2002;16:408.
[45] Bolon B, Bucci TJ, Warbritton AR, Chen JJ, Mattison DR, Heindel JJ. Differential follicle counts as a screen for chemically induced ovarian toxicity in mice: results from continuous breeding bioassays. Fundam Appl Toxicol 1997;39:1–10.
[46] Parker RM. Testing for reproductive toxicity. In: Hood RD, editor. Developmental and reproductive toxicology. 2nd ed. Boca Raton: CRC Press (Taylor & Francis group); 2006. p. 425–87.

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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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2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB) is widely used as an ultraviolet (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/d. Male and female rats were dosed beginning 28 d 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 d, and females were dosed for a total of 55–69 d 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 10 females were killed on Days 4–6 after parturition. Five rats/sex treated at 0 and 250 mg/kg/d for 56 d were then kept without treatment for 14 d (recovery period). No deaths were 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 an albumin/globulin ratio at 25 mg/kg/d and higher and alkaline phosphatase levels at 250 mg/kg/d were noted in males. The absolute and relative weights of the liver were significantly increased in males at 25 mg/kg/d and higher. Significantly increased serum albumin and absolute and relative liver weight were also found in males at 250 mg/kg/d 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.

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Keywords Repeated-dose toxicity, Reproductive and developmental toxicity, UV absorber, Benzotriazole, Rat.

INTRODUCTION

Benzotriazole ultraviolet (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 the 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 5,000 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 the maternal administration of DBHCB on Days 5–19 of pregnancy caused no adverse effects in dams and fetuses at doses up to 1,000 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 by 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-h light/dark cycle, and ventilation with 15 air changes/h. 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 by 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 high-performance liquid chromatography (HPLC) analysis, and it was kept in a dark, 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, cool place under airtight conditions had been confirmed for up to 14 d. During use, the formulations were maintained under these conditions for no more than seven days and were 97.3–100.1% of the target concentration.

The initial numbers of the rats were 15/sex at 0 (control) and 250 mg/kg/d, and 10/sex at 2.5 and 25 mg/kg/d. Male and female rats were dosed once-daily

beginning 28 d 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 d, 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 d of administration, and ten females were killed on Days 4–6 after parturition. The remaining five rats/sex treated at 0 and 250 mg/kg/d for 56 d were kept without treatment for 14 d (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/d and higher, but not in females, even at 1,000 mg/kg/day, after the administration of DBHCB for 14 d 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 nonadministration period. The body weight was recorded twice a week in males, and twice a week during the premating 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 premating 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 an intraperitoneal injection of sodium pentobarbital. Blood samples were analyzed for the following hematological parameters by 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 an 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 of 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 days (PNDs) 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 by 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 an F test. If the variance ratio was equivalent, the groups were compared by a 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 the 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 the 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 PNDs 0 or 4 between the control and DBHCB-treated groups. External and internal examinations revealed no morphological anomalies in the 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/d and shorter APTT at 25 and 250 mg/kg/d were observed in males. The number of neutrophils was significantly increased, at 250 mg/kg/d, in males. In females, the only significant change was a lowered number of eosinophils, at 25 and 250 mg/kg/d. At the end of the recovery period, significantly increased numbers of platelets and neutrophils, as well as an increased neutrophil ratio, were observed in males at 250 mg/kg/d, 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/d, as well as decreased levels of creatinine at 25 mg/kg/d and higher, were observed. Additionally, males presented decreased levels of total bilirubin, and increased levels of ALP, at 250 mg/kg/d, were observed. The levels of total protein were significantly increased at 25 mg/kg/d. A significantly increased albumin percentage and A/G ratio and decreased

