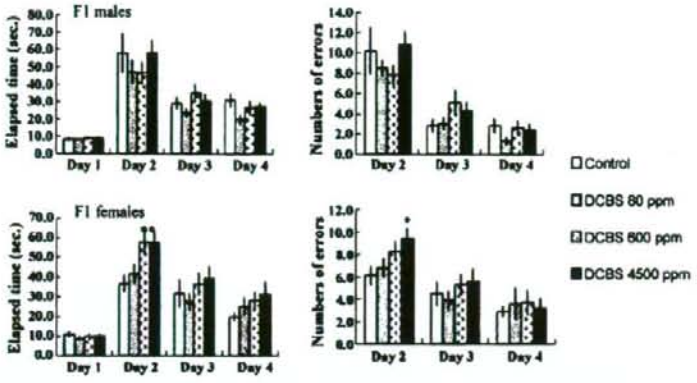

Fig. 3. Performance in water-filled multiple T-maze in F1 males and females. Each rat was allowed to swim in a straight channel on day 1, and then tested in the maze for the next consecutive three days (days 2–4). Values are given as the mean ± S.E.M. *Significantly different from the control, $p < 0.05$.

nificantly decreased absolute weights of the spleen and adrenal gland, and increased relative weights of the brain, thyroid, liver, kidney and testis were detected at 4500 ppm in males. A significant increase in the absolute weights of the brain at 80 and 600 ppm and the pituitary at 80 ppm, and decrease in the relative weight of the spleen at 80 and 600 ppm was observed in F0 females. Significantly decreased absolute weight of the spleen, and increased relative weights of the brain, kidney, and adrenal gland were found at 4500 ppm in females (data not shown).

3.8. Organ weights (F1 weanlings and adults)

The organ weights of male and female F1 weanlings are presented in Table 6. The body weight at the scheduled sacrifice was significantly lowered in males and females at 4500 ppm. The relative weights of the kidney at 80 ppm and the liver at 600 ppm were significantly higher in males. Significant decreases in the absolute weights of the brain, thymus, liver, kidney, adrenal gland, epididymis, and ventral prostate, and decrease in both the absolute and relative weights of the spleen, and increase in the relative weights of the brain, liver and testis were all observed at 4500 ppm in males. A significantly increased relative weight of the kidney at 80 ppm and decreased absolute weight of the ovary at 600 ppm was found in females. The absolute weights of the brain, thymus, liver, kidney, spleen, adrenal, ovary and uterus, and the relative weight of the spleen were significantly lowered at 4500 ppm in females. In this group, significantly higher relative weights of the brain and liver were also observed in females.

Table 7 shows the organ weights of male F1 adults at the scheduled terminal sacrifice. The absolute and relative weights of the thymus were significantly lower at 80 ppm in males. A significantly decreased absolute weight of the brain, decreased absolute and relative weights of the seminal vesicle, increased relative weight of the kidney, and increased absolute and relative weights of the liver were noted at 4500 ppm in males.

The organ weights of female F1 adults at the scheduled terminal sacrifice are shown in Table 8. The absolute weight of the brain at 80 and 600 ppm, and the relative weights of the liver and kidney, and the absolute and relative weights of the adrenal gland at 4500 ppm were significantly increased.

3.9. Organ weights (F2 weanlings)

Table 9 presents the organ weights of male F2 weanlings. The body weight at sacrifice was significantly reduced at 4500 ppm. A significant decrease in the absolute and relative weight of the spleen was observed at 80 ppm. The relative weights of the liver and kidney were significantly higher at 600 ppm. At 4500 ppm, a significantly decreased absolute weight of the adrenal gland, decreased absolute and relative weights of the thymus and spleen, and increased relative weights of the brain, liver, and kidney were noted in males.

Table 9 also presents the organ weights of female F2 weanlings. A significant decrease in the body weight at sacrifice was found at 4500 ppm. The relative weight of the thymus was significantly lower at 80 ppm. Significantly increased relative weights of the liver and kidney, and reduced absolute and relative weights of the uterus were found at 600 ppm. At 4500 ppm, significantly decreased absolute weights of the brain and spleen, and absolute and relative weights of the thymus and uterus, and increased relative weights of the brain, liver and kidney were noted in females.

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

A significantly higher percent of lymphocytes was observed in male F0 adults at 4500 ppm and in female F1 adults at 600 ppm. In female F0 and male F1 adults, no significant difference was noted in the WBC or differential leukocyte count between the control and DCBS-treated groups. There were no significant changes in biochemistry parameters such as total protein, albumin and globulin in male and female F0 and F1 adult rats (data not shown).

Table 6
Organ weight of male and female F1 weanlings

	DCBS (ppm)			
	0 (control)	80	600	4500
No. of male F1 weanlings examined	22	24	24	24
Body weight (g)	92.2 ± 8.0[a]	88.9 ± 6.2	88.1 ± 9.1	78.2 ± 7.2**
Brain (g)	1.69 ± 0.07[c]	1.69 ± 0.05	1.70 ± 0.08	1.63 ± 0.07**
	1.84 ± 0.13[d]	1.91 ± 0.13	1.94 ± 0.15	2.09 ± 0.17**
Thymus (mg)	359 ± 68	350 ± 53	365 ± 52	278 ± 37**
	388 ± 52[d]	393 ± 50	416 ± 61	357 ± 48
Liver (g)	4.16 ± 0.49	4.09 ± 0.37	4.09 ± 0.44	3.83 ± 0.51*
	4.51 ± 0.26[d]	4.60 ± 0.25	4.64 ± 0.15*	4.89 ± 0.28**
Kidney[b] (g)	1.023 ± 0.111	1.040 ± 0.079	1.003 ± 0.135	0.894 ± 0.069**
	1.110 ± 0.073[d]	1.171 ± 0.064*	1.137 ± 0.079	1.146 ± 0.073
Spleen (mg)	394 ± 68	352 ± 68	356 ± 61	278 ± 41**
	425 ± 52[d]	395 ± 63	405 ± 59	357 ± 48**
Adrenal[b] (mg)	25.5 ± 2.6	25.5 ± 3.3	25.0 ± 3.3	22.4 ± 2.9**
	27.8 ± 2.9[d]	28.7 ± 3.0	28.4 ± 3.1	28.8 ± 4.0
Testis[b] (mg)	561 ± 77	542 ± 64	541 ± 88	529 ± 88
	608 ± 61[d]	610 ± 55	612 ± 58	677 ± 95**
Epididymis[b] (mg)	78.6 ± 9.4	77.3 ± 9.5	75.9 ± 11.6	71.3 ± 10.2*
	85.5 ± 9.8[d]	86.9 ± 8.4	86.4 ± 11.3	91.3 ± 11.7
Ventral prostate (mg)	49.2 ± 9.8	47.5 ± 7.8	43.7 ± 10.1	42.3 ± 8.9*
	53.3 ± 9.1[d]	53.4 ± 7.8	49.6 ± 10.7	54.0 ± 9.5
No. of female F1 weanlings examined	22	24	24	24
Body weight (g)	85.9 ± 7.8	82.5 ± 6.4	82.8 ± 7.2	74.3 ± 6.7**
Brain (g)	1.62 ± 0.07	1.65 ± 0.05	1.64 ± 0.05	1.57 ± 0.05**
	1.90 ± 0.14[d]	2.01 ± 0.17	2.00 ± 0.16	2.13 ± 0.16**
Thymus (mg)	361 ± 77	327 ± 49	350 ± 60	281 ± 43**
	418 ± 65[d]	398 ± 60	423 ± 63	379 ± 56
Liver (g)	3.72 ± 0.44	3.52 ± 0.35	3.65 ± 0.40	3.43 ± 0.40*
	4.33 ± 0.34[d]	4.27 ± 0.22	4.41 ± 0.29	4.62 ± 0.31**
Kidney[b] (g)	0.954 ± 0.108	0.967 ± 0.081	0.940 ± 0.114	0.850 ± 0.082**
	1.110 ± 0.068[d]	1.173 ± 0.055**	1.133 ± 0.065	1.148 ± 0.088
Spleen (mg)	338 ± 58	323 ± 47	316 ± 53	249 ± 32**
	392 ± 43[d]	392 ± 54	382 ± 55	337 ± 49**
Adrenal[b] (mg)	23.8 ± 2.6	24.5 ± 2.7	23.1 ± 2.9	21.5 ± 2.4*
	27.8 ± 2.8[d]	29.8 ± 3.6	27.9 ± 2.6	29.1 ± 3.6
Ovary[b] (mg)	23.2 ± 3.3	22.2 ± 3.4	20.5 ± 3.2*	20.3 ± 3.2*
	27.1 ± 3.3[d]	27.0 ± 4.0	24.8 ± 4.3	27.5 ± 4.7
Uterus (mg)	58.2 ± 14.5	55.8 ± 7.6	62.1 ± 12.3	48.4 ± 11.8*
	67.9 ± 15.8[d]	67.9 ± 9.9	75.2 ± 14.1	65.0 ± 14.1

[a] Values are given as the mean ± S.D.
[b] Values are represented as the total weights of the organs of both sides.
[c] Absolute organ weight.
[d] 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, $p < 0.01$.

