Table 2 Reproductive and developmental findings for F0 parents/F1 offspring of rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. pairs	6	6	6	6	6
Copulation index[b]					
Male/female (%)	100/100	100/100	100/100	100/100	100/100
Pre-coital interval (days)[a]	2.2 ± 0.8	2.3 ± 1.2	3.2 ± 0.8	3.0 ± 0.9	2.7 ± 1.2
Fertility index[c]					
Male/female (%)	100/100	100/100	100/100	100/100	100/100
Gestation index (%)[d]	100	100	100	100	100
Gestation length (days)[a]	22.2 ± 0.4	22.2 ± 0.4	22.2 ± 0.4	22.0 ± 0.0	22.2 ± 0.4
No. implantations[a]	16.0 ± 1.8	15.0 ± 0.9	16.3 ± 1.2	13.5 ± 2.0*	12.8 ± 1.2**
Delivery index (%)[a,e]	95.8 ± 8.0	96.7 ± 3.7	95.8 ± 5.3	95.6 ± 8.1	86.7 ± 21.1
No. pups delivered[a]	15.3 ± 2.2	14.5 ± 1.0	15.7 ± 1.8	13.0 ± 2.6	11.2 ± 3.1*
Sex ratio of F1 pups[f]	0.467	0.448	0.564	0.526	0.463
Viability index (%)[a]					
PND 0[g]	100 ± 0	100 ± 0	100 ± 0	100 ± 0	91.2 ± 12.9
PND 4[h]	99.1 ± 2.3	97.9 ± 3.3	95.9 ± 5.3	90.6 ± 12.2	72.1 ± 40.8
PND 21[i]	97.9 ± 5.1	97.9 ± 5.1	100.0 ± 0.0	89.6 ± 25.5	83.3 ± 40.8
Male pup body weight during lactation (g)[a]					
PND 0	6.8 ± 0.4	6.7 ± 0.7	6.3 ± 0.4	6.2 ± 0.6	6.5 ± 0.7
PND 4	10.6 ± 0.9	10.3 ± 0.8	9.6 ± 0.6	9.1 ± 0.7**	9.1 ± 2.2[j]
PND 7	18.7 ± 1.3	17.7 ± 1.3	17.6 ± 1.3	14.5 ± 2.2**	13.3 ± 3.7[j]**
PND 14	39.2 ± 3.0	36.2 ± 3.0	37.3 ± 2.9	33.0 ± 4.0	26.3 ± 7.2[j]**
PND 21	67.0 ± 4.6	61.1 ± 6.1	62.8 ± 3.2	55.7 ± 7.6*	44.1 ± 9.9[j]**
Female pup body weight during lactation (g)[a]					
PND 0	6.4 ± 0.4	6.4 ± 0.5	6.0 ± 0.3	5.8 ± 0.6	6.2 ± 0.5
PND 4	10.1 ± 1.1	9.9 ± 0.7	9.0 ± 0.6	8.7 ± 0.7	8.5 ± 1.9
PND 7	18.2 ± 2.0	17.4 ± 0.7	16.0 ± 1.2	13.8 ± 1.3**	11.7 ± 4.2*
PND 14	38.6 ± 3.5	36.1 ± 2.1	35.0 ± 2.4	31.5 ± 4.9*	25.3 ± 7.2[k]**
PND 21	65.1 ± 5.2	60.1 ± 3.7	58.2 ± 3.3	53.5 ± 9.0*	42.5 ± 9.9[k]**

*Significantly different from the control, $P < 0.05$; **significantly different from the control, $P < 0.01$.

[a]Values are given as mean ± SD; [b]copulation index (%) (number of animals with successful copulation/number of animals paired) × 100; [c]fertility index (%) (number of animals that impregnated a female or were pregnant/number of animals with successful copulation) × 100; [d]gestation index (%) (number of females that delivered live pups/number of pregnant females) × 100; [e]delivery index (%) (number of pups delivered/number of implantations) × 100; [f]sex ratio (total number of male pups/total number of pups delivered); [g]viability index on PND 0 (number of live pups on PND 0/number of pups delivered) × 100; [h]viability index on PND 4 (number of live pups on PND 4/number of live pups on PND 0) × 100; [i]viability index on PND 21 (number of live pups on PND 21/number of live pups selected on PND 4) × 100; [j]data were obtained from five litters because one female experienced total male litter loss by day 4 of lactation; and [k]data were obtained from five litters because one female experienced total litter loss by day 9 of lactation.

PND, post natal day.

highest dose were 551 and 707 mg/kg bw in F0 males and females, respectively. One possible explanation for the discrepancy in the degree of reproductive and developmental toxicity between the present and previous studies may be the difference in administration method. Some studies have shown that gavage and feed administration result in different toxicokinetics for various chemicals (Yuan et al. 1994, 1995). Further studies are needed to clarify the difference in DCBS toxicokinetics between gavage and feed administrations.