Table 1: Reproductive and developmental findings in rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of pairs	10	10	10	10
Copulation index (%)[b]	90	100	100	100
Fertility index (%)[c]	100	100	100	100
No. of pregnant females	9	10	10	10
Precoital interval (days)[a]	4.9 ± 4.4	3.4 ± 3.8	2.7 ± 1.3	2.8 ± 1.5
Gestation index (%)[d]	100	100	100	100
Gestation length (days)[a]	21.9 ± 0.4	21.9 ± 0.3	22.0 ± 0.4	22.0 ± 0.2
No. of litters	9	10	10	10
No. of corpora lutea[a]	16.1 ± 1.9	15.7 ± 1.8	15.3 ± 1.5	16.0 ± 1.9
No. of implantations[a]	15.3 ± 1.7	14.8 ± 1.5	14.1 ± 1.2	14.2 ± 3.2
Preimplantation loss (%)[a,e]	6.1 ± 4.3	6.6 ± 8.4	7.5 ± 7.0	10.9 ± 17.3
Delivery index (%)[a,f]	91.1 ± 7.2	93.8 ± 7.0	91.0 ± 13.8	96.5 ± 5.7
No. of pups delivered[a]	14.1 ± 2.2	14.0 ± 1.9	12.8 ± 2.0	14.0 ± 3.1
No. of live pups[a]	14.0 ± 2.2	13.9 ± 1.9	12.8 ± 2.0	13.9 ± 2.9
No. of stillborn[a]	0.1 ± 0.3	0.1 ± 0.3	0	0.1 ± 0.3
Sex ratio of live pups (female/total)[a]	0.53 ± 0.09	0.50 ± 0.15	0.53 ± 0.09	0.61 ± 0.16
Viability index during lactation (%)[a,g,h]				
Day 0	99.2 ± 2.4	99.3 ± 2.3	98.8 ± 3.7	98.8 ± 2.6
Day 4	100	98.8 ± 2.6	97.6 ± 4.0	97.7 ± 3.7
Male pup weight during lactation (g)[a]				
Day 0	6.5 ± 0.5	6.5 ± 0.5	6.8 ± 0.3	6.5 ± 0.4
Day 4	9.3 ± 1.1	9.4 ± 0.9	10.2 ± 0.7	9.6 ± 1.4
Female pup weight during lactation (g)[a]				
Day 0	6.0 ± 0.4	6.2 ± 0.5	6.3 ± 0.4	6.1 ± 0.4
Day 4	8.9 ± 1.0	9.0 ± 0.8	9.7 ± 0.7	9.1 ± 1.5

[a]Values are given as the mean ± SD.
[b]Copulation index (%) = (no. of females with successful copulation/no. of females paired) × 100.
[c]Fertility index (%) = (no. of females pregnant/no. of females with successful copulation) × 100.
[d]Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.
[e]Preimplantation loss (%) = ((no. of corpora lutea − no. of implantations)/no. of corpora lutea) × 100.
[f]Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.
[g]Viability index on postnatal day 0 (%) = (no. of live pups on postnatal day 0/no. of pups delivered) × 100.
[h]Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of live pups on postnatal day 0) × 100.

α2-globulin percentage were found in males at 25 and 250 mg/kg/d, as well as a decreased percentage of β-globulin at 2.5 mg/kg/d and higher. In females, the levels of total cholesterol were significantly decreased at 2.5 and 25 mg/kg/d. 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/d in males. In females, parameters remained unchanged in all DBHCB-treated groups.

