(2000 Hz, 25 dB) as the conditioned stimulus (CS) for 5 s. A rat crossing to the opposite side of the shuttle box during the buzzer period would successfully avoid the electric shock (3 mA) that followed the buzzer. If the rat had not yet crossed to the opposite compartment of the shuttle box after the 5 s buzzer period, an electric shock was applied for 10 s, as the unconditioned stimulus. A 30 s intertrial interval preceded the next presentation of the CS. Each rat was tested for 60 min a day for three consecutive days. The rate of successfully conditioned responses for every 10 and 60 min was calculated.

One male (4m) and one female (4f) pups selected from each dam were maintained as reserve animals for replacements or additional tests.

2.4.3. Necropsy of offspring

Humane sacrifice was performed on PND 22 for pups (1m and 1f), on PND 70 for pups (2m, 3m, 2f and 3f), and on PNDs 103–126 for pups (4m and 4f) of each dam. The external surfaces of pups were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. For histopathological examinations, half of pups in each group (10–11/sex/group) killed on PNDs 22 or 70 were perfused with heparinized phosphate-buffered solution and paraformaldehyde-phosphate buffered solution, and the brain, spinal cord in the thoracic and lumbar regions, and sciatic nerve were removed and stored in 10% neutral buffered formalin. Histopathological evaluations were performed on the cerebrum, cerebellum, medulla oblongata, pons, spinal cord and sciatic nerve of male and female pups in the control and highest dose groups after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin. The remaining pups (8–11/sex/group) killed on PNDs 22 or 70 were subjected to weighing of the brain, liver, kidney, spleen and adrenal.

2.5. Statistical analysis

Statistical analysis of offspring before weaning was carried out using the litter as the experimental unit. The initial body weight, body weight gain and food and water consumptions of maternal rats, numbers of implantations and pups per litter, organ weight, pup weight, day of completion of developmental landmarks, latency of reflex response, distance traveled by pups, rate of avoidance response were analyzed with Bartlett's test [10] for homogeneity of variance at the 5% level of significance. If it was homogeneous, the data were analyzed using Dunnett's multiple comparison test [11] to compare the mean of the control group with that of each dosage group, and if it was not homogeneous, the mean rank of the PS80-treated groups was compared with that of the control group with the Dunnett-type test which was used for the gestation length, delivery index, incidence of pups with malformations, viability index of pups and rate of pups that completed reflex responses to compare the mean rank of groups treated with PS80 and the control group. The fecundity index and gestation index were analyzed by chi-square test. The 0.05 level of probability was used as the criterion for significance.

3. Results

3.1. Findings in dams

No feces were found in one female on days 20–21 of pregnancy and on day 0 of lactation at 7.5%. During lactation, loose stools in 18 females on days 2–21, and scattering of offspring on days 0–1, scant or no feces on days 1–2, reddish brown soiled perianal region on days 12 and 20 and death of all offspring on day 2 in one female each were observed at 7.5%. No clinical signs of toxicity were noted at 1.0% and below (data not shown).

The body weights of maternal rats during pregnancy and lactation are shown in Fig. 1. A significantly lower body weight was observed on days 3, 10, 12–20 of pregnancy and days 0, 2–18 of lactation at 7.5%. In this group, body weight gain was also significantly decreased during pregnancy.

A significant decrease in food consumption on all measuring days during pregnancy and lactation was noted at 7.5%. No

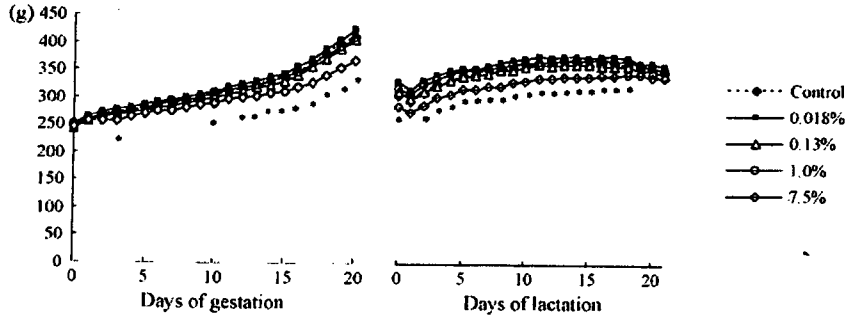

Fig. 1. Body weight of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

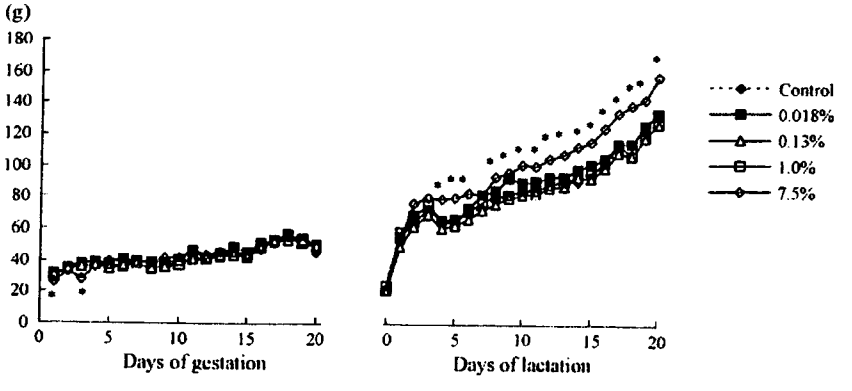

Fig. 2. Water consumption of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

significant changes in food consumption were found at 1.0% and below (data not shown).

The water consumption of maternal rats during pregnancy and lactation are shown in Fig. 2. At 7.5%, water consumption was significantly decreased on days 1 and 3 of pregnancy and increased on days 5–7 and 9–21 of lactation. No significant changes in water consumption were found at 1.0% and below. The least water consumption was observed on day 0 of lactation and the most water consumption was observed on day 21 of lactation in all groups.

The average daily intakes of PS80 during pregnancy were 0.024, 0.171, 1.314 and 10.576 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The average daily intake of PS80 during lactation were 0.046, 0.321, 2.521 and 23.908 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The average daily intakes of PS80 throughout the administration period were 0.035, 0.245, 1.864 and 16.783 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The least intake of PS80 was noted on day 0 of lactation and the most intake of PS80 was noted on day 21 of lactation in all groups. The ranges of average daily intakes of PS80 based on each day of the administration period were 0.012–0.071, 0.093–0.499, 0.837–3.765 and 5.869–36.431 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively.

At necropsy of dams, dilatation of the cecum in seven females and a significant increase in the relative weight, but not in the absolute weight, of the kidney, were observed at 7.5%. No changes in gross pathology or in absolute and relative weights were detected in any organs at 1.0% and below (data not shown).

3.2. Reproductive/developmental findings

The reproductive findings in maternal rats are presented in Table 1. One female at 7.5% showed total litter loss by day 2 of lactation, one female at 0.018 and 1.0% and two females at 0.13% were not impregnated, and one pregnant female at 1.0%, died on day 22 of pregnancy; however, no significant differences were noted in the fecundity index, gestation index and length of gestation between control and PS80-treated groups.

The developmental findings are shown in Table 2. The number of pups born was significantly reduced at 7.5%. There were no significant effects of treatment of PS80 on the numbers of implantations, pups born alive and dead pups, delivery index and sex ratio of pups and the viability index of pups before weaning. A fetus with acaudate and anal atresia was observed at 0.018%, but no fetuses with external malformations were found in other groups. Although no significant changes in the body weight of male and female pups were observed on PNDs 0, 4 and 7 in PS80-treated groups, significantly reduced body weights were noted on PNDs 14 and 21 at 7.5%. No PS80-related clinical signs of toxicity were found during the pre-weaning period.

The body weights after weaning in male and female offspring of rats given PS80 during pregnancy and lactation are shown in Table 3. At 7.5%, a significantly reduced body weight was noted on PNDs 28, 35, 42, 49 and 56 in males and on PNDs 28 and 35 in females.

One male at 1.0% died on PND 23; however, there were no compound-related clinical signs of toxicity or adverse effects on the survival rate in male and female weaned rats (data not shown).

3.3. Developmental landmarks in offspring

Physical development in male and female pups is presented in Table 4. There was no significant difference in the age of male and female pups that displayed pinna unfolding, fur appearance, incisor eruption, or eye opening. Body weight at the age of fur appearance in males and eye opening in both sexes was significantly reduced at 7.5%.

Data on sexual development in male and female pups is shown in Table 5. No significant differences in age at preputial separation in males or vaginal opening in females, or body weight at the age of preputial separation or vaginal opening were found between control and PS80-treated groups.

Examination of reflex ontogeny revealed no significant difference between control and PS80-treated groups in the latency of response, i.e., the time taken by the subject to complete reflex response, or the incidence of pups completing reflex response. All male and female pups in all groups, except for one male pup at 1.0%, showed completion of the reflex response when testing surface righting reflex on PND 5 and negative geotaxis reflex on

Table 1
Reproductive findings in rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of females copulated	22	22	22	22	22
No. of pregnant females	22	21	20	21	22
No. of non-pregnant females	0	1	2	1	0
Fecundity index (%)[a]	100	95.5	90.9	95.5	100
No. of deaths during pregnancy	0	0	0	1	0
Gestation length (days)[b]	21.9 ± 0.4	21.8 ± 0.4	21.9 ± 0.4	21.9 ± 0.4	21.7 ± 0.5
No. of females with live born	22	21	20	20	22
Gestation index (%)[c]	100	100	100	95.2	100
No. of females with totally litter loss	0	0	0	0	1

[a] Fecundity index (%) = (no. of pregnant females/no. of females confirmed mating) × 100.
[b] Values are given as the mean ± S.D.
[c] Gestation index (%) = (no. of females with live pups born/no. of pregnant females) × 100.