Regarding the development of offspring, decreases in the numbers of implantations and pups delivered and lowered body weights of male and female pups were noted at 6000 p.p.m. and higher. These findings indicate that the dose level of 6000 p.p.m. used in this study was potent enough to adversely affect the survival and growth of pups. Reduced weight of the spleen was also observed in male and female weanlings. These findings also suggest that the immune system may be a target of DCBS toxicity. Other changes in the weights of organs, such as the brain and liver in male weanlings and the brain, liver and uterus in female weanlings are unlikely to be due to the toxic effects of DCBS because the degree of changes was relatively small, no dose-dependency was shown, no changes were noted in the absolute or relative weight, and also

Table 3 Absolute and relative organ weights of F0 female rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. females	6	6	6	6	5
Body weight (g)†	331 ± 18	316 ± 16	320 ± 11	306 ± 14*	274 ± 20**
Brain (g)†	2.10 ± 0.05‡	2.11 ± 0.08	2.10 ± 0.05	2.06 ± 0.10	2.06 ± 0.03
	0.63 ± 0.03§	0.67 ± 0.04	0.66 ± 0.02	0.67 ± 0.04	0.76 ± 0.05**
Pituitary (mg)†	13.3 ± 1.6‡	13.4 ± 2.4	15.4 ± 0.9	13.9 ± 1.9	12.9 ± 2.6
	4.03 ± 0.44§	4.24 ± 0.65	4.81 ± 0.32*	4.53 ± 0.46	4.70 ± 0.66
Thyroid (mg)†	18.3 ± 3.6‡	17.6 ± 3.5	17.7 ± 4.3	18.8 ± 2.7	17.5 ± 3.6
	5.52 ± 0.87§	5.55 ± 0.99	5.51 ± 1.18	6.15 ± 0.94	6.39 ± 1.02
Thymus (mg)†	255 ± 47‡	205 ± 63	237 ± 45	186 ± 89	116 ± 60**
	77.1 ± 14.4§	65.0 ± 19.6	74.2 ± 13.1	60.1 ± 26.5	41.7 ± 19.9*
Liver (g)†	13.03 ± 0.83‡	12.51 ± 0.71	13.42 ± 1.18	13.69 ± 0.68	12.18 ± 1.60
	3.94 ± 0.21§	3.97 ± 0.23	4.20 ± 0.27	4.48 ± 0.09**	4.46 ± 0.59
Kidney (g)†	2.34 ± 0.16‡	2.38 ± 0.13	2.35 ± 0.10	2.20 ± 0.12	2.51 ± 0.41
	0.71 ± 0.04§	0.75 ± 0.05	0.74 ± 0.04	0.72 ± 0.03	0.92 ± 0.18**
Spleen (mg)†	682 ± 74‡	589 ± 68	600 ± 89	493 ± 24**	459 ± 46**
	206 ± 20§	187 ± 19	188 ± 31	161 ± 5**	168 ± 15**
Adrenal (mg)†	75.5 ± 11.0‡	81.8 ± 12.9	77.0 ± 8.8	72.0 ± 8.8	88.2 ± 8.3
	22.9 ± 3.2§	26.0 ± 3.9	24.1 ± 2.7	23.5 ± 2.8	32.4 ± 3.8**
Ovary (mg)†	109 ± 18‡	113 ± 17	101 ± 5	101 ± 10	75 ± 23**
	32.9 ± 3.8§	36.1 ± 6.8	31.6 ± 2.4	32.9 ± 3.9	27.1 ± 6.4
Uterus (mg)†	513 ± 68‡	465 ± 73	489 ± 101	414 ± 71	369 ± 183
	156 ± 24§	148 ± 26	153 ± 32	135 ± 22	132 ± 56

*Significantly different from the control, $P < 0.05$; **significantly different from the control, $P < 0.01$.

†Values are given as the mean ± S.D; ‡absolute organ weight; §relative organ weight (organ weight [g or mg]/100 g body weight).

Table 4 Absolute and relative organ weights for F1 male weanlings of rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. males	6	6	6	6	5
Body weight (g)†	67.1 ± 6.7	62.5 ± 4.5	63.8 ± 4.2	55.3 ± 8.9*	43.8 ± 10.6**
Brain (g)†	1.70 ± 0.05‡	1.63 ± 0.12	1.59 ± 0.04	1.51 ± 0.05**	1.45 ± 0.11**
	2.55 ± 0.21§	2.61 ± 0.24	2.50 ± 0.15	2.78 ± 0.42	3.44 ± 0.74*
Thymus (mg)†	257 ± 44‡	219 ± 33	265 ± 45	246 ± 36	190 ± 65
	382 ± 50§	351 ± 57	415 ± 59	449 ± 60	424 ± 50
Liver (g)†	2.56 ± 0.35‡	2.65 ± 0.29	2.69 ± 0.38	2.37 ± 0.38	1.72 ± 0.49**
	3.80 ± 0.17§	4.22 ± 0.20*	4.20 ± 0.37	4.30 ± 0.33*	3.90 ± 0.22
Spleen (mg)†	372 ± 63‡	276 ± 53**	296 ± 32*	250 ± 45**	148 ± 36**
	556 ± 84§	442 ± 80*	466 ± 56	452 ± 32*	337 ± 31**

*Significantly different from the control, $P < 0.05$; **significantly different from the control. $P < 0.01$.