Table 2: Hematological findings in male and female rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of male rats	5	5	5	5
RBC (10^6/mL)	8.18 ± 0.32[a]	7.95 ± 0.31	8.07 ± 0.30	7.63 ± 0.36*
WBC (10^3/mL)	9.41 ± 1.06	8.22 ± 2.94	8.10 ± 2.37	8.94 ± 1.13
Hematocrit value (%)	45.6 ± 1.9	44.3 ± 0.9	44.7 ± 2.2	42.7 ± 1.7
Hemoglobin concentration (g/dL)	15.2 ± 0.4	14.9 ± 0.5	15.1 ± 0.9	14.2 ± 0.7
Platelet count (10^3/mL)	1063 ± 110	1145 ± 134	1202 ± 119	1205 ± 108
MCV (fL)	55.7 ± 2.3	55.8 ± 1.5	55.4 ± 0.9	55.9 ± 0.7
MCH (pg)	18.7 ± 0.7	18.7 ± 0.8	18.7 ± 0.4	18.6 ± 0.3
MCHC (g/dL)	33.5 ± 0.7	33.5 ± 0.7	33.8 ± 0.4	33.3 ± 0.4
Reticulocyte ratio (%)	2.60 ± 0.34	2.74 ± 0.57	3.00 ± 0.40	3.02 ± 0.44
PT (sec)	8.52 ± 0.42	9.50 ± 0.97	9.20 ± 0.57	8.50 ± 0.58
APTT (sec)	20.1 ± 0.8	20.9 ± 0.7	18.3 ± 1.0**	18.2 ± 0.7**
No. of female rats	5	5	5	5
RBC (10^6/mL)	6.81 ± 0.49[a]	6.90 ± 0.36	6.82 ± 0.14	6.50 ± 0.24
WBC (10^3/mL)	5.95 ± 0.96	6.19 ± 1.38	6.34 ± 1.46	5.05 ± 0.71
Hematocrit value (%)	40.2 ± 2.1	41.1 ± 1.7	39.4 ± 1.2	39.6 ± 2.3
Hemoglobin concentration (g/dL)	13.4 ± 0.7	14.0 ± 0.8	13.1 ± 0.4	13.4 ± 0.8
Platelet count (10^3/mL)	1468 ± 237	1518 ± 44	1496 ± 208	1503 ± 157
MCV (fL)	59.1 ± 2.4	59.6 ± 1.8	57.8 ± 2.1	60.9 ± 1.5
MCH (pg)	19.7 ± 0.8	20.3 ± 0.5	19.3 ± 0.7	20.5 ± 0.5
MCHC (g/dL)	33.3 ± 0.2	34.0 ± 0.6	33.4 ± 0.7	33.7 ± 0.4
Reticulocyte ratio (%)	6.48 ± 2.55	4.88 ± 1.04	4.48 ± 1.28	6.28 ± 2.55
PT (sec)	7.38 ± 0.29	7.28 ± 0.19	7.42 ± 0.27	6.94 ± 0.32
APTT (sec)	18.6 ± 1.2	19.1 ± 1.9	18.8 ± 0.3	14.7 ± 3.4

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

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/d 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/d, 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/d.

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/d group.

Table 3: Blood biochemical findings in male and female rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of male rats	5	5	5	5
ASAT (IU/L)	116 ± 26[a]	92 ± 18	136 ± 28	121 ± 23
ALAT (IU/L)	38.8 ± 3.7	39.2 ± 2.9	58.2 ± 25.5*	48.8 ± 7.5
ALP (IU/L)	539 ± 57	476 ± 78	617 ± 178	943 ± 150**
Total bilirubin (mg/dL)	0.052 ± 0.008	0.048 ± 0.016	0.046 ± 0.013	0.024 ± 0.009**
BUN (mg/dL)	20.7 ± 1.2	19.7 ± 2.6	21.8 ± 1.9	21.3 ± 3.8
Creatinine (mg/dL)	0.312 ± 0.053	0.274 ± 0.022	0.226 ± 0.037**	0.248 ± 0.022**
Total cholesterol (mg/dL)	68.0 ± 6.9	58.4 ± 12.8	64.0 ± 7.3	61.2 ± 16.5
Glucose (mg/dL)	186 ± 14	173 ± 14	190 ± 15	198 ± 27
Total protein (g/dL)	5.60 ± 0.10	6.04 ± 0.27	6.26 ± 0.41**	5.92 ± 0.034
Albumin (%)	51.5 ± 2.3	53.3 ± 1.8	58.6 ± 2.5**	61.0 ± 1.7**
A/G ratio	1.07 ± 0.10	1.14 ± 0.09	1.42 ± 0.14**	1.57 ± 0.11**
α1-Globulin (%)	20.4 ± 2.7	20.7 ± 2.5	19.1 ± 2.9	18.1 ± 1.2
α2-Globulin (%)	9.4 ± 0.5	9.0 ± 0.3	7.8 ± 0.2**	7.6 ± 0.4**
β-Globulin (%)	14.5 ± 0.9	12.9 ± 1.0**	10.6 ± 0.6**	9.0 ± 0.3**
γ-Globulin (%)	4.2 ± 1.0	4.2 ± 0.3	4.0 ± 0.8	4.2 ± 0.8
No. of female rats	5	5	5	5
ASAT (IU/L)	130 ± 11[a]	113 ± 37	106 ± 15	104 ± 23
ALAT (IU/L)	59.0 ± 9.1	42.8 ± 7.8	49.4 ± 9.9	60.2 ± 15.3
ALP (IU/L)	215 ± 29	185 ± 71	184 ± 56	194 ± 59
Total bilirubin (mg/dL)	0.058 ± 0.016	0.074 ± 0.030	0.044 ± 0.011	0.056 ± 0.013
BUN (mg/dL)	26.1 ± 8.2	17.3 ± 5.3	19.8 ± 4.1	18.9 ± 5.0
Creatinine (mg/dL)	0.308 ± 0.044	0.290 ± 0.040	0.330 ± 0.029	0.282 ± 0.028
Total cholesterol (mg/dL)	79.6 ± 16.8	58.4 ± 3.2*	57.6 ± 13.3*	64.2 ± 12.9
Glucose (mg/dL)	109 ± 16	109 ± 13	120 ± 7	115 ± 24
Total protein (g/dL)	5.74 ± 0.31	5.60 ± 0.27	5.54 ± 0.36	5.50 ± 0.22
Albumin (%)	55.0 ± 1.8	54.2 ± 2.1	55.5 ± 0.8	55.4 ± 1.8
A/G ratio	1.23 ± 0.09	1.19 ± 0.10	1.25 ± 0.04	1.25 ± 0.09
α1-Globulin (%)	17.8 ± 2.1	19.2 ± 1.4	17.8 ± 2.2	17.6 ± 1.3
α2-Globulin (%)	8.8 ± 1.2	8.8 ± 0.9	7.9 ± 0.8	8.3 ± 0.3
β-Globulin (%)	13.5 ± 0.9	13.3 ± 0.9	13.7 ± 0.8	13.4 ± 1.0
γ-Globulin (%)	4.9 ± 1.2	4.4 ± 0.4	5.1 ± 0.5	5.3 ± 0.4