Table 2
Developmental findings in rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of litters	22	21	20	21[a]	22
No. of implantations[b]	16.1 ± 1.7	15.4 ± 2.7	15.1 ± 1.6	15.8 ± 1.4	15.1 ± 2.1
Total no. of pups born[b]	15.4 ± 1.8	14.8 ± 2.4	14.2 ± 2.1	14.6 ± 1.2	13.9 ± 1.7*
No. of pups born alive[b]	14.7 ± 1.8	14.6 ± 2.3	13.7 ± 2.0	14.5 ± 1.2	13.6 ± 2.0
No. of dead pups[b]	0.7 ± 1.2	0.1 ± 0.4	0.5 ± 0.7	0.2 ± 0.7	0.3 ± 0.8
Delivery index (%)[c]	95.8	96.0	94.1	92.9	92.8
Proportion of male pups (%)[d]	54.2	49.2	53.6	47.7	44.7
Viability index before weaning (%)					
Postnatal day 0[e]	95.5	99.1	96.6	99.0	97.6
Postnatal day 4[f]	98.6	98.2	98.5	98.7	93.8
Postnatal day 21[g]	100	100	100	100	100
Body weight of male pups before weaning (g)[h]					
Postnatal day 0	6.4 ± 0.5	6.5 ± 0.5	6.5 ± 0.5	6.5 ± 0.4	6.1 ± 0.8
Postnatal day 4	10.1 ± 1.0	10.6 ± 1.3	10.4 ± 1.3	10.4 ± 0.8	9.8 ± 1.1
Postnatal day 7	16.9 ± 1.4	17.6 ± 1.5	17.1 ± 1.9	17.4 ± 1.1	16.2 ± 1.8
Postnatal day 14	35.3 ± 1.6	36.5 ± 2.4	34.8 ± 2.5	35.6 ± 1.8	32.4 ± 3.3*
Postnatal day 21	58.0 ± 3.6	59.0 ± 4.1	57.0 ± 3.5	57.1 ± 3.0	50.4 ± 5.1*
Body weight of female pups before weaning (g)[h]					
Postnatal day 0	6.0 ± 0.5	6.2 ± 0.5	6.2 ± 0.5	6.1 ± 0.3	5.8 ± 0.7
Postnatal day 4	9.6 ± 1.0	10.2 ± 1.1	9.9 ± 1.3	9.9 ± 0.8	9.5 ± 1.0
Postnatal day 7	16.1 ± 1.4	17.0 ± 1.6	16.3 ± 1.9	16.6 ± 1.1	15.7 ± 1.8
Postnatal day 14	34.0 ± 1.9	35.2 ± 2.3	33.5 ± 2.3	34.4 ± 1.4	31.5 ± 3.1*
Postnatal day 21	55.0 ± 3.7	56.4 ± 4.0	54.5 ± 3.1	55.1 ± 2.0	49.2 ± 4.7*
External examination of pups[h]					
No. of pups (litters) examined	339 (22)	310 (21)	284 (20)	278 (19)	306 (22)
No. of pups with malformations	0	1[i]	0	0	0

[a] One female, who delivered four live pups on day 23 of pregnancy, was euthanized on day 0 after delivery, and her data were excluded.
[b] Values are expressed as the mean ± S.D.
[c] Delivery index (%) = (no. of pups born/no. of implantations) × 100.
[d] Proportion of male pups = (no. of male pups/total no. of pups) × 100.
[e] Viability index on postnatal day 0 (%) = (no. of pups born alive/total no. of pups born) × 100.
[f] Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of pup born alive) × 100.
[g] Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live on postnatal day 4 after cull) × 100.
[h] External examinations were performed on all pups born on postnatal day 0.
[i] One live pup had acaudate and anal atresia.
* Significantly different from the control, $p < 0.05$.

PND 8. As for the sensory function of offspring, all male and female pups in all groups completed pupillary reflex, Preyer's reflex, pain response and mid-air righting reflex when tested on PNDs 23–26 and 62–64 (data not shown).

3.4. Locomotor activity in offspring

Locomotor activity of male and female pups during the pre-weaning period is presented in Fig. 3. No significant differences in the distance traveled by male and female pups during the nocturnal period (20:00 and 2:00) and diurnal period (8:00 and 14:00) were found between control and PS80-treated groups when locomotor activity was determined on PNDs 14–15. Although a significantly higher activity was observed in male pups in the 1.0% group at 2:00 on PND 18, no significant changes in activity were noted in males and females at any other test time on PNDs 17–18. There were no significant differences between control and PS80-treated groups in locomotor activity of male and female offspring at any test time on PNDs 20–21.

After weaning, no significant differences in the distance traveled by male and female offspring were detected at any test time when activity was determined on PNDs 33–37 and PNDs 60–66 (data not shown).

3.5. Conditioned avoidance response in offspring

The rate of successfully conditioned responses for every 10 min test period on PNDs 23–27 is presented in Fig. 4. On the first day of the test, the rate of successful responses for 60 min was lower in males and females at 7.5%, and a significantly decreased rate was noted in males during the last 10 min and in females during the first and third 10 min test periods. However, there were no significant changes in the rate of successful responses in any 10 min test periods in males and females of any PS80-treated groups on the second- and third-day of the test. No significant changes in the total rate of successfully responses for 60 min were found in male and female pups in any PS80-treated groups on any test days.

Table 3
Body weight after weaning in offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of male offspring	63	61	60	55	60
Body weight of male offspring (g)[a]					
Postnatal day 28	101 ± 7	102 ± 8	98 ± 6	100 ± 6	92 ± 9**
Postnatal day 35	161 ± 12	163 ± 13	157 ± 10	160 ± 10	151 ± 13**
Postnatal day 42	225 ± 15	230 ± 16	221 ± 14	225 ± 14	217 ± 15**
Postnatal day 49	289 ± 20	297 ± 20	285 ± 18	289 ± 18	281 ± 17*
Postnatal day 56	346 ± 24	356 ± 24	342 ± 22	345 ± 24	336 ± 22*
Postnatal day 63	390 ± 27	400 ± 27	384 ± 26	388 ± 27	379 ± 25
Postnatal day 70	424 ± 32	436 ± 30	418 ± 30	422 ± 32	414 ± 33
No. of female offspring	63	62	58	57	63
Body weight of female offspring (g)[a]					
Postnatal day 28	90 ± 7	93 ± 6	90 ± 5	90 ± 5	85 ± 7**
Postnatal day 35	135 ± 9	138 ± 11	135 ± 9	135 ± 7	130 ± 10*
Postnatal day 42	171 ± 13	175 ± 13	172 ± 12	171 ± 10	168 ± 13
Postnatal day 49	196 ± 14	204 ± 15*	200 ± 15	199 ± 12	196 ± 15
Postnatal day 56	222 ± 17	230 ± 17*	226 ± 17	222 ± 14	221 ± 18
Postnatal day 63	239 ± 19	248 ± 19*	246 ± 22	243 ± 17	241 ± 19
Postnatal day 70	254 ± 20	264 ± 22*	262 ± 22	258 ± 18	257 ± 21

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

Table 4
Physical development in offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No of litters examined	22	21	20	19	21
Age at pinna unfolding (days)[a]					
Male	2.9 ± 0.6	3.0 ± 0.5	3.0 ± 0.5	2.9 ± 0.7	3.1 ± 0.5
Female	2.8 ± 0.5	2.9 ± 0.5	3.0 ± 0.5	3.1 ± 0.6	3.1 ± 0.6
Body weight at pinna unfolding (g)[a]					
Male	8.6 ± 0.7	9.3 ± 1.3	9.0 ± 1.0	8.9 ± 0.5	8.8 ± 1.0
Female	8.1 ± 0.8	8.8 ± 1.0	8.6 ± 1.0	8.5 ± 0.5	8.4 ± 0.9
Age at fur appearance (days)[a]					
Male	9.0 ± 0.4	9.0 ± 0.4	9.0 ± 0.6	9.1 ± 0.5	9.0 ± 0.5
Female	9.1 ± 0.4	9.1 ± 0.4	9.2 ± 0.6	9.2 ± 0.5	9.1 ± 0.6
Body weight at fur appearance (g)[a]					
Male	22.2 ± 1.5	23.2 ± 1.8	21.9 ± 1.8	22.5 ± 1.6	20.9 ± 2.2*
Female	21.5 ± 1.5	22.3 ± 1.7	21.2 ± 1.8	22.0 ± 1.5	20.5 ± 2.3
Age at incisor eruption (days)[a]					
Male	10.0 ± 0.8	10.1 ± 0.5	10.1 ± 0.5	10.1 ± 0.7	10.1 ± 0.9
Female	10.0 ± 0.6	10.0 ± 0.7	10.1 ± 0.4	9.9 ± 0.6	10.0 ± 1.0
Body weight at incisor eruption (g)[a]					
Male	24.6 ± 2.2	25.7 ± 1.6	24.4 ± 2.5	25.0 ± 1.8	23.1 ± 3.2
Female	23.5 ± 1.6	24.3 ± 1.7	23.6 ± 2.5	24.0 ± 1.3	22.4 ± 3.0
Age at eye opening (days)[a]					
Male	15.3 ± 0.5	15.2 ± 0.7	15.3 ± 0.5	15.4 ± 0.7	15.3 ± 0.6
Female	15.4 ± 0.5	15.2 ± 0.5	15.2 ± 0.5	15.3 ± 0.6	15.2 ± 0.6
Body weight at eye opening (g)[a]					
Male	37.9 ± 1.6	39.4 ± 2.4	37.7 ± 2.6	38.5 ± 2.0	35.1 ± 3.1*
Female	36.9 ± 1.6	37.6 ± 2.0	36.0 ± 2.1	37.3 ± 1.7	34.1 ± 3.1*