3.11. Serum hormone levels (F0 and F1 adults)

No significant changes in any serum hormone levels of male and female F0 adults were noted between the control and DCBS-treated groups (data not shown).

Serum hormone levels of male and female F1 adult rats are shown in Fig. 4. Although significantly higher levels of testosterone at 80 ppm and LH at 600 ppm were observed in F1 males, no significant changes were noted in any hormone levels in F1 males at 4500 ppm. There were no significant changes in any

Table 7
Organ weight of male F1 adults

	DCBS (ppm)			
	0 (control)	80	600	4500
No. of male F1 adults examined	24	24	24	24
Body weight (g)	630.7 ± 74.7[a]	605.1 ± 47.7	614.2 ± 52.5	622.6 ± 51.8
Brain (g)	2.26 ± 0.10[c]	2.29 ± 0.06	2.26 ± 0.06	2.21 ± 0.09*
	0.363 ± 0.038[d]	0.380 ± 0.028	0.370 ± 0.030	0.356 ± 0.027
Pituitary gland (mg)	13.6 ± 1.4	13.9 ± 1.3	13.9 ± 1.1	14.0 ± 1.6
	2.17 ± 0.23[d]	2.30 ± 0.25	2.27 ± 0.17	2.26 ± 0.26
Thyroid[b] (mg)	24.9 ± 4.9	23.3 ± 4.7	23.8 ± 4.5	24.6 ± 4.9
	3.95 ± 0.66[d]	3.86 ± 0.78	3.88 ± 0.69	3.95 ± 0.67
Thymus (mg)	346 ± 116	269 ± 54*	331 ± 83	316 ± 62
	54.8 ± 17.0[d]	44.5 ± 8.9*	53.9 ± 12.7	50.9 ± 9.8
Liver (g)	20.80 ± 3.73	19.69 ± 2.32	21.19 ± 2.06	22.82 ± 3.37*
	3.28 ± 0.29 [d]	3.25 ± 0.19	3.46 ± 0.28	3.65 ± 0.28**
Kidney[b] (g)	3.70 ± 0.52	3.66 ± 0.23	3.69 ± 0.36	3.91 ± 0.43
	0.586 ± 0.041[d]	0.606 ± 0.042	0.602 ± 0.047	0.629 ± 0.044**
Spleen (mg)	909 ± 129	845 ± 141	847 ± 124	869 ± 162
	145 ± 16[d]	139 ± 18	138 ± 17	139 ± 17
Adrenal[b] (mg)	60.5 ± 9.8	60.3 ± 7.1	61.8 ± 7.2	61.3 ± 13.1
	9.6 ± 1.5[d]	10.0 ± 1.0	10.1 ± 1.3	9.8 ± 2.0
Testis[b] (g)	3.60 ± 0.35	3.61 ± 0.27	3.60 ± 0.27	3.78 ± 0.32
	0.575 ± 0.062[d]	0.601 ± 0.073	0.589 ± 0.066	0.610 ± 0.062
Epididymis[b] (mg)	1348 ± 138	1342 ± 67	1327 ± 111	1346 ± 118
	215 ± 24[d]	223 ± 21	217 ± 22	217 ± 19
Seminal vesicle (g)	2.30 ± 0.23	2.19 ± 0.28	2.21 ± 0.22	2.07 ± 0.26**
	0.368 ± 0.047[d]	0.364 ± 0.054	0.362 ± 0.039	0.333 ± 0.045*
Ventral prostate (mg)	838 ± 174	812 ± 181	822 ± 190	784 ± 168
	133 ± 24[d]	134 ± 28	134 ± 29	127 ± 31

[a] Values are given as the mean ± S.D.
[b] Values are represented as the total weights of the organs of both sides.
[c] Absolute organ weight.
[d] 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, $p < 0.01$.

serum hormone levels of female F1 adults between the control and DCBS-treated groups.

3.12. Sperm parameters (F0 and F1 adults)

Table 10 shows the sperm parameters in F0 and F1 adult males. No significant changes in sperm counts, percentage of motile sperm and progressively motile sperm, swimming speed and pattern, or percentage of morphologically abnormal sperm were noted in F0 adults between the control and DCBS-treated groups. A significant decrease in the mean lateral head displacement was found at 4500 ppm in F1 males.

4. Discussion

A two-generation reproductive toxicity study was performed to further evaluate the potential effects of DCBS on reproduction and development in rats.

The deaths and clinical signs observed in the present study are not thought to be attributable to the administration of DCBS, because the incidences of deaths and clinical signs were very low and inconsistent across generations, and these occurrences are not uncommon in toxicological studies.

The decreased food consumption in F0 males and females at 4500 ppm was accompanied by decreases in the body weight and body weight gain. However, lowered food consumption in F1 males at 80, 600 and 4500 ppm was occasional, inconsistent, and unaccompanied by changes in body weight or body weight gain. It seems likely that DCBS adversely affects the body weight and food consumption in F0 rats at 4500 ppm, but not in F1 rats.

Although a few F0 and F1 adults showed reproductive difficulties, necropsy and the histopathology of reproductive organs revealed no evidence of reproductive failure in these rats. Two F1 females showing abnormal estrous cycles remained in diestrus for 10–11 days, suggesting they were pseudopregnant. No significant changes in reproductive indices were noted in any

Table 8
Organ weight of female F1 adults

	DCBS (ppm)			
	0 (control)	80	600	4500
No. of female F1 adults examined	22	22	21	23
Body weight (g)	331.9 ± 32.5[a]	331.2 ± 28.5	331.3 ± 23.1	330.2 ± 30.8
Brain (g)	2.08 ± 0.08[c]	2.17 ± 0.08**	2.15 ± 0.08*	2.08 ± 0.08
	0.632 ± 0.056[d]	0.658 ± 0.056	0.651 ± 0.043	0.633 ± 0.060
Pituitary gland (mg)	15.9 ± 2.0	16.1 ± 2.4	15.8 ± 1.8	16.1 ± 1.9
	4.83 ± 0.73[d]	4.90 ± 0.79	4.78 ± 0.52	4.89 ± 0.66
Thyroid[b] (mg)	19.0 ± 3.9	18.2 ± 2.7	17.7 ± 3.5	19.4 ± 4.1
	5.72 ± 0.98[d]	5.51 ± 0.70	5.35 ± 1.08	5.89 ± 1.15
Thymus (mg)	251 ± 69	212 ± 47	261 ± 65	211 ± 63
	75.3 ± 18.4[d]	64.1 ± 14.2	79.2 ± 20.2	64.0 ± 18.7
Liver (g)	14.55 ± 1.66	14.18 ± 2.14	14.32 ± 1.49	15.83 ± 2.11
	4.39 ± 0.28[d]	4.28 ± 0.49	4.33 ± 0.41	4.81 ± 0.59**
Kidney[b] (g)	2.37 ± 0.30	2.39 ± 0.22	2.40 ± 0.21	2.53 ± 0.26
	0.713 ± 0.046[d]	0.723 ± 0.040	0.726 ± 0.063	0.771 ± 0.080**
Spleen (mg)	632 ± 73	599 ± 63	609 ± 80	639 ± 115
	191 ± 18[d]	181 ± 15	184 ± 19	194 ± 37
Adrenal[b] (mg)	70.0 ± 9.7	73.5 ± 10.9	73.4 ± 9.3	77.5 ± 8.9*
	21.2 ± 3.2[d]	22.2 ± 3.1	22.2 ± 3.0	23.6 ± 3.2*
Ovary[b] (mg)	110.6 ± 13.0	109.1 ± 16.3	108.5 ± 12.5	108.2 ± 13.4
	33.4 ± 2.9[d]	33.0 ± 4.5	32.8 ± 3.2	32.8 ± 3.3
Uterus (mg)	927 ± 191	928 ± 128	976 ± 185	949 ± 192
	280 ± 54[d]	283 ± 48	295 ± 52	288 ± 52

[a] Values are given as the mean ± S.D.
[b] Values are represented as the total weights of the organs of both sides.
[c] Absolute organ weight.
[d] 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, $p < 0.01$.