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

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 d 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

Table 4: Organ weights of male rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of male rats	5	5	5	5
Body weight (g)	451 ± 35[a]	463 ± 26	454 ± 37	437 ± 11
Brain (g)	2.06 ± 0.07[b]	2.09 ± 0.06	2.06 ± 0.11	2.00 ± 0.09
	0.461 ± 0.034[c]	0.453 ± 0.034	0.457 ± 0.061	0.459 ± 0.030
Heart (g)	1.41 ± 0.07[b]	1.52 ± 0.11	1.44 ± 0.16	1.42 ± 0.11
	0.314 ± 0.011[c]	0.329 ± 0.018	0.312 ± 0.018	0.325 ± 0.024
Thymus (mg)	391 ± 90[b]	401 ± 104	412 ± 174	396 ± 88
	87.4 ± 22.7[c]	86.2 ± 18.6	89.4 ± 31.5	90.8 ± 20.5
Liver (g)	14.81 ± 1.43[b]	16.46 ± 1.70	20.11 ± 3.76*	24.11 ± 2.60**
	3.28 ± 0.13[c]	3.54 ± 0.20	4.41 ± 0.55*	5.52 ± 0.66**
Kidney (g)	3.17 ± 0.25[b]	3.49 ± 0.31	3.50 ± 0.40	3.34 ± 0.18
	0.706 ± 0.082[c]	0.753 ± 0.051	0.769 ± 0.045	0.763 ± 0.045
Spleen (mg)	853 ± 82[b]	957 ± 205	908 ± 218	790 ± 62
	190 ± 19[c]	206 ± 38	199 ± 35	181 ± 17
Adrenal (mg)	61.2 ± 9.5[b]	62.1 ± 9.2	61.5 ± 6.9	50.8 ± 3.2
	13.6 ± 2.3[c]	13.4 ± 2.1	13.6 ± 2.2	11.6 ± 0.6
Testis (g)	3.23 ± 0.14[b]	3.39 ± 0.17	3.01 ± 0. 28	3.00 ± 0.25
	0.720 ± 0.062[c]	0.732 ± 0.051	0.666 ± 0.073	0.686 ± 0.058
Epididymis (g)	1.26 ± 0.07[b]	1.27 ± 0.06	1.23± 0.14	1.24 ± 0.13
	0.281 ± 0.027[c]	0.274 ± 0.010	0.271 ± 0.018	0.284 ± 0.028
Seminal vesicle (g)	1.71 ± 0.18[b]	1.69 ± 0.14	1.70 ± 0.21	1.60 ± 0.12
	0.383 ± 0.060[c]	0.365 ± 0.040	0.376 ± 0.040	0.366 ± 0.027
Prostate (g)	1.37 ± 0.099[b]	1.25 ± 0.10	1.42 ± 0.34	1.39 ± 0.19
	0.305 ± 0.035[c]	0.270 ± 0.025	0.309 ± 0.057	0.319 ± 0.038