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

Table 5
Sexual maturation of offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
Male preputial separation					
No. of male pups examined	22	21	20	18	21
Age (days)[a]	40.5 ± 1.5	40.5 ± 1.2	40.9 ± 1.3	40.3 ± 1.1	41.0 ± 1.3
Body weight (g)[a]	206 ± 32	205 ± 24	207 ± 22	205 ± 25	202 ± 28
Female vaginal opening					
No. of female pups examined	22	21	20	19	21
Age (days)[a]	33.3 ± 1.9	33.6 ± 1.6	33.9 ± 1.4	32.8 ± 0.9	33.4 ± 2.2
Body weight (g)[a]	124 ± 10	130 ± 14	127 ± 9	121 ± 9	121 ± 16

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

The rate of successfully conditioned responses for every 10 min test period on PNDs 60–67 is shown in Fig. 5. No significant changes were found in males and females of any PS80-treated groups when conditioned avoidance responses were determined on PNDs 60–67.

3.6. Necropsy and histopathology in offspring

There were no compound-related gross lesions in males and females at necropsy on PND 22. Table 6 shows absolute and relative organ weights on postnatal day 22 in male and female offspring. There were no significant differences in absolute and relative weights of the brain, liver, spleen, adrenal or kidney in male and female pups between control and PS80-treated groups.

No histopathological changes in the cerebrum, cerebellum, medulla oblongata, pons, spinal cord in the thoracic and lumbar regions, and sciatic nerve were noted in 22-day-old males and females of the control and 7.5% groups (data not shown).

No compound-related gross lesions were found in males and females at necropsy on PND 70 and on PNDs 103–126. There were no significant differences in the absolute and relative weights of the brain, liver, spleen, adrenal or kidney in 70-day-old male and female pups between control and PS80-treated groups. Although slight mononuclear cell infiltration in the choroid plexus was observed in the cerebrum in one male in the control group, no other histopathological changes in the cerebrum, cerebellum, medulla oblongata, pons, spinal cord in the thoracic and lumbar regions, and sciatic nerve were found in 70-day-old males and females of control and 7.5% groups (data not shown).

4. Discussion

A developmental neurotoxicity study was performed to evaluate the potential functional and morphological effects of PS80 on the developing nervous system of offspring of rats given PS80 during pregnancy and lactation. This study was designed to assess both continuous parameters, such as body weight and food and water consumption, and parameters at specific times, pre-weaning, adolescence and young adult periods, such as physical development, reflex ontogeny, sexual maturation, motor activity, motor and sensory function, learning, and pathological findings, and to further assess reproductive and developmental endpoints.

In the present study, loose stool during lactation was observed in many dams given drinking water containing PS80 at 7.5%. In a previous 2-year breeding study, diarrhea was observed in rats

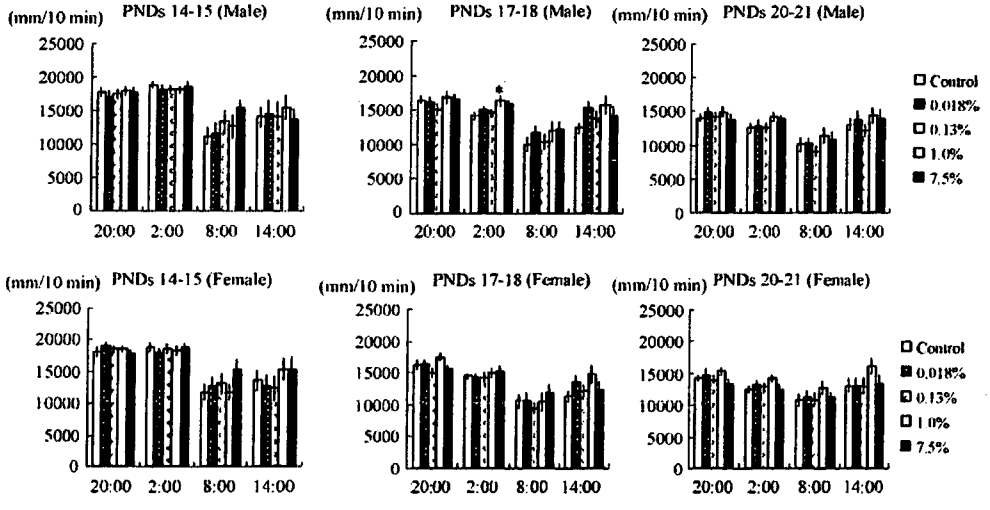

Fig. 3. Locomotor activity in pre-weaning offspring of rats given polysorbate 80 during pregnancy and lactation. Values are given as the mean ± S.E.M. *Significantly different from the control, $p < 0.05$.

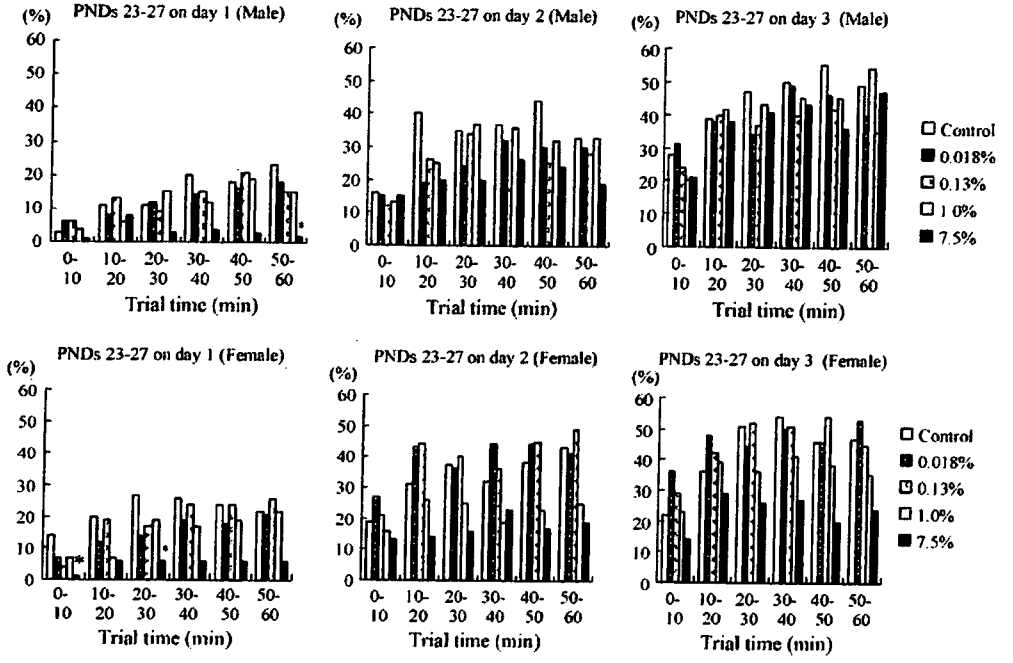

Fig. 4. The rate of successful responses in conditioned avoidance test on postnatal days 23–27 in offspring of rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

fed a diet containing PS80 at 10% and higher [12]. The diarrhea observed in feeding studies with polysorbates seems to result from having high concentrations of the unabsorbed poloxyethylene sorbitan moiety within the intestinal lumen [13,14]. The decrease in body weight and body weight gain accompanied by decreased food and water consumption was also noted at 7.5%; however, no significant findings in clinical observations, body weight, body weight gain, or food and water consumption were detected at 1.0% and below. Reduced water consumption may be due to slight characteristic scent and unpleasant and slightly bitter taste of PS80 [15]. However, lower water consumption was noted only on 2 days at the highest dose. These findings do not indicate poor palatability of PS80 in water and dose–dependent taste aversion. PS80 seems to be dosed successfully by this route. Dilatation of the cecum was also observed at 7.5%. Although increased relative weight of the kidney was