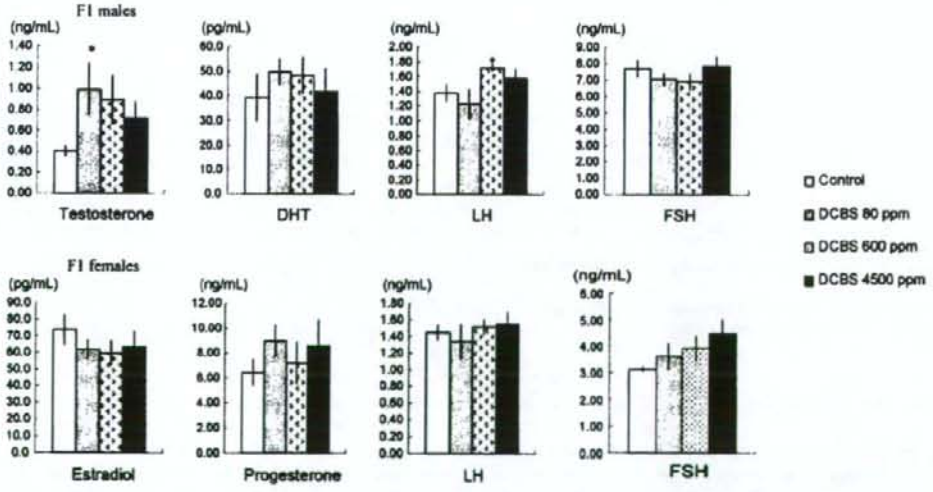

Fig. 4. Serum hormone levels in F1 males and females. The actual measurement of DHT was below the lower limit of quantification (<25.0 pg/mL) in one F1 male each in the control and 4500 ppm groups. The actual measurement of LH was below the lower limit of quantification (<0.80 ng/mL) in one F1 male and one F1 female in the 80 ppm group. Values are given as the mean ± S.E.M. *Significantly different from the control, $p < 0.05$.

Table 9
Organ weight of male and female F2 weanlings

	DCBS (ppm)			
	0 (control)	80	600	4500
No. of male F2 weanlings examined	22	22	21	23
Body weight (g)	90.8 ± 8.7[a]	91.4 ± 13.1	89.1 ± 6.5	80.0 ± 7.8**
Brain (g)	1.67 ± 0.08[c]	1.69 ± 0.09	1.70 ± 0.08	1.63 ± 0.09
	1.85 ± 0.14[d]	1.87 ± 0.22	1.92 ± 0.13	2.05 ± 0.18**
Thymus (mg)	355 ± 56	325 ± 64	361 ± 47	283 ± 52**
	392 ± 58[d]	355 ± 47	406 ± 48	354 ± 55*
Liver (g)	4.08 ± 0.47	4.12 ± 0.66	4.21 ± 0.44	3.74 ± 0.54
	4.49 ± 0.23[d]	4.50 ± 0.26	4.72 ± 0.27**	4.66 ± 0.42**
Kidney[b] (g)	1.006 ± 0.102	1.009 ± 0.147	1.022 ± 0.089	0.923 ± 0.133
	1.109 ± 0.037[d]	1.105 ± 0.066	1.146 ± 0.049*	1.152 ± 0.117*
Spleen (mg)	383 ± 61	350 ± 83*	356 ± 46	286 ± 52**
	422 ± 49[d]	381 ± 53*	400 ± 50	357 ± 54**
Adrenal[b] (mg)	25.5 ± 2.8	24.1 ± 3.4	24.2 ± 3.8	22.7 ± 3.4*
	28.1 ± 2.8[d]	26.5 ± 2.6	27.2 ± 4.3	28.5 ± 4.2
Testis[b] (mg)	548 ± 106	516 ± 103	528 ± 82	525 ± 98
	602 ± 89[d]	563 ± 69	590 ± 65	653 ± 91
Epididymis[b] (mg)	79.5 ± 12.5	72.9 ± 12.3	72.7 ± 10.0	71.3 ± 11.2
	87.6 ± 10.6[d]	80.4 ± 12.6	81.7 ± 10.6	89.0 ± 10.0
Ventral prostate (mg)	50.9 ± 16.6	44.6 ± 10.4	47.0 ± 10.3	42.6 ± 12.2
	55.6 ± 15.0[d]	48.9 ± 9.9	52.7 ± 10.1	52.9 ± 13.1
No. of female F2 weanlings examined	22	22	20	23
Body weight (g)	83.6 ± 9.5	87.2 ± 10.8	82.4 ± 6.5	74.6 ± 7.9**
Brain (g)	1.62 ± 0.08	1.66 ± 0.07	1.66 ± 0.05	1.57 ± 0.07*
	1.96 ± 0.20[d]	1.92 ± 0.19	2.03 ± 0.16	2.11 ± 0.18*
Thymus (mg)	364 ± 50	326 ± 66	348 ± 68	283 ± 56**
	439 ± 63[d]	373 ± 56**	424 ± 80	379 ± 63**
Liver (g)	3.71 ± 0.47	3.87 ± 0.50	3.80 ± 0.37	3.57 ± 0.46
	4.44 ± 0.18[d]	4.44 ± 0.23	4.61 ± 0.19*	4.78 ± 0.26**
Kidney[b] (g)	0.915 ± 0.093	0.983 ± 0.137	0.960 ± 0.111	0.885 ± 0.101
	1.096 ± 0.046[d]	1.129 ± 0.085	1.164 ± 0.083**	1.187 ± 0.061**
Spleen (mg)	340 ± 63	331 ± 55	320 ± 46	274 ± 40**
	407 ± 58[d]	380 ± 46	389 ± 56	370 ± 52
Adrenal[b] (mg)	23.6 ± 2.9	23.3 ± 4.0	22.2 ± 3.3	21.6 ± 3.0
	28.4 ± 3.3[d]	26.7 ± 3.3	27.0 ± 4.0	29.0 ± 3.9
Ovary[b] (mg)	22.0 ± 3.9	22.5 ± 2.8	20.9 ± 3.1	21.4 ± 2.9
	26.6 ± 5.2[d]	26.0 ± 3.0	25.5 ± 4.4	29.0 ± 4.3
Uterus (mg)	61.8 ± 18.9	58.1 ± 11.9	50.0 ± 10.0*	46.6 ± 12.9**
	73.3 ± 17.2[d]	67.0 ± 13.5	60.7 ± 11.5*	62.3 ± 15.0*

[a] Values are given as the mean ± S.D.
[b] Values are represented as the total weights of the organs of both sides.
[c] Absolute organ weight.
[d] 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, $p < 0.01$.

generation even at the highest dose of 4500 ppm. Our previous screening test revealed that DCBS given by gavage to rats from day 14 before mating to day 3 of lactation caused significant decreases in the gestation index, numbers of corpora lutea, implantations, pups born and pups born alive, live birth index, and viability index at 400 mg/kg bw per day [10]. This dose also caused severe maternal toxicity and a total loss of pups until PND 4. No maternal or reproductive/developmental toxicity was detected at 100 mg/kg bw per day and below in our previous study. In the present feeding study, the mean daily intakes of DCBS were 416 and 417 mg/kg bw per day for the highest dose in F0 and F1 females, respectively. Consideration of these