†Values are given as mean ± S.D; ‡absolute organ weight; §relative organ weight (organ weight [g or mg]/100 g body weight).

because the changes seem to be secondary effects of the lowered body weight. In the present study, external and internal morphological examinations of offspring were performed, but no skeletal examinations were conducted. To accurately evaluate prenatal developmental toxicity including teratogenicity, it is necessary to interrupt pregnancy 12–24 h before the expected term either by hysterectomy or the necropsy of maternal animals (Wilson 1965).

The adverse effects of DCBS on reproduction and development noted in the present feeding study are almost consistent with the findings of our previous gavage study (Ema *et al.* 2007), which showed decreased numbers of implantations and pups delivered and decreased body weight of the pups at higher doses. These endpoints appear to be affected at multiple points of the female reproductive and developmental process. The decreased number of implantations

Table 5 Absolute and relative organ weights for F1 female weanlings of rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. females	6	6	6	6	5
Body weight (g)†	65.7 ± 7.2	61.1 ± 3.4	59.9 ± 4.6	54.0 ± 9.6*	42.8 ± 9.6**
Brain (g)†	1.60 ± 0.09‡	1.56 ± 0.07	1.53 ± 0.03	1.50 ± 0.05*	1.37 ± 0.08**
	2.46 ± 0.25§	2.56 ± 0.16	2.57 ± 0.18	2.84 ± 0.39	3.34 ± 0.78**
Thymus (mg)†	272 ± 46‡	253 ± 33	252 ± 27	243 ± 51	216 ± 82
	415 ± 56§	415 ± 57	422 ± 37	456 ± 101	491 ± 92
Liver (g)†	2.58 ± 0.31‡	2.47 ± 0.27	2.42 ± 0.42	2.27 ± 0.43	1.71 ± 0.49**
	3.93 ± 0.14§	4.03 ± 0.22	4.02 ± 0.41	4.19 ± 0.13	3.96 ± 0.29
Spleen (mg)†	360 ± 57‡	296 ± 16	267 ± 60*	247 ± 50**	163 ± 59**
	548 ± 66§	484 ± 9	442 ± 72*	456 ± 37*	371 ± 58**
Uterus (mg)†	44.7 ± 6.6‡	41.3 ± 6.1	35.7 ± 2.1*	42.0 ± 6.9	32.4 ± 4.8**
	68.9 ± 14.0 Temp.§	67.7 ± 9.8	60.0 ± 7.4	78.5 ± 10.8	77.3 ± 10.3

*Significantly different from the control. $P < 0.05$; ** significantly different from the control. $P < 0.01$.

†Values are given as the mean ± S.D; ‡absolute organ weight; §relative organ weight (organ weight [g or mg]/100 g body weight).

was the most striking effect in the present study. In our previous study, a decreased number of corpora lutea was noted in female rats given DCBS (Ema *et al.* 2007). Therefore, it is likely that the decreased number of implantations can be attributed to the decreased number of corpora lutea. The present study does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the endpoints. To further evaluate the reproductive and developmental toxicity of DCBS in rats, a two-generation reproductive toxicity study should be performed.

ACKNOWLEDGMENTS

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Reproductive Toxicology

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Evaluation of developmental neurotoxicity of polysorbate 80 in rats

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Abstract

The developmental neurotoxicity of polysorbate 80 (PS80) was evaluated in rats. Crl:CD(SD) rats were given drinking water containing PS80 at 0, 0.018, 0.13, 1.0, or 7.5% (0, 0.035, 0.245, 1.864, or 16.783 ml/kg bw/day) on day 0 of pregnancy through day 21 after delivery. Pregnant rats were allowed to deliver spontaneously. Potential adverse effects of pre- and post-natal exposure on the development and function of the nervous system in offspring of rats given PS80 were examined. Maternal body weight was lowered at 7.5%. Number of pups born was lowered at 7.5%. There were no compound-related effects on locomotor activity of offspring on postnatal days (PNDs) 14–15, 17–18, 20–21 and 33–37. No compound-related changes were found in developmental landmarks, sexual maturation, or reflex responses. Although decreased rate of avoidance responses was noted on PNDs 23–27 in male and female offspring at 7.5%, no compound-related changes were found in performance in the conditioned avoidance response on PNDs 60–67. Histopathological examinations of the brain revealed no toxicological changes. Lowered body weight was observed in male and female offspring at 7.5%. The NOAEL in this study was considered to be 1.0% (1.864 ml/mg/kg bw/day).
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Keywords: Polysorbate 80; Tween 80; Developmental neurotoxicity; Behavior; Developmental landmarks; Rat

1. Introduction

Polysorbate 80 (PS80, CAS No. 9005-65-6, polyoxyethylene (20) sorbitan monooleate, commercially also known as Tween® 80) is a mixture of polyoxyethylene ethers of mixed partial oleic acid esters of sorbitol anhydrides and related compounds [1]. PS80 is very soluble in water and soluble in ethanol. PS80 is widely used in biochemical applications including, solubilizing proteins, isolating nuclei from cells in cell culture, growing tubercule bacilli, and emulsifying and dispersing substances in medicinal and food products [2]. PS80 is often used in foods as an emulsifier in ice cream, frozen custard, ice milk, fruit sherbet, and nonstandardized frozen desserts. PS80 is also used in yeast-defoamer formulations and as a solubilizing and depersing agent in pickles and pickle products [1]. Exposure of the general population to PS80 is mainly through its use as a food additive.