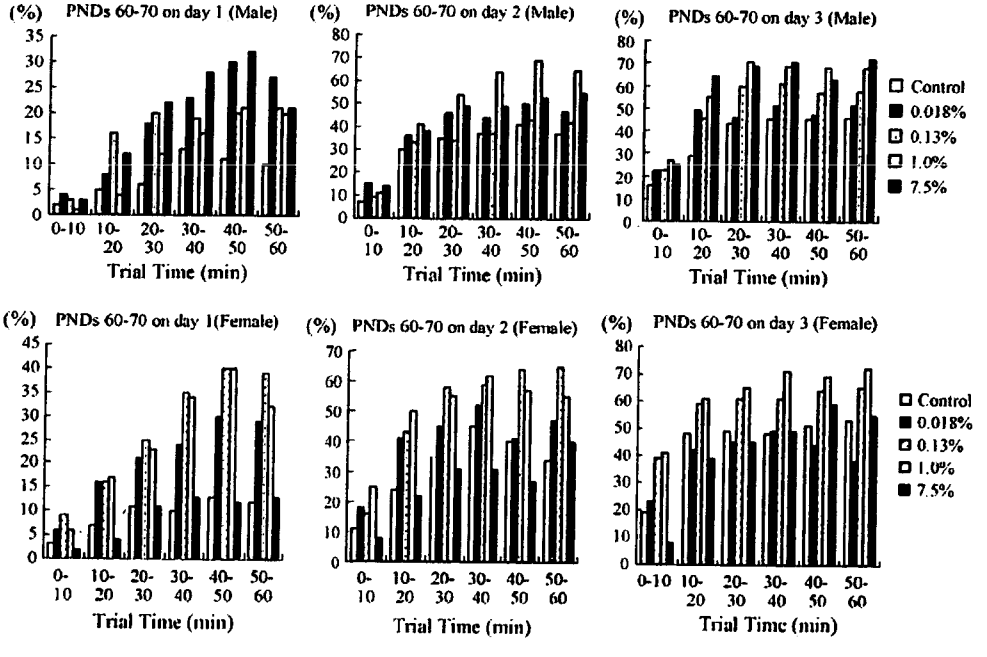

Fig. 5. The rate of successful responses in conditioned avoidance test on postnatal days 60–70 in offspring of rats given polysorbate 80 during pregnancy and lactation.

Table 6
Absolute and relative organ weights on postnatal day 22 in male and female offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of male pups	11	11	11	9	10
Body weight (g)[a]	58.9 ± 6.8	61.1 ± 5.1	60.3 ± 4.2	62.2 ± 3.6	55.0 ± 6.3
Brain (g)[a]	1.49 ± 0.05[b]	1.55 ± 0.06	1.53 ± 0.06	1.53 ± 0.05	1.49 ± 0.07
	2.56 ± 0.29[c]	2.55 ± 0.18	2.54 ± 0.18	2.47 ± 0.17	2.75 ± 0.33
Liver (g)[a]	2.45 ± 0.43	2.38 ± 0.38	2.25 ± 0.23	2.44 ± 0.21	2.21 ± 0.38
	4.18 ± 0.77[c]	3.88 ± 0.37	3.74 ± 0.29	3.92 ± 0.23	3.99 ± 0.33
Spleen (mg)[a]	266 ± 57	308 ± 65	302 ± 52	290 ± 32	274 ± 45
	449 ± 59[d]	502 ± 91	500 ± 66	466 ± 35	495 ± 37
Adrenal (mg)[a]	21 ± 4	23 ± 3	22 ± 5	22 ± 4	21 ± 4
	35.5 ± 5.7[d]	38.0 ± 4.8	36.8 ± 8.1	35.2 ± 6.6	38.9 ± 9.5
Kidney (mg)[a]	720 ± 97	746 ± 80	725 ± 61	738 ± 49	666 ± 53
	1222 ± 84[d]	1219 ± 70	1202 ± 45	1187 ± 76	1218 ± 72
No. of female pups	11	11	11	9	10
Body weight (g)[a]	55.2 ± 6.3	56.5 ± 6.0	55.8 ± 3.9	57.5 ± 4.3	52.4 ± 5.6
Brain (g)[a]	1.45 ± 0.05[b]	1.47 ± 0.06	1.45 ± 0.06	1.46 ± 0.04	1.43 ± 0.08
	2.65 ± 0.28[c]	2.63 ± 0.29	2.61 ± 0.21	2.54 ± 0.16	2.75 ± 0.25
Liver (g)[a]	2.09 ± 0.38	2.15 ± 0.34	2.21 ± 0.43	2.23 ± 0.21	2.07 ± 0.27
	3.77 ± 0.35[c]	3.78 ± 0.26	3.99 ± 0.86	3.87 ± 0.14	3.95 ± 0.25
Spleen (mg)[a]	263 ± 44	295 ± 56	266 ± 30	265 ± 51	262 ± 36
	479 ± 80[d]	519 ± 75	477 ± 40	458 ± 64	501 ± 50
Adrenal (mg)[a]	23 ± 4	21 ± 4	22 ± 5	22 ± 6	19 ± 2
	41.5 ± 6.6[d]	37.7 ± 7.2	39.0 ± 8.8	38.6 ± 10.1	36.4 ± 5.5
Kidney (mg)[a]	717 ± 90	724 ± 72	711 ± 65	726 ± 66	676 ± 52
	1299 ± 92[d]	1282 ± 57	1277 ± 99	1262 ± 82	1294 ± 74

[a] Values are expressed as the mean ± S.D.
[b] Absolute organ weight.
[c] Relative organ weight = organ weight (g)/100 g body weight.
[d] Relative organ weight = organ weight (mg)/100 g body weight.

observed at 7.5%, this change was not thought to have toxicological meaning because of no changes in the gross pathology or in absolute weight. These findings indicate that the NOAEL for general toxicity in maternal rats was 1.0% (1.864 ml/kg bw/day).

In previous studies, the reproductive and developmental effects of PS80 were investigated in rats and mice given relatively high doses of PS80. No adverse effects on reproductive and developmental outcome were noted in rats fed a diet containing PS80 at 2% through three generations [16]. Fertility and offspring survival were diminished in a 2-year breeding study using rats fed a diet containing PS80 at 20%, but not at 5 or 10% [17]. A prenatal developmental toxicity study revealed no clear adverse effects in dams and fetuses of rats given PS80 at 500 and 5000 mg/kg bw/day by gavage on days 6–15 of pregnancy [18]. Administration of PS80 at 2500 mg/kg bw/day by gavage on days 8–12 of pregnancy caused no adverse effects on dams or offspring of mice [19]. In these previous studies, no detailed information on reproductive and developmental parameters was reported. Although a few females showed reproductive difficulties in PS80-treated groups in the present study, necropsy of the reproductive organs revealed no evidence of reproductive failure in these rats. No changes in the fecundity index, gestation length or gestation index were noted in any PS80-treated groups; however, the number of pups born was significantly decreased at 7.5%. One possible explanation for this decrease may be the slight decrease in the number of implantations. It is not known whether the decreased number of implantations was attributable to a decreased number of corpora lutea or an increase of number of pre-implantation embryonic loss, because the dams were sacrificed 21 days after delivery and the number of corpora lutea was not determined in the present study. No information on the adverse effects of PS80 on formation of the corpora lutea, implantation process and pre-implantation embryonic loss is available in previous reproductive and developmental toxicity studies of PS80 [16–19]. In the present study, acaudate and anal atresia were found in one pup at 0.018%; however, the incidence of malformations was very low, not dose-related and not significantly different from that in the control group. The external malformations observed in the present study were of the types that occur spontaneously among control rat fetuses reported in the literature [20–23]; therefore, it seems unlikely that the morphological changes in pups observed in the present

study indicate a teratogenic response. Maternal administration of PS80 at 7.5% caused the low body weight of male and female offspring during the pre-weaning period, and these changes were accompanied with the decreased weight of maternal rats. Body weight of offspring during the post-weaning period was also lower at 7.5%. The effect on pup weight showed up later but may actually have been present at birth in the 7.5% group, because the smaller number of litter mates per dam might have heavier body weight of pups in this group. These findings indicate that the dose level of 7.5% used in this study was potent enough to have adverse effects on growth of the offspring.

In the present study, the body weights at the age of completed fur appearance in male pups and eye opening in male and female pups were reduced at 7.5%; however, no significant changes were found in the age of completed these developmental landmarks. No changes were detected in reflex ontogeny and sensory function in male and female offspring given PS80. In addition, PS80 did not cause any changes in indicators of the onset of sexual maturity. It seems unlikely that PS80 affects the functional development and sexual maturation of offspring.

In the previous developmental neurotoxicity study of PS80 [6], daily locomotor activity and diurnal locomotor activity were increased in male offspring of rats given PS80 at 0.125% in their drinking water. The locomotor activity of male pups was determined during the pre-weaning period using a cage consisting of two sections, a home cage section with exploration holes that allowed movement of the pups back and forth to a second section, the exploration cage while restricting the movement of the dam to the home cage [24]. The OECD Draft Test Guideline 426 Developmental Neurotoxicity Study [7] noted that motor activity should be monitored during the pre-, peri- and post-weaning periods, including the young adult period, by an automated activity recording apparatus and that the animals should be tested individually. Monitoring nocturnal activity in rodents is important for toxicological studies [25,26], because rodents are more active during the nocturnal period [27–29] and neurotoxicants may be more effective during this period. In the present study, the motor activity of pups was individually determined during diurnal and nocturnal periods in the pre-, and peri-weaning, and young adult periods using automated activity recording apparatus. Although higher activity was detected in male pups at 2:00 on PND 18 in the 1.0% group, this change was discontinuous, inconsistent across sexes and not dose-related; therefore, this change was not thought to be due to the administration of PS80 and had no toxicological significance. No changes in locomotor activity were observed in PS80-treated pups of both sexes during other test periods. These findings indicate that PS80 is ineffective on locomotor activity in male and female pups of rats fed this compound during pregnancy and lactation.