Table 10
Sperm parameters in F0 and F1 males

	DCBS (ppm)			
	0 (control)	80	600	4500
F0 males				
No. of animals	24	23	24	24
No. of testicular sperm ($\times 10^6$)				
Per testis	184.1 ± 29.3[a]	187.7 ± 28.3	184.2 ± 32.7	180.8 ± 35.4
Per g testis	111.4 ± 13.2	110.7 ± 15.7	110.6 ± 17.1	106.1 ± 18.8
No. of epididymal sperm ($\times 10^6$)				
Per cauda	268.5 ± 47.6	276.2 ± 40.3	269.9 ± 56.8	263.7 ± 62.8
Per g cauda	856.4 ± 94.4	838.9 ± 99.4	850.3 ± 122.1	844.2 ± 191.3
Percent motile	88.1 ± 9.3	92.6 ± 8.2	93.2 ± 5.9	89.4 ± 10.2
Percent progressive	70.9 ± 17.4	77.3 ± 15.3	77.4 ± 12.1	70.5 ± 22.2
Mean path velocity (μm/s)	159.6 ± 20.8	159.8 ± 19.2	162.7 ± 22.0	156.8 ± 25.3
Straight line average velocity (μm/s)	112.1 ± 22.5	114.1 ± 20.0	116.1 ± 19.3	110.5 ± 29.2
Mean curvilinear velocity (μm/s)	365.7 ± 53.4	370.1 ± 42.5	372.3 ± 49.8	358.4 ± 56.3
Mean lateral head displacement (μm)	20.1 ± 1.1	19.9 ± 1.1	20.0 ± 1.3	19.9 ± 1.0
Mean beat cross frequency (Hz)	27.9 ± 1.5	27.4 ± 1.5	27.6 ± 2.2	28.3 ± 2.3
Mean straightness (%)[b]	69.3 ± 6.6	70.7 ± 5.7	71.0 ± 4.3	69.5 ± 8.6
Mean linearity (%)[c]	30.4 ± 2.8	30.7 ± 3.0	31.3 ± 2.5	30.6 ± 4.0
Total abnormal sperm ratio (%)	1.1 ± 0.6	1.2 ± 0.8	2.4 ± 3.5	2.0 ± 2.4
Tailless sperm (%)	1.0 ± 0.6	1.2 ± 0.8	2.2 ± 3.5	1.8 ± 2.0
F1 males				
No. of animals	24	24	24	24
No. of testicular sperm ($\times 10^6$)				
Per testis	194.5 ± 23.0[a]	181.1 ± 21.3	186.3 ± 22.5	201.0 ± 33.3
Per g testis	115.3 ± 9.5	108.4 ± 14.3	111.1 ± 11.3	113.6 ± 15.0
No. of epididymal sperm ($\times 10^6$)				
Per cauda	273.6 ± 40.0	254.0 ± 40.4	256.2 ± 46.0	250.3 ± 55.4
Per g cauda	849.9 ± 69.4	821.5 ± 106.8	827.2 ± 93.3	807.0 ± 127.5
Percent motile	92.3 ± 5.0	92.9 ± 4.0	93.3 ± 5.6	93.0 ± 7.4
Percent progressive	81.8 ± 8.1	81.8 ± 4.9	83.9 ± 6.4	82.7 ± 8.2
Mean path velocity (μm/s)	175.2 ± 9.8	171.7 ± 11.2	172.4 ± 11.4	171.3 ± 13.9
Straight line average velocity (μm/s)	126.9 ± 10.2	123.9 ± 10.3	126.0 ± 10.5	125.7 ± 12.6
Mean curvilinear velocity (μm/s)	399.5 ± 19.8	391.5 ± 28.6	395.1 ± 28.6	393.6 ± 29.8
Mean lateral head displacement (μm)	21.3 ± 0.9	20.9 ± 0.8	20.8 ± 0.8	20.5 ± 1.0*
Mean beat cross frequency (Hz)	26.4 ± 1.6	26.8 ± 1.4	26.1 ± 1.6	27.0 ± 1.8
Mean straightness (%)[b]	72.5 ± 3.3	72.1 ± 2.7	73.3 ± 2.9	73.5 ± 2.8
Mean linearity (%)[c]	32.0 ± 2.1	31.9 ± 2.0	32.1 ± 1.8	32.2 ± 1.5
Total abnormal sperm ratio (%)	1.4 ± 1.3	1.1 ± 0.8	1.2 ± 1.7	1.6 ± 1.9
Tailless sperm (%)	1.3 ± 1.2	0.9 ± 0.8	1.0 ± 1.6	1.5 ± 1.8

[a] Values are given as the mean ± S.D.
[b] Mean straightness (%) = straight line average velocity/mean path velocity × 100.
[c] Mean linearity (%) = straight line average velocity/mean curvilinear velocity × 100.
* Significantly different from the control, $p < 0.05$.

findings suggests that the highest dose of DCBS in the present study may be very close to the dose that induces severe maternal and reproductive toxicity. However, the possibility remains that the difference in the degree of toxicity may be due to differences in administration method. There are some examples showing that gavage and feed administration result with differences in the toxicokinetics of chemicals [20,21]. Further studies are needed to clarify the relationship between maternal and reproductive/developmental toxicity.

Regarding developmental parameters, lowered body weights of male and female pre-weaning F1 and F2 pups were noted at 4500 ppm. These findings indicate that the dose level of 4500 ppm used in this study was potent enough to have adverse effects on the growth of pups. It is noted that there are strong correlations between developmental landmark parameters and pup body weight data, and that pup body weight data is consistently a more sensitive indicator of the developmental status of the offspring [22,23]. Although delayed completion of incisor eruption was noted in male and female F1 pups at 80 ppm and in male and female F2 pups at 80 and 4500 ppm, the delayed completion of incisor eruption was not dose-dependent and the difference from the control value was very slight. Therefore, it is unlikely that the delay of incisor eruption observed in the present study was compound-related or toxicologically significant. There were

no significant changes in indices of pre-weaning functional development in the DCBS-treated groups. The AGD is also a developmental landmark for the differentiation of the external genitalia and is commonly used as a hormonally sensitive parameter of sexual differentiation in rodents [24]. The AGD per cube root of body weight ratio provides a more appropriate adjustment when it is necessary to normalize AGD to body weight [17]. No changes were observed in the AGD per cube root of body weight ratio at any doses of DCBS in any generation. The data on the AGD indicate a lack of effect of DCBS on AGD. These findings on pre-weaning developmental parameters suggest that DCBS adversely affects the growth of offspring, but not the pre-weaning landmarks of development or reflex ontogeny. An increase in the frequency of fetuses with internal hydrocephalus was reported in rats given *N*-cyclohexyl-2-benzothiazolesulfenamide, a structurally similar compound, during organogenesis in rats [25]. However, no significantly increased incidence of pups with anomalies was detected even at the highest dose in the present and previous studies of DCBS [10,15]. Regarding post-weaning landmarks of development, delays of preputial separation at 4500 ppm and vaginal opening at 600 and 4500 ppm were observed in the present study. Although the body weight at the age of preputial separation was not different between the control and DCBS-treated groups, a higher body weight at the age of vaginal opening was found at 600 and 4500 ppm in females. Preputial separation and vaginal opening indicate the onset of sexual maturity, and the body weight is correlated with the occurrence of these events [23]. Ashby and Lefevre [26] described that delays in preputial separation can only be interpreted with confidence when they are not accompanied by losses in body weight, or when the expected delay in preputial separation due to a loss of body weight has been exceeded. They also noted that measurement of delays in preputial separation may be of value in cases of large delays, but delays of 1–2 days are difficult to interpret with confidence [26]. In the present study, the delay of preputial separation at 4500 ppm was slight (1.5 days) and was not accompanied by a change in body weight, and the age of preputial separation was within the range of the background control data (40.3–42.8 days) for the last seven years in the laboratory performed current study. It is likely that the delay in preputial separation at 4500 ppm is related to general delays in development. In female rats, the age at vaginal opening is the most commonly measured marker of puberty, and vaginal opening is an estrogen-dependent event that results from an increase in the blood estradiol levels [27]. Although the delay of female vaginal opening at 600 and 4500 ppm was slight (1.5–1.6 days), the age of vaginal opening was over the range of the background control data (29.6–31.0 days) for the last seven years in the laboratory performed present study. In the present study at 600 and 4500 ppm, the heavier body weight was noted at the completion of vaginal opening. Therefore, the possibility that the delay in vaginal opening may have toxicological meaning is not completely ruled out. Other hormone-dependent events including estrous cyclicity and AGD, as well as serum hormone levels at the scheduled terminal necropsy were not changed in the DCBS-treated groups. Moreover, DCBS did not affect the reproductive performance. However, decreased weight of the uterus was found in F1 weanlings at 4500 ppm and F2 weanlings at 600 and 4500 ppm. It has been noted that variations in the weights of the reproductive organs, which are strongly dependent on endocrine status, can be considered a key parameter in the identification of endocrine effects [28–30]. These findings suggest that DCBS may have endocrine effects. Further studies are needed to clarify the effects of DCBS on endocrine endpoints.