Several reports on neurobehavioral toxicity of PS80 are available. Varma et al. [3] reported that PS80 caused a decreased

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locomotor activity and hyperthermia at 2 ml/kg, and exhibition of paralytic activity at 10 ml/kg after oral administration, and decreased locomotor activity, depression and potentiation of the penobarbitone sleeping time at 2 ml/kg after intraperitoneal administration in mice. They concluded that intraperitoneal doses generally showed more pronounced effects than oral doses, and PS80 did not show any neuropharmacological effects in a dose not more than 1 ml/kg when given either intraperitoneally or orally [3]. PS80 also caused behavioral and neurochemical changes in cats after intraperitoneal administration [4,5]. Intraperitoneal injection of 0.1% saline solution of PS80 in a volume of 3 ml/kg three times every 12 h decreased the carbachol-induced growing response and increased the content of 5-hydroxyindoleacetic acid in the hypothalamus in cats [4]. As for the developmental neurotoxicity of PS80, Brubaker et al. [6] reported that locomotor activity was enhanced in pre-weaning male offspring of rats received drinking water containing PS80 at 1.25 ml/l (0.125%) during the pre-mating, mating, pregnancy and lactation periods. However, their study did not provide enough information on all aspects of developmental neurotoxicity due to the use of one dose group and the

selection of endpoints. Only pre-weaning locomotor activity in male offspring was determined, and no other parameters were evaluated in their study. The present study was therefore conducted to further evaluate the developmental neurotoxicity of PS80, including locomotor activity, in rats using a study design similar to the OECD Draft Proposal for New Guideline 426, Developmental Neurotoxicity Study [7].

2. Materials and methods

This study was performed in accordance with the principles for Good Laboratory Practice [8]. This study was conducted also in compliance with the "Law of Humane Treatment and Management of Animals" [9] and "Guidance for Animal Care and Use" of Ina Research Inc. and in accordance with the protocol reviewed by the Institutional Animal Care and Use Committee of Ina Research Inc. fully accredited by AAALAC International [Accredited Unit No. 001107].

2.1. Animals and housing conditions

Crl:CD(SD) rats were used throughout this study. Rats of this strain were chosen because they are the most commonly used in reproductive and developmental toxicity studies and historical control data are available. Male rats at 10 weeks of age and female rats at 9 weeks of age were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The males and females were acclimated to the laboratory for 7 days, prior to the start of the experiment, and rats found to be in good health were selected for use. Vaginal smears of each female rat were recorded, and rats showing regular estrous cycles were used in the experiment. Animals were reared on a basal diet (NMF; Oriental Yeast Co. Ltd., Tokyo, Japan) and water ad libitum, and were maintained in an air-conditioned room at 21.0–25.0 °C with a relative humidity of 40–70%, a 12-h light (7:00–19:00)/dark (19:00–7:00) cycle, and ventilation of 16 air changes/h. Virgin female rats were mated overnight with male rats. The day when sperm was detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats, weighing 215–324 g, were distributed into five groups of 22 females to equalize the body weights among groups. Rats were housed individually, except during the acclimation, mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared on sterilized wooden chips (Sun Flake: Charles River Laboratories Japan, Inc.).

2.2. Chemical and dosing

Polysorbate 80 (PS80) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The PS80 used in this study was a technical grade (Lot no. EWP7301/code no. 162-21771, saponification value: 49.3, hydroxyl value: 70.1), and was kept in a dark and cool place. The stability of the PS80 was verified by analysis before and after the study. Rats were given PS80 in their drinking water at a concentration of 0 (control), 0.018, 0.13, 1.0, or 7.5% on day 0 of pregnancy through day 21 after delivery. The dosage levels were determined based on the results of our previous dose-finding study, in which decreased body weight gain and food and water consumption at 10.0% and higher, slight decrease in the body weight gain and food consumption at 7.5%, and no adverse effects at 5.0% and below were observed in female rats given PS80 in their drinking water for 14 days (data not shown). Dosed water preparations were formulated by mixing and dissolving PS80 into an appropriate amount of distilled water (Otsuka Pharmaceutical Factory, Inc., Naruto, Japan) for each water concentration. Rats were given PS80 at a constant water concentration. The control rats were given only water. The stability of formulations at room temperature has been confirmed for up to 7 days. During use, formulations were maintained at room temperature for not more than 5 days, and were 100.4–108.6% of the target concentration.

2.3. Observations of dams

All pregnant rats were observed daily for clinical signs of toxicity. Maternal body weight and water consumption were recorded daily, and food consumption

was recorded every 3 or 4 days. Female rats were checked for signs of parturition before and after noon from days 20 to 25 of pregnancy to determine the time of delivery. The day on which parturition was completed by 16:00 was designated as day 0 after delivery. The females were allowed to deliver spontaneously and nurse their pups until day 21 after delivery. Parental female rats were euthanized by exsanguination under isoflurane anesthesia on day 21 after delivery. The external surfaces of rats were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. For each female, the number of uterine implantation sites was recorded, and the weights of the brain, liver, kidney, spleen, and adrenal were determined.