Although a decreased rate of successfully conditioned avoidance responses was found at 7.5% in males and females on PNDs 23–27, no changes were found in both sexes of any PS80-treated groups on PNDs 60–67. It is likely that PS80 at 7.5% caused a transient suppression of conditioned avoidance responses in pups of both sexes. However, necropsy and histopathological examinations, including the nervous system, revealed no evidence of developmental disorders in pups on PNDs 22 and 70. The magnitude of decrease in body weight of pups was more pronounced during the younger stage than the older stage. It is noted that light body weight mice performed worse than heavy body weight mice in a learning task [30]. The possibility remains that the lowered conditioned avoidance response determined on PNDs 23–27 may be due to the reduced body weight of pups.

As for growth retardation of offspring, it is known that there are strong positive correlations between developmental landmark parameters and the weight of pups [31] and the best indicator of physical development is body weight [32] in experimental animal studies. Neurobehavioral teratology studies of some organic solvents have shown that decreased birth weight and functional impairment can be caused by the same chemicals at the same dosage levels [33]. In humans, the association of intrauterine growth retardation has been amply demonstrated with respect to neurological dysfunction [34]. Furthermore, human infants who show evidence of growth retardation have a 33–50% likelihood of having a learning disability [33,35]. These reports indicate that developmental neurotoxicity parameters are often associated with growth retardation, which is also an important parameter in developmental neurotoxicity studies. In the present study, transient decrease of successful responses in the conditioned avoidance test and reduced body weight were found at 7.5%, but no neuropathological changes were detected. The exposure of pups during the lactation period may be partly indirect via maternal milk and partly direct. Rat pups may gradually start to drink treated water from around PND 14, and on a mg-test-substance per kg-body-weight basis may actually be consuming a higher dose than adults during their second week of the lactation period [36]. It is needed to clarify the exposure levels of pups to PS80 produced adverse effects and clarify whether the adverse effects are attributable to the direct effects of PS80 on the developing nervous system or secondary effects via growth retardation.

In the present study, the toxicological effects were noted at 7.5% (16.783 ml/kg bw/day) and the NOAEL in this study was considered to be 1.0% (1.864 ml/kg bw/day). The value of the NOAEL is equivalent to 2013 mg/kg bw/day. It is estimated that daily intake of polysorbates from food is 12–111 mg/human in European and American countries [37]. The estimated human intake of polysorbates is equivalent to 0.20–1.85 mg/kg bw/day and is well below the NOAEL in this study.

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A 52-Week Repeated Dose Toxicity Study of Ultraviolet Absorber 2-(2′-Hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole in Rats

Mutsuko Hirata-Koizumi,[1] Hidehiro Ogata,[2] Toshio Imai,[1] Akihiko Hirose,[1] Eiichi Kamata,[1] and Makoto Ema[1]

[1]Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan
[2]Safety Assessment Laboratory, Panapharm Laboratories Co., Ltd., Kumamoto, Japan

A 52-week repeated dose toxicity study of an ultraviolet absorber, 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), was conducted according to OECD TG 452 under GLP. CD(SD)IGS rats were given HDBB by gavage at 0, 0.1, 0.5, or 2.5 mg/kg/day in males and 0, 0.5, 2.5, or 12.5 mg/kg/day in females. No substance-related deaths or clinical signs of toxicity were observed in any group; however, a lowered body weight was found from day 36 to the end of the 52-week administration period at 2.5 mg/kg in males. At the completion of the dosing period, a decrease in red blood cells at 0.5 mg/kg and higher, and in hematocrit at 2.5 mg/kg, was detected in males. Blood biochemical changes, including increases in the levels of alkaline phosphatase and glucose and the A/G ratio, were also found at 0.5 mg/kg and higher in males and at 12.5 mg/kg in females. At necropsy, absolute and relative liver weight was increased at 0.5 mg/kg and higher in males and at 12.5 mg/kg in females. Histopathological changes were observed in the liver; centrilobular hypertrophy of hepatocytes at 0.5 mg/kg and higher in males, and at 12.5 mg/kg in females, and altered hepatocellular foci at 0.5 mg/kg and higher, and cystic degeneration and lipofuscin deposition in hepatocytes at 2.5 mg/kg in males. Based on these findings, the no observed adverse effect level was concluded to be 0.1 mg/kg/day in male rats and 2.5 mg/kg/day in female rats.

Keywords Benzotriazole UV absorber, Chronic toxicity, Rat, Gender-related difference.

Address correspondence to Makoto Ema, Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; E-mail: ema@nihs.go.jp

INTRODUCTION

Ultraviolet (UV) absorbers are added to plastics to prevent polymer degradation due to UV rays, such as loss of strength, reduced flexibility and electric properties, discoloration, scratching, and loss of gloss (Commerce Online, 2007; Tenkazai.com, 2007). Currently, many kinds of UV absorbers are used: benzotriazoles, benzophenones, salicylates, cyanoacrylates, nickels, triazines, etc. Among them, benzotriazole UV absorbers are known to have the most excellent absorption capacity with a full spectrum of UV absorption and are, therefore, used in a variety of polymers.

2-(2′-Hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (CAS No. 3846-71-7; HDBB) is a benzotriazole UV absorber added at ~0.02%–2% mainly to unsaturated polyester resin, polycarbonate, vinyl chloride resin, polyacrylic acid ester, polyacetal, polyolefin, polymethacrylic acid ester, and polyamide (METI, 2006). From these resins, plastic resin products, such as building materials and automobile components, are manufactured. In addition, HDBB is also used in printing or sensitive materials and coating compounds, all intended for UV absorption.

In spite of such widespread use, no reliable data were available on the toxicity of HDBB; therefore, this chemical was selected as an object substance in an existing chemical testing program by the Japanese Government (MHLW, 2003; 2006). Previously, we reported the result of a 28-day repeated dose toxicity study of HDBB conducted under this program (Hirata-Koizumi et al., 2007). In this study, CD(SD)IGS rats were administered HDBB by gavage at a dose of 0.5, 2.5, 12.5, or 62.5 mg/kg/day. As a result, adverse effects, mainly on the liver and heart, were found at all doses in males and at 12.5 mg/kg and higher in females. Anemic changes and histopathological changes in the kidneys and thyroids were also observed at the higher dose. These changes remained after the 14-day recovery period. The no observed adverse effect level (NOAEL) for females was concluded to be 2.5 mg/kg/day based on the induction of hypertrophy and increased mitosis of hepatocytes, and the degeneration and hypertrophy of the myocardium at 12.5 mg/kg. On the other hand, the NOAEL for males could not be determined because hypertrophy and decreased incidence of fatty change of hepatocytes and bile duct proliferation were noted at the lowest dose of 0.5 mg/kg. Considering the toxic effects observed at a relatively low dose and the incomplete recovery, more severe damage induced by longer exposure was a concern; therefore, a chronic toxicity study was performed under the Japanese existing chemical testing program. We here report the details of the results of a 52-week repeated dose toxicity study in rats.

MATERIALS AND METHODS

This study was performed in compliance with the OECD Guideline 452 "Chronic Toxicity Studies" (OECD, 1981) and in accordance with the principles

for Good Laboratory Practice (OECD, 1998; EA, MHW and MITI, 2000) at the Safety Assessment Laboratory, Panapharm Laboratories Co., Ltd. (Kumamoto, Japan).

Chemicals

HDBB was obtained from Shipro Kasei Kaisha, Ltd. (Osaka, Japan). The HDBB (Lot no. S4-034-1) used in this study was 100% pure, based on analysis using liquid chromatography, and it was kept at room temperature. The purity and stability during the study were verified by analysis before and after animal experiments. HDBB was dissolved in corn oil once or twice a week and kept in a dark, cool place until dosing since stability under these conditions was confirmed for up to eight days. The concentrations of formulations were confirmed to be 98.0%–102.0% of the target by analysis using high-performance liquid chromatography (HPLC). All other reagents used in this study were of specific purity grade.

Animals

Crj: CD (SD) IGS rats (SPF, five weeks old) were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). After a seven- or eight-day acclimation, they were subjected to treatment at six weeks of age. Rats found to be in good health were selected and assigned to four groups of 20 males and 20 females by stratified random sampling based on body weight.

All animals were maintained in an air-conditioned room at 21–27°C, with a relative humidity of 47%–60%, a 12-h light/dark cycle, and ventilation with 13–15 air changes/h. They were housed individually, except during the acclimation period, in stainless steel hanger cages. A basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and sodium-hypochlorite-added well water were provided *ad libitum*.

This experiment was approved by the Ethical Committee for Animal Experiments of Panapharm Laboratories, Co., Ltd. and performed in accordance with the Guidance for Animal Experiments of Panapharm Laboratories, Co., Ltd.

Experimental Design

Male and female rats were given HDBB once-daily by gavage for 52 weeks at 0 (vehicle control), 0.1, 0.5, or 2.5 mg/kg/day and at 0, 0.5, 2.5, or 12.5 mg/kg/day, respectively. The dosage levels were determined based on the results of our previous 28-day repeated dose toxicity study in rats given HDBB by gavage at 0.5, 2.5, 12.5, or 62.5 mg/kg/day, in which adverse effects, mainly on the liver and hearts, were found at all doses in males, and at 12.5 mg/kg and more in females (Hirata-Koizumi et al., 2007). The volume of each dose was

adjusted to 5 mL/kg of body weight, based on the latest body weight. At the end of the 13-week administration period, 10 males and 10 females from each group were euthanized for the assessment of hematology, blood biochemistry, organ weights, and macroscopic and microscopic findings. The remaining animals in all groups (10 rats/sex/dose) were fully examined at the completion of the 52-week administration period.