[a]Values are given as the mean ± SD.
[b]Absolute organ weight.
[c]Relative organ weight = organ weight (g or mg)/100 g body weight.
*Significantly different from the control, $p < 0.05$.
**Significantly different from the control, $p < 0.01$.

developmental parameters regarding embryonic/fetal/neonatal survival and growth and morphological development of offspring were not affected by the 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 1,000 mg/kg/d (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/d 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

Table 5: Organ weights of female rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of female rats	5	5	5	5
Body weight (g)	282 ± 33[a]	290 ± 14	276 ± 15	283 ± 21
Brain (g)	1.96 ± 0.04[b]	1.96 ± 0.06	1.97 ± 0.09	1.94 ± 0.06
	0.706 ± 0.100[c]	0.677 ± 0.044	0.716 ± 0.068	0.688 ± 0.058
Heart (g)	1.06 ± 0.14[b]	1.00 ± 0.04	0.98 ± 0.08	1.01 ± 0.10
	0.376 ± 0.019[c]	0.346 ± 0.012	0.357 ± 0.022	0.358 ± 0.035
Thymus (mg)	219 ± 40[b]	272 ± 60	247 ± 87	253 ± 64
	77.6 ± 7.8[c]	93.6 ± 18.6	90.2 ± 32.9	90.2 ± 26.5
Liver (g)	9.89 ± 1.64[b]	8.99 ± 0.67	9.16 ± 0.69	9.69 ± 0.54
	3.51 ± 0.37[c]	3.10 ± 0.19*	3.32 ± 0.10	3.43 ± 0.20
Kidney (g)	2.16 ± 0.28[b]	2.07 ± 0.14	1.98 ± 0.21	2.03 ± 0.03
	0.770 ± 0.067[c]	0.713 ± 0.033	0.715 ± 0.057	0.721 ± 0.049
Spleen (mg)	716 ± 178[b]	713 ± 125	666 ± 172	749 ± 62
	252 ± 44[c]	246 ± 47	240 ± 52	265 ± 16
Adrenal (mg)	95.7 ± 15.2[b]	85.0 ± 10.3	85.3 ± 10.3	89.5 ± 4.0
	34.1 ± 5.23[c]	29.3 ± 3.6	30.9 ± 3.3	31.7 ± 1.6
Ovary (mg)	95.9 ± 10.4[b]	96.4 ± 6.2	95.6 ± 11.6	104.9 ± 18.8
	34.5 ± 5.9[c]	33.2 ± 1.9	34.7 ± 4.2	36.9 ± 4.5

[a]Values are given as the mean ± SD.
[b]Absolute organ weight.
[c]Relative organ weight = organ weight (g or mg)/100 g body weight.
*Significantly different from the control, $p < 0.05$.

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/d 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 noted only in males, indicating a sex difference in the toxicity of DBHCB.

The no observed adverse effect level (NOAEL) for repeated-dose toxicity of DBHCB is considered to be 2.5 mg/kg/d in male rats, based on the increased levels of albumin and weight of the liver, and 250 mg/kg/d, the highest dose used in the present study, in female rats. Our findings indicate that male rats have more than a 100-fold greater susceptibility to DBHCB toxicity than female rats. Previously, we showed sex differences in toxicity in the 28-d 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 a UV absorber (Hirata-Koizumi et al., 2007, 2008a). In the 28-d repeated-dose toxicity study, using rats given HDBB by gavage at 0, 0.5, 2.5,

12.5, or 62.5 mg/kg/d, 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., 2007). 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/d in males and 0, 0.5, 2.5, or 12.5 mg/kg/d in females, toxic effects were observed in the liver at 0.5 mg/kg/d and higher in males and 12.5 mg/kg/d in females (Hirata-Koizumi et al., 2008a).

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 gonadectomized 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., 2008b). We also reported no sex differences in susceptibility to the toxic effects of HDBB in preweaning rats (Hirata-Koizumi et al., 2008c). 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 preweaning 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.

CONCLUSIONS

In conclusion, the administration of DBHCB during premating, 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/d and higher, as well as ALP levels at 250 mg/kg/d in males, but no change in females. These findings indicate a sex difference in the toxicity of DBHCB in rats.