2.4. Observations of offspring

The day of birth was designated as postnatal day (PND) 0. On PND 0, total litter size and the numbers of live and dead pups were counted, and pups were counted, sexed, and examined grossly on PND 0. All pups were observed daily for clinical signs of toxicity, and individually weighed on PNDs 0, 4, 7, 14 and 21. On PND 4, each of the litters was randomly adjusted to eight pups comprising of four males (1m, 2m, 3m and 4m) and four females (1f, 2f, 3f and 4f). Litters of less than eight pups were not used in the experiment. All pups were observed daily for pinna unfolding beginning on PND 2, fur appearance and incisor eruption beginning on PND 8, and eye opening beginning on PND 12. Body weights of pups were recorded on the day of completion of these developmental landmarks. Pups were weaned on PND 21.

2.4.1. Functional/behavioral observations during the pre-weaning period

One male (1m) and one female (1f) pup selected from all dams in each group was evaluated for surface righting reflex on PND 5, and negative geotaxis reflex on PND 8. Locomotor activity of offspring (1m and 1f) on PNDs 14–15, 17–18, and 20–21 at 20:00, 2:00, 8:00 and 14:00 was determined in the open field. Subject rats were placed individually in a box (26 cm in length and width, and 20 cm height) in a 3 × 3 matrix, consisting of a black acrylic plate, with a camera directly overhead and were allowed to explore freely for 10 min. The distance traveled by each monitored rat was recorded with video-based tracking software (BigBrother, Actimetrics, Inc., Wilmette, IL). Locomotor activity was determined under white noise (60 dB) to attenuate external sound, and light at 166–300 lx during the diurnal period and an infrared lamp during the nocturnal period.

2.4.2. Functional/behavioral observations during the adolescent and young adult periods

All remaining male (2m, 3m and 4m) and female (2f, 3f and 4f) pups of each dam were observed daily for clinical signs of toxicity, and individually weighed on PNDs 28, 35, 42, 49, 56 and 70.

Male (2m) and female (2f) pups selected from all dams in each group were evaluated for pupillary reflex, Preyer's reflex, pain response and mid-air righting on PNDs 23–26 and 62–64, and locomotor activity was determined on PNDs 33–37 and 60–66. An open-field with a box (39 cm in length and width, and 30 cm height) in a 2 × 2 matrix was used to evaluate locomotor activity in post-weaning offspring. Other procedures for the determination of locomotor activity were the same as described above for pre-weaning pups. Offspring (2m and 2f) were observed daily for male preputial separation beginning on PND 35 or female vaginal opening beginning on PND 25. The body weight of the respective rats was recorded on the day of preputial separation or vaginal opening.

Conditioned avoidance response was determined on PNDs 23–27 in male (3m) and female (3f) pups of half of the dams in each group, and on PNDs 60–67 in male (3m) and female (3f) pups of the other half of the dams in each group. The shuttle box (40 cm in length, 20 cm width, and 20 cm height), which consisted of transparent acrylic plastic panels, was divided into two equal compartments by a roller (40 and 55 mm in diameter for pups on PNDs 23–27 and PNDs 60–67, respectively). A rat placed in one compartment could get over the roller and cross to the other side. The grid floor of each compartment consisted of stainless steel rods spaced at 10 mm (for pups on PNDs 23–27) or 13 mm (for pups on PNDs 60–67) center to center. An electric shock could be delivered through the grid floor of the occupied compartment from a shock generator/scrambler (MU Co., Chino, Japan). A subject rat was given 2 min to adapt to the shuttle box after its introduction into one compartment. The trial began with a warning buzzer

(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
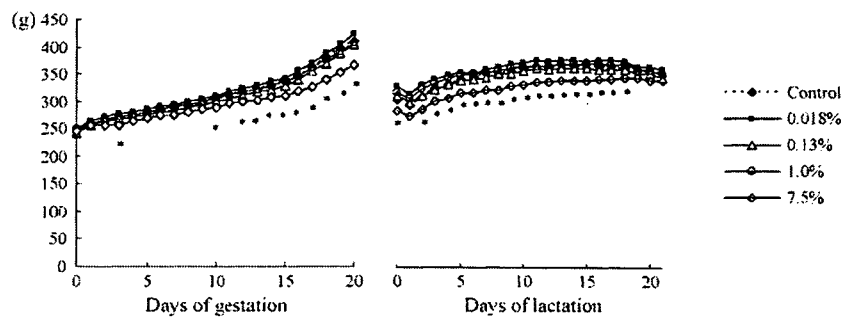

Fig. 1. Body weight of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.
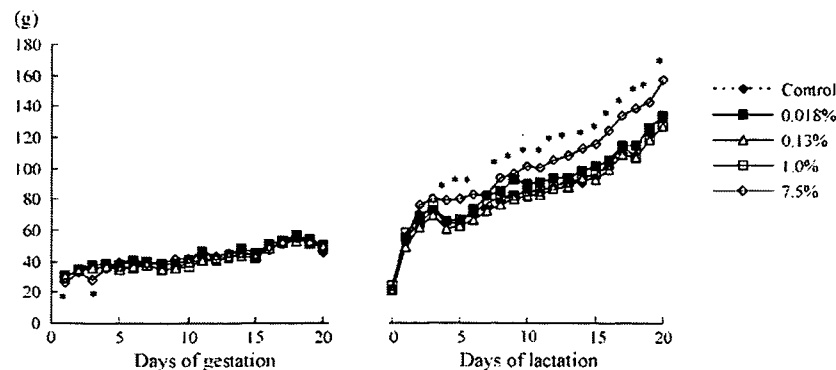