All animals were observed daily before and after dosing for clinical signs of toxicity. Body weight and food consumption were recorded weekly for the first 13 weeks of the administration period, and once every four weeks for the remainder of the dosing period. At weeks 13 and 52 of the dosing period, fresh urine was collected. It was examined microscopically for urinary sediment and analyzed for dipstick parameters, such as occult blood, pH, protein, glucose, ketone bodies, bilirubin, and urobilinogen. In addition, a 24-h urine sample was also collected for the determination of sodium, potassium, and chlorine levels, color, specific gravity, osmotic pressure, and volume of urine.

Prior to necropsy at the end of the 13- and 52-week dosing periods, blood was collected from the caudal vena cava in the abdomen under deep anesthesia by the intraperitoneal (i.p.) injection of pentobarbital sodium after overnight starvation. One portion of the blood was treated with ethylenediaminetetraacetic acid (EDTA)-2K and examined for hematological parameters, such as red blood cell count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count, platelet count, reticulocyte count, and differential leukocyte count. Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured using plasma separated from another blood sample treated with 3.8% sodium citrate. Serum from the remaining portions of blood was analyzed for blood biochemistry (total protein, protein fraction ratio, albumin-globulin (A/G) ratio, glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase (ALP)., calcium, inorganic phosphorus, sodium, potassium, and chlorine).

Following the collection of blood, all animals were sacrificed by exsanguination, and organs and tissues of the entire body were macroscopically observed. The brain, pituitary, thymus, thyroids (including parathyroids), heart, lungs (including bronchus), liver, spleen, kidneys, adrenals, testes, epididymides, ovaries, and uterus were then excised and weighed. The trachea, pancreas, lymph nodes (mandibular and mesenteric), tongue, sublingual gland, submandibular gland, parotid gland, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, eyeballs, optic nerve, Harderian gland, spinal cord (pectoral and lumbar part), sciatic nerve, seminal vesicles, prostates, vagina, mammary gland, aorta (thoracic), bone (sternum and femur including bone marrow), skeletal muscle (biceps femoris

muscle), and skin (hypogastric) as well as the above organs were fixed in 10% neutral-buffered formalin solution (following Bouin's fixation for testes and epididymides, and 2.5% glutaraldehyde fixation for eyeballs, optic nerve, and Harderian gland). Histopathological examination of these organs was conducted for all animals found dead or moribund, and for scheduled-sacrifice animals in the control and highest dose groups. In addition, the livers of males in the lowest dose group and of both sexes in the middle-dose group were examined, since test substance–related changes were found in the higher group. Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin and eosin.

Data Analysis

Parametric data, such as body weight, food consumption, urinalysis findings (sodium, potassium, chlorine, specific gravity, osmotic pressure, and volume), hematological and blood biochemical findings, and organ weights were analyzed by Bartlett's test (Bartlett, 1937) for homogeneity of distribution. When homogeneity was recognized, Dunnett's test (Dunnett, 1964) was conducted for comparison between control and individual treatment groups. If not homogenous, data were analyzed using Steel's multiple comparison test (Steel, 1959). For dipstick parameters, color, and sediment of urine, the grades were converted into numeric values, for which Steel's multiple comparison test (Steel, 1959) was conducted. Macroscopic and histopathological findings were analyzed using Fisher's exact test (Fisher, 1973) and Mann-Whitney's U test (Mann and Whitney, 1947), respectively. These analyses were all conducted by a two-tailed test with a significance level of 1% and 5%.

RESULTS

One male at 2.5 mg/kg was found dead on day 54 of the administration period. Two males at 0.1 mg/kg were also found dead on days 231 or 357 of the administration period. In addition, one female at 12.5 mg/kg was found moribund and was, therefore, euthanized on day 354 of the administration period.

In animals surviving to completion of the 13- or 52-week administration period, no substance-related clinical signs of toxicity were observed; however, body weight was significantly lowered from day 36 to the end of the 52-week dosing period at 2.5 mg/kg in males. A significant increase in food consumption was also detected on days 120, 204–288, and 364 of the dosing period in this group of males.

Examination at Completion of the 13-Week Administration Period

With urine analysis, a significant increase in osmotic pressure and specific gravity was detected at 2.5 mg/kg in males. No changes were noted in other parameters of urinalysis in any HDBB-treated groups (data not shown).

In hematological examination, a significant decrease in hemoglobin and hematocrit at 0.5 mg/kg and higher, decrease in red blood cell count, and increase in platelet count at 2.5 mg/kg was found in males (Table 1). In females, a significant decrease in hematocrit and MCV was noted at 12.5 mg/kg (Table 2). Blood biochemical examination revealed a significant increase in serum levels of glucose, BUN, and ALP at 0.5 mg/kg and higher in males (Table 3) and of total protein at 12.5 mg/kg in females (Table 4). A significant change in the serum protein fraction, such as an increase in albumin and decrease in α_2- and β-globulin at 0.5 mg/kg and higher in males, and at 12.5 mg/kg in females, and a decrease in α_1-globulin at 0.5 mg/kg and higher in males, was also found with a significant increase in the A/G ratio at 0.5 mg/kg and higher in males, and at 12.5 mg/kg in females. There were no substance-related changes in other blood biochemical parameters, including total bilirubin level (data not shown).

At necropsy, enlargement of the liver was observed in five of nine males at 2.5 mg/kg and in one of ten females at 12.5 mg/kg, and the absolute and relative liver weight was significantly increased at 0.5 mg/kg and higher in males, and at 12.5 mg/kg in females (Tables 5 and 6). A significant increase in

Table 1: Hematological findings in male rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.1	0.5	2.5
At completion of the 13-week administration period				
No. of animals	10	10	10	9
Red blood cells (10^4/μL)	855 ± 27	870 ± 29	828 ± 43	807 ± 22**
Hemoglobin (g/dL)	15.6 ± 0.4	15.5 ± 0.5	15.0 ± 0.6*	14.3 ± 0.6**
Hematocrit (%)	43.3 ± 1.6	43.2 ± 1.2	41.6 ± 1.8*	40.0 ± 1.3**
MCV (fL)	50.6 ± 1.6	49.6 ± 1.1	50.3 ± 0.9	49.6 ± 2.6
MCH (pg)	18.3 ± 0.5	17.8 ± 0.5	18.1 ± 0.5	17.7 ± 1.0
MCHC (g/dL)	36.1 ± 0.7	35.9 ± 0.4	36.0 ± 0.7	35.7 ± 0.5
Reticulocyte (10^4/μL)	15.8 ± 2.8	16.3 ± 1.8	16.2 ± 3.2	14.8 ± 3.6
Platelet count (10^4/μL)	103.4 ± 11.2	108.7 ± 8.2	112.9 ± 16.0	130.5 ± 27.1*
PT (s)	15.2 ± 2.7	14.9 ± 1.1	15.1 ± 1.5	14.3 ± 1.4
APTT (s)	24.6 ± 1.9	24.1 ± 1.9	23.0 ± 1.5	23.5 ± 3.1
At completion of the 52-week administration period				
No. of animals	10	8	10	10
Red blood cells (10^4/μL)	840 ± 68	780 ± 145	754 ± 133*	778 ± 66*
Hemoglobin (g/dL)	14.0 ± 1.1	13.1 ± 2.7	12.7 ± 2.1	12.9 ± 1.1
Hematocrit (%)	44.2 ± 2.9	41.3 ± 7.4	40.3 ± 5.7	40.7 ± 3.6*
MCV (fL)	52.7 ± 2.1	53.1 ± 1.2	53.9 ± 4.5	52.3 ± 2.3
MCH (pg)	16.7 ± 0.8	16.7 ± 0.7	16.9 ± 1.0	16.6 ± 0.7
MCHC (g/dL)	31.7 ± 0.8	31.5 ± 1.5	31.3 ± 1.1	31.8 ± 0.3
Reticulocyte (10^4/μL)	18.2 ± 8.4	20.1 ± 9.8	27.1 ± 20.4	15.7 ± 3.3
Platelet count (10^4/μL)	106.5 ± 12.6	110.2 ± 28.5	123.7 ± 28.5	140.1 ± 13.6**
PT (s)	13.5 ± 1.0	13.8 ± 1.0	14.5 ± 1.9	21.8 ± 9.0**
APTT (s)	21.5 ± 1.5	20.9 ± 2.7	21.2 ± 2.6	29.5 ± 9.3

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.