Regarding the behavioral tests, the only significant change in the T-maze test was observed in females on day 2 of the test. Longer elapsed times at 600 and 4500 ppm and more errors at 4500 ppm were detected in females. There are behavioral functions not classically hormone-mediated and expressed by both sexes such as learning capacities, exploration activity, novelty seeking and anxiety levels that show both qualitative and quantitative differences in the two sexes [30]. The reduced activity, as well as the other effects on neuromuscular function, could be at least partially the result of lower body weight [31] and it has been found that light body weight caused worse performance in a learning task [32]. In the present study, the spontaneous activity, swimming ability in the straight channel and body weight at the time of the T-maze test was not different in F1 females between the control and DCBS-treated groups. Thus, it seems likely that DCBS may have transiently affected learning ability in the T-maze at the highest dose administered.

The changes in weight of the organs, such as the brain, thymus, kidney, and spleen that were observed at 80 and/or 600 ppm are not thought to be due to administration of DCBS, because changes occurred sporadically and not in a dose-dependent manner. The changes in the weights of the adrenal, thyroid, and male and female reproductive organs, except for the uterus, at 600 and/or 4500 ppm seem unlikely to be attributable to administration of DCBS because of inconsistent changes across ages, sexes and generations. No consistent DCBS-related effects on serum hormone levels or sperm parameters were also detected across generations. Decreased absolute weights and/or increased relative weights of the liver except for in female F0 adults, the spleen in F0 adults, and the brain and kidney in F0 and F1 adults and F1 and F2 weanlings at 4500 ppm seem to be due to secondarily lowered body weight, but not due to the direct effects of DCBS on the organs. Decreased absolute and/or relative weights of the thymus and spleen in the weanlings are supported by the results of our previous study in which atrophy of the thymus and spleen was observed at 400 mg/kg bw per day [10]. These findings may suggest that one of target systems of DCBS toxicity is the immune system in weanlings. In the present study, however, no DCBS-related histopathological changes were detected. The discrepancy in histopathological findings between the previous and present studies could be explained by a difference in the toxicokinetics of chemicals due to differences in administration method. No DCBS-related findings were found in the hematological and blood biochemical examinations. In general, the effects of DCBS on organ weights were more pronounced in weanlings than adults. These phenomena suggest that DCBS may be more toxic before weaning than after weaning, and this possibility is supported by the lowered body weight of pups during the pre-weaning period, but not post-weaning.

Table 11
Summary of relevant findings in rat two-generation reproductive toxicity study of DCBS (80, 600 and 4500 ppm)

	F0						F1						F2					
	Male			Female			Male			Female			Male			Female		
	80	600	4500	80	600	4500	80	600	4500	80	600	4500	80	600	4500	80	600	4500
Lowered body weight			+			+			+			+			+			+
Decreased food consumption			+			+												
Delayed vaginal opening											+	+						
Worse performance in water T-maze											+	+						
Reduced spleen weight									+			+			+			+
Reduced thymus weight									+			+			+			+
Reduced uterine weight												+					+	+

In conclusion, the results of the two-generation reproductive toxicity study described here provide a more comprehensive toxicity profile of DCBS than has been previously reported. Relevant findings obtained from the present rat two-generation reproductive toxicity study of DCBS are summarized in Table 11. The NOAEL in the present study is considered to be 80 ppm (5.2 mg/kg bw per day) in rats.

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Lack of Gender-Related Difference in the Toxicity of 2-(2′-Hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole in Preweaning Rats

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In our previous toxicity studies using young rats, we showed that an ultraviolet absorber, 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), principally affected the liver, and male rats had nearly 25 times higher susceptibility to the toxic effects than females. In the present study, the toxicity of HDBB was investigated in preweaning rats. HDBB was administered by gavage to male and female CD(SD) rats from postnatal days 4 to 21 at a dose of 0, 0.1, 0.5, 2.5, or 12.5 mg/kg/day. No substance-related deaths, clinical signs of toxicity, or body-weight changes were observed. Increased levels of albumin, AST and ALP in both sexes, BUN in males, and LDH in females were found at 12.5 mg/kg. Liver weights increased at 2.5 mg/kg and above in both sexes. Histopathologically, hepatocellular findings, such as nucleolar enlargement, anisokaryosis, increased mitosis, and/or hypertrophy, were observed at 2.5 mg/kg and above in both sexes. These results indicate no gender-related differences in the susceptibility to the toxic effects of HDBB in preweaning rats.

Keywords Benzotriazole UV absorber, Preweaning rat, Gender-related difference, Hepatotoxicity.

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INTRODUCTION

A number of reports have been published on gender-related differences in the toxic effects of chemicals in rats (Agarwal et al., 1982; Coleman et al., 1990; McGovren et al., 1981; Muraoka and Itoh, 1980; Nishino et al., 1998; Ogirima et al., 2006; Raheja et al., 1983). For example, fluoranthene, a polycyclic aromatic hydrocarbon, showed greater effects on male rats than females, especially on the kidneys, in a subchronic toxicity study (Knuckles et al., 2004). In contrast, female rats exhibited greater susceptibility to hypothalamic cholinesterase inhibitory and hypothermic effects of a carbamate cholinesterase inhibitor, rivastigmine (Wang et al., 2001). Such gender-related variations are also reported in humans, mostly for medicines (Harris et al., 1995). Examples include more severe adverse effects, but with greater improvement in response, to antipsychotic drugs such as chlorpromazine and fluspirilene in women.

Previously, we reported that male and female rats showed markedly different susceptibilities to the toxicity of 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), which is an ultraviolet absorber used in plastic resin products, such as building materials and automobile components (METI, 2006). In a 28-day repeated-dose toxicity study, male and female rats were administered HDBB by gavage, and adverse effects on the liver, heart, blood, kidneys, and thyroids were found (Hirata-Koizumi et al., 2007). The no observed adverse effect level (NOAEL) for females was 2.5 mg/kg/day based on histopathological changes in the liver and heart detected at 12.5 mg/kg, but the NOAEL for males could not be determined because hepatic changes were noted even at the lowest dose of 0.5 mg/kg. In the 52-week repeated-dose toxicity study, chronic oral administration of HDBB principally affected the liver, and the NOAEL was concluded to be 0.1 mg/kg/day in males and 2.5 mg/kg/day in females (Hirata-Koizumi et al., 2008a), showing that male rats have approximately 25 times higher susceptibility to HDBB toxicity than females.

For such gender differences in toxic responses, sexual hormones are likely to play important roles. In fact, Wang et al. (2001) reported that orchidectomy completely abolished the above-mentioned sex differences in hypothalamic cholinesterase inhibition induced by rivastigmine, and testosterone treatment to gonadectomized males and females decreased the cholinesterase inhibitory effects of rivastigmine; therefore, it is apparent that testosterone interferes with the effects of rivastigmine. On the other hand, estrogen has been shown to act as a dopamine antagonist (Harris et al., 1995), which is considered to contribute, at least in part, to sex differences in response to antipsychotic drugs.

In order to investigate the role of sex steroids in the mediation of sex differences in the susceptibility to the toxic effects of HDBB, we recently performed a 28-day repeated-dose toxicity study using male and female

castrated rats (Hirata-Koizumi et al., 2008b). As expected, castration markedly reduced the sexual variation in HDBB toxicity, but some difference, less than five times, remained between male and female castrated rats. It is speculated that the determinants of susceptibility to HDBB toxicity are already differentiated between sexes by four weeks of age, when the castration was performed; therefore, in the present study, we determined the sexual difference in the susceptibility to HDBB toxicity in preweaning rats.