Fig. 2. Water consumption of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

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)[b]					
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, preweaning, 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
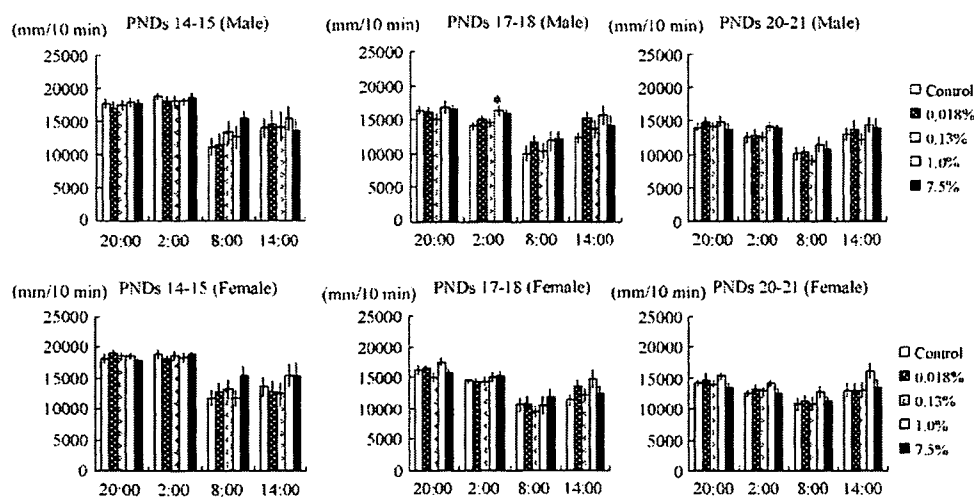

Fig. 3. Locomotor activity in pre-weaning offspring of rats given polysorbate 80 during pregnancy and lactation. Values are given as the mean ± S.E.M. *Significantly different from the control, p < 0.05.
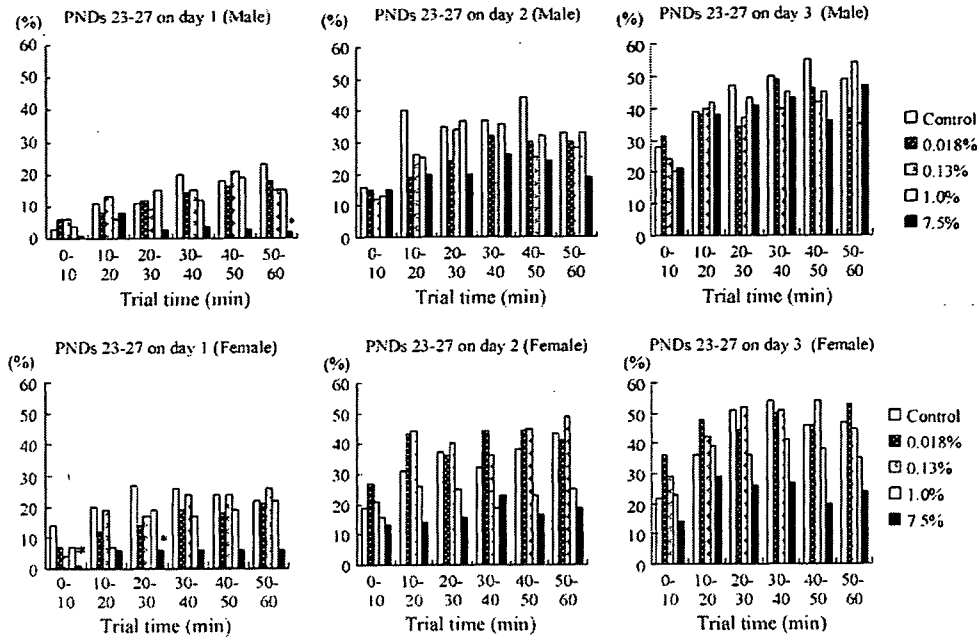
Fig. 4. The rate of successful responses in conditioned avoidance test on postnatal days 23–27 in offspring of rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control. $p < 0.05$.