Table 2: Hematological findings in female rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.5	2.5	12.5
At completion of the 13-week administration period				
No. of animals	10	10	10	10
Red blood cells ($10^4/\mu L$)	768 ± 38	793 ± 40	762 ± 23	753 ± 25
Hemoglobin (g/dL)	13.9 ± 0.5	14.1 ± 0.6	13.8 ± 0.4	13.4 ± 0.5
Hematocrit (%)	40.1 ± 1.7	40.7 ± 2.2	39.5 ± 0.9	38.1 ± 1.2*
MCV (fL)	52.2 ± 1.1	51.3 ± 0.7	51.9 ± 1.3	50.6 ± 1.0**
MCH (pg)	18.1 ± 0.4	17.7 ± 0.4	18.1 ± 0.5	17.7 ± 0.5
MCHC (g/dL)	34.6 ± 0.5	34.6 ± 0.6	35.0 ± 0.3	35.1 ± 0.5*
Reticulocyte ($10^4/\mu L$)	16.5 ± 3.4	13.9 ± 1.9	14.8 ± 3.4	13.7 ± 1.6
Platelet count ($10^4/\mu L$)	106.1 ± 12.1	110.4 ± 6.8	117.4 ± 11.6	106.2 ± 9.9
PT (s)	11.7 ± 0.5	11.7 ± 0.3	11.7 ± 0.3	11.8 ± 0.4
APTT (s)	19.2 ± 1.5	19.7 ± 0.9	19.0 ± 1.6	19.2 ± 1.5
At completion of the 52-week administration period				
No. of animals	10	10	10	9
Red blood cells ($10^4/\mu L$)	707 ± 100	708 ± 62	730 ± 55	673 ± 115
Hemoglobin (g/dL)	13.2 ± 1.4	13.5 ± 0.8	13.5 ± 1.0	12.3 ± 1.5
Hematocrit (%)	40.3 ± 3.8	41.0 ± 2.5	41.3 ± 3.0	37.3 ± 4.4
MCV (fL)	57.5 ± 4.3	58.1 ± 2.3	56.6 ± 2.4	56.1 ± 4.8
MCH (pg)	18.8 ± 1.0	19.1 ± 0.7	18.5 ± 0.8	18.4 ± 1.4
MCHC (g/dL)	32.7 ± 0.9	33.0 ± 0.5	32.7 ± 0.6	32.9 ± 0.4
Reticulocyte ($10^4/\mu L$)	14.9 ± 8.9	16.4 ± 9.6	13.9 ± 5.8	17.1 ± 15.1
Platelet count ($10^4/\mu L$)	90.2 ± 10.0	94.2 ± 14.7	101.5 ± 13.9	105.6 ± 11.9*
PT (s)	12.3 ± 0.8	12.9 ± 0.7	12.5 ± 0.5	12.1 ± 0.5
APTT (s)	18.4 ± 0.9	18.5 ± 0.9	17.7 ± 1.4	17.7 ± 1.2

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.

the relative weight of the brain, heart, kidneys, and testes was also found at 2.5 mg/kg in males, but the absolute weight was not significantly changed. On histopathology, centrilobular hypertrophy of hepatocytes, accompanied with eosinophilic granular cytoplasm, was observed in the liver (Tables 7 and 8). The incidence was significantly increased at 2.5 mg/kg in males and at 12.5 mg/kg in females.

Examination at Completion of the 52-Week Administration Period

Urinalysis revealed a significant increase in osmotic pressure at 0.5 mg/kg and higher in males, while it was significantly decreased at 12.5 mg/kg in females. A significant increase in urine volume was also detected at 12.5 mg/kg in females (data not shown).

On hematological examination, a significant decrease in the red blood cell count at 0.5 mg/kg and higher, and in hematocrit at 2.5 mg/kg in males, and increase in platelet count at 2.5 mg/kg in males, and at 12.5 mg/kg in females was found (Tables 1 and 2). In addition, PT was significantly prolonged at 2.5 mg/kg in males. In the blood biochemical examination, a significant

Table 3: Blood biochemical findings in male rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.1	0.5	2.5
At completion of the 13-week administration period				
No. of animals	10	10	10	9
Total protein (g/dL)	5.8 ± 0.3	5.8 ± 0.2	5.7 ± 0.5	5.8 ± 0.5
A/G ratio	1.22 ± 0.12	1.30 ± 0.09	1.67 ± 0.23**	2.09 ± 0.27**
Protein fraction ratio				
α_1-Globulin (%)	18.7 ± 1.6	17.9 ± 1.6	15.6 ± 1.3**	12.1 ± 2.4**
α_2-Globulin (%)	7.1 ± 0.7	6.8 ± 0.6	5.9 ± 0.6**	5.6 ± 0.6**
β-Globulin (%)	15.2 ± 0.8	14.4 ± 0.6	11.5 ± 1.0**	9.9 ± 0.7**
γ-Globulin (%)	4.2 ± 0.5	4.3 ± 0.6	4.6 ± 0.8	5.0 ± 1.4
Albumin (%)	54.8 ± 2.3	56.6 ± 1.6	62.4 ± 2.9**	67.4 ± 3.0**
ALP (IU/L)	164 ± 23	216 ± 57	373 ± 60**	619 ± 115**
Glucose (mg/dL)	121 ± 9	120 ± 7	154 ± 13**	151 ± 9**
BUN (mg/dL)	12.3 ± 1.1	11.8 ± 1.7	14.2 ± 1.7*	14.8 ± 1.8**
At completion of the 52-week administration period				
No. of animals	10	8	10	10
Total protein (g/dL)	5.8 ± 0.2	5.8 ± 0.3	5.8 ± 0.5	5.8 ± 0.2
A/G ratio	1.01 ± 0.21	1.01 ± 0.29	1.42 ± 0.31**	1.75 ± 0.30**
Protein fraction ratio				
α_1-Globulin (%)	19.2 ± 2.2	18.2 ± 1.8	15.2 ± 2.4**	13.4 ± 2.0**
α_2-Globulin (%)	7.5 ± 0.5	7.1 ± 1.4	6.1 ± 1.3*	5.0 ± 1.1**
β-Globulin (%)	17.9 ± 2.3	18.5 ± 4.5	15.3 ± 3.0	12.7 ± 2.2**
γ-Globulin (%)	5.7 ± 2.3	6.9 ± 3.1	5.2 ± 1.7	5.8 ± 1.2
Albumin (%)	49.7 ± 5.4	49.3 ± 8.4	58.1 ± 5.4**	63.2 ± 4.7**
ALP (IU/L)	141 ± 42	165 ± 56	364 ± 87**	565 ± 137**
Glucose (mg/dL)	125 ± 27	115 ± 11	139 ± 17	125 ± 16
BUN (mg/dL)	9.1 ± 1.5	8.8 ± 0.9	10.4 ± 1.9	12.8 ± 1.5**

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **Significantly different from the control, $p < 0.01$.

increase in the levels of ALP at 0.5 mg/kg and higher in males, and at 12.5 mg/kg in females, of BUN at 2.5 mg/kg in males, and of glucose at 12.5 mg/kg in females was found (Tables 3 and 4). For the serum protein fraction ratio, a significant increase in albumin and decrease in α_1- and α_2-globulin at 0.5 mg/kg and higher, and a decrease in β-globulin at 2.5 mg/kg was detected in males. The A/G ratio was significantly increased at 0.5 mg/kg and higher in males. No substance-related changes were found in other blood biochemical parameters, including total bilirubin level (data not shown).

At necropsy, enlarged liver was observed in seven of ten males at 0.5 mg/kg, nine of ten males at 2.5 mg/kg, and five of nine females at 12.5 mg/kg, and light gray macules were grossly detected in the liver of two of ten males at 2.5 mg/kg and of one of nine females at 12.5 mg/kg. Absolute and relative liver weight was significantly increased at 0.5 mg/kg and higher in males, and at 12.5 mg/kg in females (Tables 5 and 6). A significant increase in the relative weight of the brain, pituitary, thyroids, lungs, heart, kidneys, testes, and epididymides at 2.5 mg/kg in males was also found, but no statistically significant

Table 4: Blood biochemical findings in female rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.5	2.5	12.5
At completion of the 13-week administration period				
No. of animals	10	10	10	10
Total protein (g/dL)	6.2 ± 0.4	6.3 ± 0.2	6.4 ± 0.4	6.7 ± 0.5*
A/G ratio	1.78 ± 0.16	1.87 ± 0.22	1.93 ± 0.19	2.24 ± 0.31**
Protein fraction ratio				
α_1-Globulin (%)	13.8 ± 1.0	12.9 ± 1.7	12.6 ± 1.6	12.9 ± 1.8
α_2-Globulin (%)	5.6 ± 0.8	5.6 ± 0.2	5.5 ± 0.6	4.7 ± 0.5*
β-Globulin (%)	12.6 ± 0.9	12.4 ± 1.2	12.1 ± 1.4	9.9 ± 0.8**
γ-Globulin (%)	3.9 ± 0.8	4.3 ± 1.0	4.2 ± 1.0	3.6 ± 1.1
Albumin (%)	64.0 ± 2.0	64.9 ± 2.8	65.7 ± 2.2	68.9 ± 2.9**
ALP (IU/L)	92 ± 30	107 ± 25	101 ± 31	136 ± 81
Glucose (mg/dL)	119 ± 13	117 ± 10	118 ± 15	130 ± 10
BUN (mg/dL)	14.5 ± 1.7	14.3 ± 1.7	13.6 ± 1.1	14.1 ± 1.8
At completion of the 52-week administration period				
No. of animals	10	10	10	9
Total protein (g/dL)	6.4 ± 0.3	6.7 ± 0.2	6.7 ± 0.3	6.5 ± 0.5
A/G ratio	1.79 ± 0.25	1.69 ± 0.17	1.73 ± 0.17	2.00 ± 0.19
Protein fraction ratio				
α_1-Globulin (%)	13.5 ± 1.6	14.2 ± 1.6	12.8 ± 1.4	12.1 ± 1.0
α_2-Globulin (%)	4.8 ± 0.6	4.8 ± 0.5	5.0 ± 0.9	4.1 ± 0.4
β-Globulin (%)	13.2 ± 1.5	13.5 ± 0.7	13.6 ± 1.6	12.2 ± 1.2
γ-Globulin (%)	4.6 ± 0.9	4.9 ± 1.2	5.4 ± 1.2	5.0 ± 1.2
Albumin (%)	63.9 ± 3.1	62.6 ± 2.5	63.3 ± 2.3	66.5 ± 2.1
ALP (IU/L)	57 ± 26	59 ± 16	57 ± 14	86 ± 20**
Glucose (mg/dL)	103 ± 9	110 ± 9	106 ± 16	119 ± 16*
BUN (mg/dL)	13.4 ± 2.7	12.6 ± 2.8	12.7 ± 3.1	12.1 ± 2.0

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.

change was noted in the absolute weight. As observed at the end of the 13-week administration period, centrilobular hypertrophy of hepatocytes with eosinophilic granular cytoplasm was observed on histopathological examination, and the incidence was significantly increased at 0.5 mg/kg and higher in males, and at 12.5 mg/kg in females (Tables 7 and 8). In addition, significant increases in the incidence of cystic degeneration and lipofuscin deposition in hepatocytes at 2.5 mg/kg, and of altered hepatocellular foci (clear cell foci) at 0.5 mg/kg and higher were found in the liver of males.