MATERIALS AND METHODS

This study was performed at Shin Nippon Biomedical Laboratories, Ltd., Drug Safety Research Laboratories (SNBL DSR; Kagoshima, Japan) in 2006–2007. The experiment was approved by the Institutional Animal Care and Use Committee of SNBL DSR and was performed in accordance with the ethics criteria contained in the bylaws of the Committee.

Animals and Housing Conditions

Eleven-week-old male and 10-week-old female Crl:CD(SD) rats were purchased from Hino Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan) and individually housed in stainless steel cages suspended over a cage board. After a seven-day acclimation, females were cohabited overnight with one male each. Females with vaginal plugs were regarded as pregnant, and this day was designated as Day 0 of gestation. On gestation day 20, the pregnant females were transferred to aluminum cages with wooden chips as bedding (White Flake; Charles River Laboratories Japan, Inc.) and allowed to deliver spontaneously and rear their pups. The day of birth was defined as postnatal day (PND) 0. On PND 4, the sex of the pups was determined, and the litters were adjusted randomly to four males and four females. Five litters were selected and randomly assigned to each of five dose groups, including control groups; the initial number of pups for treatment was 20/sex/group.

Throughout the study, the animals were maintained in an air-conditioned room at 21.5–22.4°C, with a relative humidity of 43–55%, a 12-h light/dark cycle, and ventilation with 15 air changes/hour. A basal diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water, which met the drinking water standard under the Water Works Law of Japan, were provided *ad libitum*.

Chemicals and Doses

HDBB (CAS No. 3846-71-7, Lot no. AY11) was 100% pure and was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); it was kept in a dark place at room temperature under airtight conditions. Dosing

solutions were prepared as a suspension in corn oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan) once or twice a week and kept cool in a dark place under airtight conditions until dosing. Stability under refrigerated conditions was confirmed for seven days in the previous 28-day repeated-dose toxicity study using young animals (Hirata-Koizumi et al., 2007).

Male and female preweaning rats were given HDBB by gavage once-daily from PNDs 4 to 21. Control rats received the vehicle only. A nutrient catheter (Type 3Fr; Atom Medical Corporation, Tokyo, Japan), attached to a disposable syringe, was used for dosing. The volume of each dose was adjusted to 10 mL/kg of body weight, based on the latest body weight.

The dosage levels of HDBB were determined to be 0.1, 0.5, 2.5, or 12.5 mg/kg/day, based on the results of our previous 28-day repeated-dose toxicity study using young rats (Hirata-Koizumi et al., 2007). In this previous study, male and female young rats were given HDBB by gavage at 0.5, 2.5, 12.5, or 62.5 mg/kg/day, and adverse effects, mainly on the liver and heart, were found at all doses in males and at 12.5 mg/kg and above in females.

Observations

All dams were observed daily for clinical signs of toxicity, and body weight was recorded on Days 0, 10, and 20 of pregnancy and on Days 0, 10, 20, and 22 after delivery. On Day 22 after delivery, they were euthanized by exsanguination under deep ether anesthesia, and the surface, organs, and tissues of the entire body were macroscopically observed.

All pups were observed daily before and three to four hours after dosing for clinical signs of toxicity. Body weight was recorded on PNDs 0, 4, 6, 8, 10, 12, 14, 16, 18, 21, and 22. On PND 22, blood was collected from the caudal vena cava in the abdomen of two male and two female pups per litter under deep ether anesthesia. Plasma separated from the blood by centrifugation was examined for total protein, albumin, glucose, total cholesterol, triglycerides, total bilirubin, blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase, calcium, inorganic phosphorus, sodium, potassium, and chlorine. Following the collection of blood, all pups (four males and four females per litter) were euthanized by exsanguination under deep ether anesthesia, and the surface, organs, and tissues of the entire body were macroscopically observed. The heart, lungs, liver, spleen, kidneys, and adrenals were then collected and weighed. The liver and heart were histopathologically examined in one male and one female per litter. The organs were fixed in 10% neutral-buffered formalin, and paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin.

Data Analysis

Body weight, blood biochemical parameters, and organ weights of pups were analyzed by Bartlett's test (Bartlett, 1937) for homogeneity of distribution ($p < 0.01$). When homogeneity was recognized, Dunnett's test (Dunnett, 1964) was conducted to compare between control and individual treatment groups ($p < 0.01$ or 0.05). If not homogenous, data were analyzed using the mean rank test of Dunnett's type (Hollander and Wolfe, 1973) ($p < 0.01$ or 0.05). Histopathological findings were analyzed using Wilcoxon's rank sum test (Wilcoxon, 1945) ($p < 0.01$ or 0.05).

RESULTS

HDBB, orally administered to pups from PNDs 4 to 21, did not induce any clinical signs of toxicity or affect the body weight of maternal rats (data not shown). At necropsy, no gross abnormality was found in the dams.

One male pup each at 0 or 0.5 mg/kg and one female pup each at 0, 0.5, or 12.5 mg/kg died, which was confirmed to be due to gavage error. No substance-related clinical signs of toxicity were found in pups of any groups. There were also no significant changes in the body weight of male and female pups, as shown in Figure 1.

Principle blood biochemical values are summarized in Table 1. In males, the levels of albumin, AST, ALP, and BUN were significantly increased at 12.5 mg/kg. In females, significant increases in the levels of albumin, AST, ALP, and LDH were found at the same dose. There were no substance-related changes in other blood biochemical parameters.

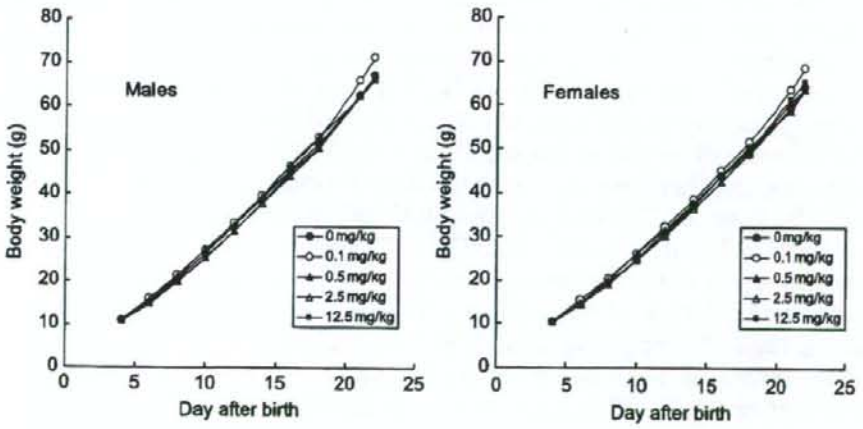

Figure 1: Body weight curves of male and female preweaning rats given HDBB by gavage.

Table 1: Principle blood biochemical values in male and female preweaning rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.1	0.5	2.5	12.5
No. of males	10	10	10	10	10
Total protein (g/dL)	4.49 ± 0.28	4.53 ± 0.22	4.48 ± 0.26	4.43 ± 0.17	4.42 ± 0.18
Albumin (g/dL)	3.62 ± 0.24	3.60 ± 0.24	3.59 ± 0.21	3.74 ± 0.27	4.04 ± 0.17**
BUN (mg/dL)	11.4 ± 1.5	14.1 ± 2.6	13.7 ± 5.3	12.9 ± 1.8	14.7 ± 2.3**
AST (IU/L)	91.4 ± 15.9	85.2 ± 4.8	88.7 ± 5.2	91.6 ± 12.2	100.2 ± 8.5*
ALT (IU/L)	34.8 ± 5.7	34.0 ± 6.3	29.4 ± 5.3	30.7 ± 5.5	35.9 ± 6.1
ALP (IU/L)	1557 ± 203	1529 ± 240	1412 ± 279	1286 ± 249	2054 ± 444**
LDH (IU/L)	198 ± 123	165 ± 16	184 ± 40	236 ± 170	326 ± 221
No. of females	10	10	10	10	10
Total protein (g/dL)	4.49 ± 0.24	4.54 ± 0.24	4.53 ± 0.28	4.55 ± 0.18	4.50 ± 0.14
Albumin (g/dL)	3.59 ± 0.28	3.66 ± 0.24	3.70 ± 0.26	3.80 ± 0.25	4.04 ± 0.16**
BUN (mg/dL)	12.5 ± 2.0	15.4 ± 1.5	13.5 ± 4.0	14.1 ± 4.1	15.5 ± 3.3
AST (IU/L)	87.3 ± 9.4	85.1 ± 8.2	86.5 ± 6.3	85.2 ± 6.6	101.3 ± 9.2**
ALT (IU/L)	30.7 ± 5.9	30.7 ± 3.6	27.1 ± 5.5	27.1 ± 4.5	35.9 ± 4.2
ALP (IU/L)	1470 ± 136	1394 ± 215	1287 ± 105	1339 ± 183	1872 ± 259**
LDH (IU/L)	175 ± 52	176 ± 36	179 ± 35	139 ± 28	370 ± 295*

Values are expressed as the mean ± SD.
BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.
*Significantly different from the control group ($p < 0.05$).
**Significantly different from the control group ($p < 0.01$).