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
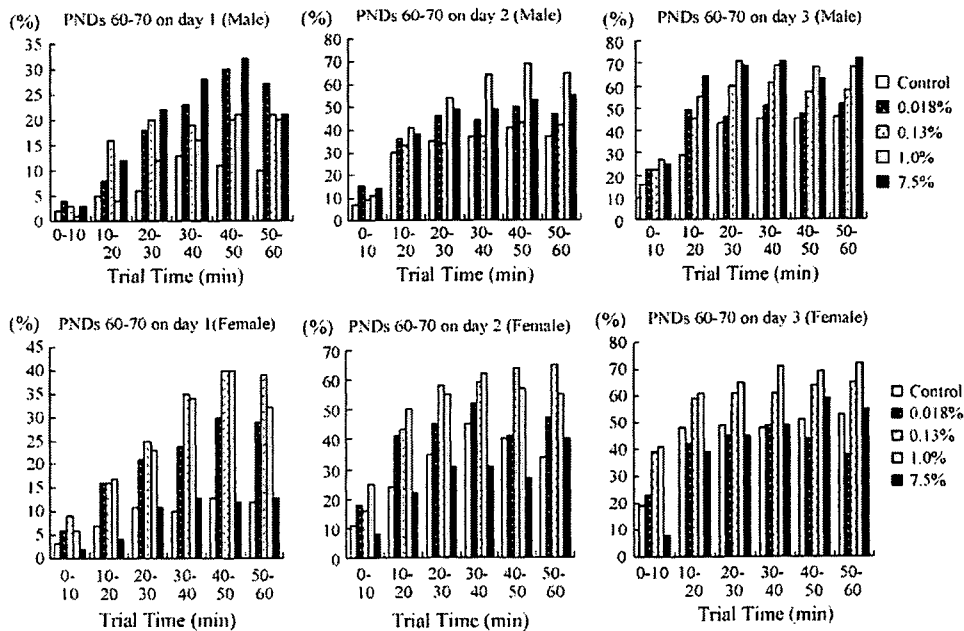

Fig. 5. The rate of successful responses in conditioned avoidance test on postnatal days 60–70 in offspring of rats given polysorbate 80 during pregnancy and lactation.

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.

Acknowledgment

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A 28-Day Repeated Dose Toxicity Study of Ultraviolet Absorber 2-(2'-Hydroxy -3',5'-di-*tert*-butylphenyl) benzotriazole in Rats

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To examine the possible repeated-dose toxicity of an ultraviolet absorber, 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole (HDBB), CD(SD)IGS rats were administered HDBB by gavage at a dose of 0 (vehicle: corn oil), 0.5, 2.5, 12.5, or 62.5 mg kg^{-1} day^{-1} for 28 days. At the completion of the administration period, a decrease in red blood cells, hemoglobin, and hematocrit was noted only in males at 2.5 mg/kg and more. Blood biochemical changes were noted at 0.5 mg/kg and more in males and at 62.5 mg/kg in females. Histopathologic changes were observed principally in the liver (vacuolar degeneration and hypertrophy of hepatocytes, bile duct proliferation, etc.) and in the heart (degeneration and hypertrophy of myocardium and cell infiltration). These changes were noted at 0.5 mg/kg and more in males and at 12.5 mg/kg and more in females. At higher doses, hypertrophy of tubular epithelium in the kidneys and diffuse follicular cell hyperplasia in the thyroids in both sexes and increased severity of basophilic tubules in the kidneys and extramedullary hematopoiesis in the spleen in males were also detected. After the 14-day recovery period, these changes mostly recovered in females but not in males. Based on these findings, no observed adverse effect level (NOAEL) was concluded to be less than 0.5 mg kg^{-1} day^{-1} in male rats and 2.5 mg kg^{-1} day^{-1} in female rats.

Keywords Benzotriazole, Gender-related difference, Rat, Repeated dose toxicity, UV absorber

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INTRODUCTION

Plastic generally ages rapidly under the effects of light, oxygen, and heat, leading to loss of strength, reduced flexibility and electric properties, discoloration, scratching, and loss of gloss (Commerce Online, 2006; Tenkazai.com, 2006). In particular, ultraviolet (UV), possessing considerable energy (e.g., approximately 70 kcal/mol at 400 nm and 110 kcal/mol at 250 nm), directly breaks polymer bonds and promotes oxidative degradation in the presence of oxygen; therefore, UV absorbers are added to plastics to improve their long-term weather resistance and stability.

Benzotriazole UV absorbers, which have a phenolic group attached to the benzotriazole structure, are known to have the most excellent absorption capacity with a full spectrum of UV absorption (Tenkazai.com, 2006), and are therefore used in a variety of polymers. In 1999, the Phenolic Benzotriazole Association voluntarily agreed to participate in the U.S. High Production Volume Chemical Challenge Program (U.S. EPA, 2001). The existing data on four benzotriazole UV absorbers (2-(2′-hydroxy-5′-methylphenyl)benzotriazole, 2-(2′-hydroxy-5′-octylphenyl)benzotriazole, 2-(2′-hydroxy-3′,5′-di-*tert*-amylphenyl)benzotriazole, and 2-(2H-benzotriazole-2-yl)-4,6-bis(1-methyl-1-phenylethyl)phenol), reviewed in this program, showed low acute mammalian toxicity, moderate toxicity with repeated exposure (effect typically in the liver and kidney), and a lack of genotoxicity in this category of chemicals.

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. Although 257.5 tons were produced in Japan from April 2002 to March 2003, only limited toxicity information as a short abstract written in Japanese, which was distributed to the Committee on Safety of Chemical Substances in Chemical Substances Council of Japan, was available (METI, 2006). HDBB was selected as an object substance in an existing chemical testing program by the Japanese Government (MHLW, 2003). In this program, a 28-day repeated-dose toxicity study of HDBB was performed using rats to obtain information on its toxicity. We report the details here.