DISCUSSION

In the present study, one male receiving the highest dose of 2.5 mg/kg died early in the dosing period. Although the cause of death was not identified on histopathological examination, it is unlikely that this death was due to treatment with HDBB because no deaths in this group occurred during the remaining dosing period. Other the deaths of two males at 0.1 mg/kg and the

Table 5: Relative organ weight in male rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.1	0.5	2.5
At completion of the 13-week administration period				
No. of animals	10	10	10	9
Body weight[a]	530.1 ± 32.1	566.3 ± 42.2	546.5 ± 40.3	450.1 ± 27.8**
Brain[b]	0.42 ± 0.02	0.40 ± 0.03	0.42 ± 0.03	0.49 ± 0.03**
Pituitary[c]	2.7 ± 0.3	2.5 ± 0.2	2.6 ± 0.2	2.8 ± 0.2
Thyroids[c]	3.8 ± 1.0	4.7 ± 0.8	4.5 ± 1.1	4.1 ± 0.7
Heart[b]	0.29 ± 0.03	0.29 ± 0.02	0.30 ± 0.02	0.33 ± 0.02**
Lungs[b]	0.29 ± 0.02	0.28 ± 0.03	0.30 ± 0.02	0.31 ± 0.03
Liver[b]	2.75 ± 0.10	2.82 ± 0.23	3.71 ± 0.21**	5.12 ± 0.72**
Kidneys[b]	0.62 ± 0.04	0.62 ± 0.02	0.67 ± 0.06	0.70 ± 0.07*
Testes[b]	0.65 ± 0.07	0.62 ± 0.07	0.61 ± 0.06	0.81 ± 0.07**
Epididymides[b]	0.26 ± 0.02	0.25 ± 0.02	0.23 ± 0.02*	0.28 ± 0.03
At completion of the 52-week administration period				
No. of animals	10	8	10	10
Body weight[a]	819.9 ± 145.4	792.5 ± 140.4	842.4 ± 136.3	614.2 ± 97.3**
Brain[b]	0.30 ± 0.04	0.31 ± 0.07	0.29 ± 0.04	0.39 ± 0.05**
Pituitary[c]	2.0 ± 0.2	2.0 ± 0.5	1.9 ± 0.3	2.8 ± 0.3**
Thyroids[c]	3.8 ± 0.9	3.9 ± 1.0	4.1 ± 0.8	4.9 ± 0.9*
Heart[b]	0.23 ± 0.02	0.25 ± 0.04	0.25 ± 0.03	0.31 ± 0.03**
Lungs[b]	0.23 ± 0.02	0.24 ± 0.05	0.23 ± 0.02	0.29 ± 0.03**
Liver[b]	2.22 ± 0.25	2.26 ± 0.20	2.95 ± 0.47**	4.13 ± 0.62**
Kidneys[b]	0.47 ± 0.05	0.48 ± 0.08	0.51 ± 0.06	0.68 ± 0.09**
Testes[b]	0.45 ± 0.06	0.47 ± 0.10	0.46 ± 0.07	0.61 ± 0.15**
Epididymides[b]	0.16 ± 0.03	0.18 ± 0.04	0.17 ± 0.02	0.22 ± 0.06*

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.
[a]Body weight after overnight starvation following the last dosing (g).
[b]g/100 g body weight.
[c]mg/100 g body weight.

moribund sacrifice of one female at 12.5 mg/kg were related to pituitary, renal, or muscular disorders, which was not observed in scheduled-sacrifice animals, and were considered incidental.

In scheduled-sacrifice animals, a lowered body weight was found at 2.5 mg/kg in males. This change was not observed even at the highest dose of 62.5 mg/kg in the previous 28-day repeated dose toxicity study of HDBB (Hirata-Koizumi et al., 2007). Since increased food consumption, blood glucose, and A/G ratio were noted in both previous 28-day and present 52-week studies, prolonged disturbance of metabolic homeostasis might affect body weight gain. Increased relative weight of the brain, heart, kidneys, testes, etc., without changes in the absolute weight, which was noted at 2.5 mg/kg in males in the present study, is considered to be due to the lowering of body weight.

Anemic changes, such as decreased red blood cell count, hematocrit, and hemoglobin, were also found at 0.5 mg/kg and higher in males in the current study. In females, slight changes indicative of anemia, such as decreased hematocrit and MCV, were noted at 12.5 mg/kg at the end of the 13-week

Table 6: Relative organ weight in female rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.5	2.5	12.5
At completion of the 13-week administration period				
No. of animals	10	10	10	10
Body weight[a]	304.1 ± 26.9	303.0 ± 31.0	297.0 ± 17.5	299.8 ± 23.1
Brain[b]	0.68 ± 0.06	0.69 ± 0.05	0.70 ± 0.03	0.70 ± 0.05
Pituitary[c]	5.6 ± 0.5	6.1 ± 0.7	6.4 ± 1.0*	6.2 ± 0.8
Thyroids[c]	5.5 ± 1.1	5.9 ± 0.8	6.5 ± 1.1*	6.2 ± 0.7
Heart[b]	0.32 ± 0.02	0.30 ± 0.02	0.32 ± 0.02	0.32 ± 0.03
Lungs[b]	0.37 ± 0.03	0.37 ± 0.02	0.36 ± 0.02	0.38 ± 0.03
Liver[b]	2.63 ± 0.14	2.63 ± 0.18	2.80 ± 0.18	3.88 ± 0.50**
Kidneys[b]	0.70 ± 0.25	0.64 ± 0.07	0.64 ± 0.05	0.66 ± 0.06
Ovaries[c]	26.1 ± 4.0	26.5 ± 3.2	26.9 ± 4.6	27.0 ± 4.0
Uterus[b]	0.19 ± 0.03	0.22 ± 0.04	0.19 ± 0.03	0.21 ± 0.03
At completion of the 52-week administration period				
No. of animals	10	10	10	9
Body weight[a]	423.2 ± 87.2	441.8 ± 71.4	481.0 ± 104.7	425.8 ± 71.4
Brain[b]	0.54 ± 0.12	0.51 ± 0.07	0.47 ± 0.10	0.52 ± 0.08
Pituitary[c]	6.6 ± 2.3	7.0 ± 3.8	7.1 ± 3.2	7.4 ± 2.4
Thyroids[c]	5.7 ± 1.1	5.5 ± 1.3	5.9 ± 1.1	6.4 ± 1.4
Heart[b]	0.28 ± 0.04	0.28 ± 0.04	0.26 ± 0.04	0.29 ± 0.03
Lungs[b]	0.33 ± 0.07	0.30 ± 0.05	0.29 ± 0.07	0.32 ± 0.05
Liver[b]	2.48 ± 0.39	2.42 ± 0.14	2.45 ± 0.32	3.54 ± 0.41**
Kidneys[b]	0.55 ± 0.08	0.54 ± 0.06	0.52 ± 0.13	0.63 ± 0.09
Ovaries[c]	16.0 ± 3.3	14.3 ± 4.4	13.5 ± 5.5	14.3 ± 2.5
Uterus[b]	0.24 ± 0.08	0.22 ± 0.06	0.22 ± 0.09	0.25 ± 0.08

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.
[a]Body weight after overnight starvation following the last dosing (g).
[b]g/100 g body weight.
[c]mg/100 g body weight.

administration period but not at the completion of the 52-week administration period. The previous 28-day study also showed anemic effects of HDBB at 2.5 mg/kg and higher in males (Hirata-Koizumi et al., 2007). Since no change in the serum bilirubin level or hemosiderin accumulation in the liver, spleen, or kidneys were found in both the present 52-week and previous 28-day studies, anemic changes seem at least not to come from the hemolytic action of HDBB. In order to clarify the mechanisms, further study is required.

In the previous 28-day study, histopathological changes in the liver and heart were observed at 0.5 mg/kg and higher in males, and at 12.5 mg/kg and higher in females (Hirata-Koizumi et al., 2007). At higher doses, histopathological changes were also found in the kidneys and thyroids. In the current study, histopathological changes were observed in the liver. At the end of the 13-week administration, the incidence of centrilobular hypertrophy of hepatocytes was increased at 2.5 mg/kg in males and at 12.5 mg/kg in females, and this change was accompanied with eosinophilic granular cytoplasm. In addition to these changes, increased incidences of altered