At necropsy, no gross abnormality was observed. Absolute and relative organ weights of scheduled sacrifice animals are shown in Table 2. In males, absolute liver weight at 12.5 mg/kg and relative weight at 2.5 mg/kg and above were significantly increased. In addition, absolute and relative weights of the lungs and spleen were significantly decreased at 12.5 mg/kg. In females, significant increases in absolute liver weight at 12.5 mg/kg and relative liver weight at 2.5 mg/kg and above, and decreases in relative spleen weight and absolute and relative adrenal weight at 12.5 mg/kg, were found. No substance-related changes were detected in other organ weights.

Histopathological findings in the liver are presented in Table 3. In males, nucleolar enlargement, anisokaryosis, and increased mitosis of hepatocytes were observed at 2.5 mg/kg and above. In the 12.5 mg/kg group, hypertrophy of hepatocytes accompanied with eosinophilic granular changes was also observed. Further, increased incidence and/or severity of decreased glycogen in hepatocytes was found at 2.5 mg/kg and above. Similarly, in females, nucleolar enlargement, anisokaryosis, and increased mitosis of hepatocytes at 2.5 mg/kg and above, and hypertrophy and eosinophilic granular change of hepatocytes at 12.5 mg/kg were detected, and the incidence and/or severity of decreased glycogen in hepatocytes was higher at 12.5 mg/kg. No substance-related histopathological changes were detected in the heart in both sexes.

Table 2: Organ weights of male and female preweaning rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.1	0.5	2.5	12.5
No. of males	19	20	19	20	20
Body weight (g)	67.2 ± 7.3	71.3 ± 6.9	67.3 ± 5.8	66.2 ± 9.6	66.2 ± 5.0
Heart (g)	0.37 ± 0.04	0.37 ± 0.04	0.36 ± 0.05	0.36 ± 0.05	0.35 ± 0.04
	(0.55 ± 0.04)	(0.52 ± 0.04)	(0.53 ± 0.05)	(0.54 ± 0.03)	(0.53 ± 0.04)
Lung (g)	0.58 ± 0.07	0.58 ± 0.04	0.53 ± 0.03*	0.59 ± 0.08	0.53 ± 0.04*
	(0.87 ± 0.07)	(0.82 ± 0.09)	(0.80 ± 0.06*)	(0.90 ± 0.09)	(0.80 ± 0.06*)
Liver (g)	2.83 ± 0.47	2.88 ± 0.34	2.75 ± 0.44	3.24 ± 0.68	4.54 ± 0.61**
	(4.19 ± 0.36)	(4.04 ± 0.26)	(4.07 ± 0.42)	(4.87 ± 0.40**)	(6.84 ± 0.53**)
Spleen (g)	0.37 ± 0.09	0.40 ± 0.05	0.34 ± 0.08	0.38 ± 0.77	0.29 ± 0.05**
	(0.55 ± 0.10)	(0.57 ± 0.06)	(0.51 ± 0.10)	(0.57 ± 0.08)	(0.44 ± 0.06**)
Kidneys (g)	0.72 ± 0.09	0.74 ± 0.06	0.72 ± 0.08	0.68 ± 0.10	0.71 ± 0.07
	(1.07 ± 0.07)	(1.04 ± 0.07)	(1.07 ± 0.08)	(1.03 ± 0.05)	(1.07 ± 0.08)
Adrenals (mg)	17.5 ± 3.7	19.3 ± 3.7	18.1 ± 3.3	21.5 ± 5.2*	17.0 ± 2.4
	(26.2 ± 5.1)	(27.3 ± 5.8)	(27.4 ± 5.8)	(32.4 ± 6.8**)	(25.6 ± 3.3)
No. of females	19	20	19	20	19
Body weight (g)	64.0 ± 7.1	68.6 ± 7.5	63.6 ± 4.7	63.6 ± 8.9	65.3 ± 4.1
Heart (g)	0.35 ± 0.05	0.35 ± 0.05	0.33 ± 0.03	0.34 ± 0.05	0.35 ± 0.04
	(0.54 ± 0.04)	(0.51 ± 0.05)	(0.52 ± 0.06)	(0.53 ± 0.04)	(0.53 ± 0.04)
Lung (g)	0.54 ± 0.08	0.54 ± 0.06	0.55 ± 0.06	0.57 ± 0.09	0.51 ± 0.05
	(0.85 ± 0.11)	(0.80 ± 0.09)	(0.86 ± 0.10)	(0.90 ± 0.12)	(0.78 ± 0.06)
Liver (g)	2.72 ± 0.47	2.77 ± 0.41	2.62 ± 0.38	3.01 ± 0.54	4.47 ± 0.39**
	(4.23 ± 0.43)	(4.02 ± 0.24)	(4.12 ± 0.44)	(4.71 ± 0.27*)	(6.84 ± 0.41**)
Spleen (g)	0.36 ± 0.12	0.37 ± 0.06	0.32 ± 0.07	0.33 ± 0.06	0.28 ± 0.07
	(0.55 ± 0.15)	(0.53 ± 0.07)	(0.50 ± 0.10)	(0.52 ± 0.08)	(0.43 ± 0.09*)
Kidneys (g)	0.70 ± 0.07	0.71 ± 0.07	0.67 ± 0.06	0.66 ± 0.09	0.72 ± 0.07
	(1.09 ± 0.05)	(1.04 ± 0.04**)	(1.05 ± 0.05)	(1.04 ± 0.05*)	(1.10 ± 0.07)
Adrenals (mg)	19.2 ± 3.7	18.8 ± 4.5	16.9 ± 2.3	19.9 ± 3.7	15.4 ± 3.5**
	(29.9 ± 4.6)	(27.5 ± 6.8)	(26.8 ± 4.2)	(31.4 ± 5.2)	(23.5 ± 4.8**)

Values are expressed as the mean ± SD.
Values in parentheses are relative organ weights (g or mg/100 g body weight).
*Significantly different from the control group ($p < 0.05$).
**Significantly different from the control group ($p < 0.01$).

Table 3: Histopathological findings in the liver of male and female preweaning rats given HDBB by gavage.

		Dose (mg/kg/day)				
	Grade	0	0.1	0.5	2.5	12.5
No. of males		5	5	5	5	5
Nucleolar enlargement in hepatocytes	±	0	0	0	1	4 **
	+	0	0	0	0	1
Anisokaryosis of hepatocytes	±	0	0	0	1	2 **
	+	0	0	0	0	3
Increased mitosis of hepatocytes	±	0	1	0	2	1
	+	0	0	0	1	3 **
	++	0	0	0	0	1
Hypertrophy of hepatocytes	±	0	0	0	0	4 **
	+	0	0	0	0	1
Eosinophilic granular change of hepatocytes	+	0	0	0	0	5**
Decreased glycogen in hepatocytes	±	1	1	2	4	2 *
	+	0	0	0	0	3
No. of females		5	5	5	5	5
Nucleolar enlargement in hepatocytes	±	0	0	0	2	4 **
	+	0	0	0	0	1
Anisokaryosis of hepatocytes	±	0	0	0	1	3 **
	+	0	0	0	0	2
Increased mitosis of hepatocytes	±	0	1	0	1	1
	+	0	0	0	2	3 **
	++	0	0	0	0	1
Hypertrophy of hepatocytes	±	0	0	0	0	3 **
	+	0	0	0	0	2
Eosinophilic granular change of hepatocytes	±	0	0	0	0	1 **
	+	0	0	0	0	4
Decreased glycogen in hepatocytes	±	1	0	2	2	3 *
	+	0	0	0	0	2

Values represent the number of animals with the finding.
±, very slight; +, slight; ++, moderate.
*Significantly different from the control ($p < 0.05$).
**Significantly different from the control ($p < 0.01$).

DISCUSSION

In the current study, the toxicity of HDBB was investigated in preweaning rats. Based on our previous results of a 28-day repeated-dose toxicity study using young rats (Hirata-Koizumi et al., 2008a), the dosage of HDBB used in this study was sufficiently high to be expected to induce adverse effects on the liver and heart. As expected, increased absolute and/or relative liver weight and histopathological changes of hepatocytes were observed at 2.5 mg/kg and above in both sexes.

Although degeneration and hypertrophy of the myocardium or cell infiltration in the heart were observed at 2.5 mg/kg and above in the previous 28-day study (Hirata-Koizumi et al., 2007), such changes were not detected even at the highest dose of 12.5 mg/kg in the present study. Considering that histopathological changes in the heart were also not found in the previous 52-week study of HDBB using young rats (Hirata-Koizumi et al., 2008a) and a 28-day study using young castrated rats (Hirata-Koizumi et al., 2008b), it could not be concluded that preweaning rats were less susceptible to the cardiac effects of HDBB than young rats. In order to investigate the toxicological effects of HDBB on the heart in more detail, the effects on cardiac function (e.g., electrocardiographic parameters, blood pressure, etc.) should be evaluated because they are considered to be more susceptible parameters than histopathology of the heart (Glaister, 1992).

In the present study, some blood biochemical parameters increased in both sexes in the 12.5 mg/kg group. The degree of change was mostly slight, but it was considered to be HDBB related because similar changes were found in previous studies of HDBB (Hirata-Koizumi et al., 2007, 2008a, 2008b). A simultaneous increase in hepatic enzymes (AST, ALP, and LDH) might result from hepatic damage caused by HDBB. Increased BUN suggests renal effects of HDBB, although histopathology of the kidneys was not examined in the present study. As a matter of fact, hypertrophy of the tubular epithelium was noted at 12.5 mg/kg and above in males and at 62.5 mg/kg in females in the previous 28-day study of HDBB using young rats (Hirata-Koizumi et al., 2007).

No effects on the lungs, spleen, and adrenals were found both in previous 28-day and 52-week studies of HDBB using young rats (Hirata-Koizumi et al., 2007, 2008a), whereas decreased weight of these organs was found in preweaning rats given HDBB. In rats, many organs develop rapidly during the early period after birth (Vidair, 2004; Walthall et al., 2005; Zoetis and Hurtt, 2005a). For example, rat lungs have no alveoli at birth, but they develop rapidly, with most lung development complete within the first two weeks after birth (Zoetis and Hurtt, 2005b). It is conceivable that immature and/or rapidly developing organs show different susceptibility from mature organs. Considering these findings together suggests that HDBB might influence these organs, specifically in the preweaning period. Further studies are required to investigate the adverse effects of HDBB on the lungs, spleen, and adrenals during the preweaning period.

Histopathological changes in the liver detected in the current study included nucleolar enlargement, anisokaryosis, increased mitosis, and hypertrophy of hepatocytes. Nucleolar enlargement of hepatocytes indicates the enhancement of protein synthesis and is identified most frequently in hepatocytes that are undergoing rapid cell proliferation (Cattley and Popp, 2002). Anisokaryosis is also considered to correlate at least partly with cell

proliferation. In the present study, nucleolar enlargement, anisokaryosis, and increased mitosis of hepatocytes were observed at 2.5 mg/kg and above in both sexes, whereas hypertrophy of hepatocytes was observed only at the highest dose of 12.5 mg/kg. On the other hand, in the previous 28-day study of HDBB using young rats, hypertrophy of hepatocytes was observed at 0.5 mg/kg and above in males and 12.5 mg/kg and above in females, and increased mitosis of hepatocytes was observed at 62.5 mg/kg and 12.5 mg/kg and above in males and females, respectively, indicating that young rats are more susceptible to the HDBB-induced hypertrophic response of hepatocytes than the mitotic response (Hirata-Koizumi et al., 2007). The higher susceptibility of preweaning rats to such proliferative changes might be associated with dramatic changes of the liver structure during the preweaning period (Alexander et al., 1997).

In previous studies using young rats (five to six weeks of age), we showed that male rats were much more susceptible to the toxic effects of HDBB than females (Hirata-Koizumi et al., 2007, 2008a). Based on histopathological findings in the liver, which is a major target of HDBB toxicity, differences in susceptibility between sexes was approximately 25 times. Subsequently, we showed that castration markedly reduced the gender-related differences in HDBB hepatotoxicity in rats (Hirata-Koizumi et al., 2008b). Comparing the histopathological findings of the liver observed in the previous 28-day studies using young intact and castrated rats, it became clear that the castration of male rats exerted no effect but that of female rats enhanced the adverse effects of HDBB on the liver, suggesting suppressive effects of estrogen on the hepatotoxicity of HDBB in rats. Despite the marked reduction of gender-related differences in the toxic effects of HDBB by castration, a difference, less than five times, remained in castrated rats. The sexual differences in castrated rats are considered to be due to the exposure to sexual hormones before four weeks of age, when castration was conducted. In the present study, following the administration of HDBB during the preweaning period, similar changes in all examined parameters were observed at the same doses in both sexes. These findings clearly show no gender-related differences in HDBB toxicity in preweaning rats, suggesting that a development at around three to six weeks of age contributes to sexual variations in HDBB toxicity, at least in part.

Gender-related differences in HDBB toxicity were found not only for hepatotoxicity, but also for the reduction of body weight, hematotoxicity, cardiac toxicity, etc., in the previous 28-day and/or 52-week studies using young rats (Hirata-Koizumi et al., 2007, 2008a). Thus, they might be caused by differences in the blood concentration of causative substances (e.g., HDBB or its metabolites) between sexes. A number of reports have been published on the sexual variations in toxicokinetic determinants, such as hepatic metabolism (Gad, 2006) and membrane transporter in various organs, including the kidneys and intestine (Morris et al., 2003). Coleman et al. (1990) reported that

higher sensitivity of male rats to hematotoxicity of dapsone, which is a major component of the multidrug regimen for the treatment of leprosy, was due to the greater capacity for the N-hydroxylation. Another example was an amino acid antitumor agent, acivicin, of which the LD_{50} was much higher in male mice than that in females. McGovren et al. (1981) showed that the plasma half-time was much longer in female mice and speculated that the sexual variation may be related to differences in the renal excretion.

For gender-related differences in toxicokinetic determinants, many mechanistic studies have been reported on the metabolic enzyme cytochrome P450 (CYP) (Waxman and Chang, 2005). In rats, a subset of CYPs is expressed in a sex-dependent fashion. It was reported that ovariectomy reduced the hepatic expression of female-specific/predominant CYPs, but this did not lead to the expression of male-specific CYP enzyme in female rats. If female-specific/predominant metabolic enzymes have an intimate involvement in the detoxication of HDBB, our previous results, showing the higher susceptibility of male young rats to HDBB toxicity than females, and increased susceptibility by castration of females, could be explained. Interestingly, in rat liver, the difference in CYP expression between sexes is not apparent until puberty (Waxman and Chang, 2005). This is consistent with our present results that there was no gender-related difference in HDBB hepatotoxicity in preweaning rats. Mode and Gustafsson (2006) reported that brain centers involved in the hypothalamo-pituitary control of hepatic sex-dependent metabolism in adults are irreversibly programmed by neonatal androgen exposure, which might explain why sexual variation in HDBB toxicity was not completely abolished by castration at four weeks of age.

In order to clarify the cause of gender differences, we are currently performing a toxicokinetic study of HDBB, which includes the identification of metabolites and the related metabolic enzyme as well as measurement of the blood concentration of HDBB both after single and repeated administration of HDBB to young and preweaning rats.

CONCLUSION

The current results showed that oral administration of HDBB to preweaning rats caused hepatotoxicity at 2.5 mg/kg and above in both sexes. The gender-related difference in toxic susceptibility to HDBB, which was observed in young rats, was not detected in preweaning rats.

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