MATERIALS AND METHODS

This study was performed in compliance with the Test Guideline of the Japanese Chemical Control Act (law concerning examination and regulation of manufacture, etc., of chemical substances), "Twenty-eight-day Repeated Dose

Toxicity Test in Mammalian Species" (EA, MHW and MITI, 1986), and in accordance with the principles for Good Laboratory Practice (OECD, 1998; EA, MHW and MITI, 2000) at the Biosafety Research Center, Foods, Drugs and Pesticides (An-pyo Center, Iwata, 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 wt% pure, and it was kept at room temperature. Test solutions were prepared as suspension in corn oil once a week and kept cool until dosing because stability for 7 days was confirmed under these conditions. The concentration was adjusted in such a way that the volume of each dose is constantly 5 mL/kg based on the latest body weight. The test solutions were confirmed to be 94.2% to 104.3% of the target concentration by analysis using high-performance liquid chromatography. All other reagents used in this study were specific purity grade.

Animals

Crj;CD (SD) IGS rats (SPF, 4 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All animals were maintained in an air-conditioned room at 21.4–25.9°C, with a relative humidity of 51–75%, a 12-h light/dark cycle, and ventilation with 20 air changes per hour. They were housed individually in stainless steel wire mesh cages with anterior surfaces of aluminum. A basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were provided *ad libitum*. Male and female rats were assigned to each dose group by stratified random sampling based on body weight. The initial numbers of rats were 10/sex in control and the highest dose group, and 5/sex in other dose groups. After 8-day acclimation, they were subjected to treatment at 5 weeks of age. This experiment was approved by the Institutional Animal Care and Use Committee of An-pyo Center and performed in accordance with the ethics criteria contained in the bylaws of the committee of An-pyo Center.

Experimental Design

The dosage levels were determined based on the findings in a 14-day dose-finding study, in which an increase in absolute and relative liver weight was observed at all doses of 100, 300, and 1000 mg kg^{-1} day^{-1}. Rats were given HDBB once daily at 0 (vehicle control), 0.5, 2.5, 12.5, or 62.5 mg kg^{-1} day^{-1} by gavage for 28 days. The day after the last dosing, five males and five females from each group were euthanized for the assessment of hematology, blood biochemistry, organ weights, and macroscopic and microscopic findings. The remaining five rats/sex at 0 and 62.5 mg/kg were kept without treatment for 14 days as a recovery period and then fully examined.

All animals were observed before and 1 h and 5 h after dosing for clinical signs of toxicity. During the recovery period, observation was made twice a day (morning and afternoon). Body weight was recorded on days 0, 7, 14, 21, and 27 of the dosing period and days 0, 7, and 13 of the recovery period. Food consumption was measured on days 7, 14, 21, and 27 of the dosing period and days 7 and 13 of the recovery period. On day 25 of the dosing period and day 11 of the recovery period, urine was collected for 3 h 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 color, sediment, osmotic pressure, and volume of the urine.

Prior to necropsy at the end of dosing and recovery periods, blood was collected from the abdominal aorta under deep ether anesthesia after overnight starvation. One portion of the blood was treated with EDTA-2K and examined for hematologic 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. Another blood sample was treated with 3.13% sodium citrate, and blood clotting parameters such as prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen were examined. Serum from the remaining portions of blood was analyzed for blood biochemistry [total protein, albumin, albumin-globulin (A/G) ratio, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase, calcium, inorganic phosphorus, sodium, potassium, chlorine]. After the collection of blood, all animals were sacrificed by exsanguination, and the surface and cavity of the body and the organs and tissues of the entire body were macroscopically observed. The brain, pituitary, thymus, thyroids (including parathyroids), heart, liver, spleen, kidneys, adrenals, testes, epididymides, and ovaries were then removed and weighed (after formalin fixation of the pituitary and thyroids). The trachea, lungs (including bronchus), pancreas, lymph nodes (mesenteric and mandibular), stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, eyeballs, spinal cord (cervical, pectoral, and lumbar part), sciatic nerve, seminal vesicles, prostates, uterus, vagina, bone marrow (femur), skeletal muscle (femur) as well as the above organs were fixed in 10% neutral-buffered formalin phosphate (after formalin acetate fixation for testes and epididymides). Histopathologic examination was conducted for all these organs of the control and the highest dose groups. In addition, the liver, heart, kidneys, spleen, and thyroids of the other groups were examined, as test-substance-related changes were found in the highest group. Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin.

Data Analysis

Parametric data such as body weight, food consumption, urinalysis findings (urine volume and osmotic pressure), hematologic and 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, the data were analyzed using Steel's multiple comparison test (Steel, 1959). For histopathologic findings, Fisher's exact test (Fisher, 1973) was performed. The 5% level of probability was used as the criterion for significance.

RESULTS

No death or clinical sings of toxicity were found in any groups. There were also no significant changes in body weight, but a significant increase in food consumption was noted on dosing days 14 and 21 in males and on dosing days 21 and 27 in females at 62.5 mg/kg. No dose-related changes were found in the findings of urinalysis.

At the end of the 28-day administration period, a significant decrease in red blood cell count, hematocrit and hemoglobin at 2.5 mg/kg and more, decrease in MCHC at 12.5 mg/kg and more, and increase in platelet count at 62.5 mg/kg were noted in males, but these changes were not found in females (Table 1). For clotting factors, a significant decrease in fibrinogen was noted at 2.5 mg/kg and more in males and at 62.5 mg/kg in females (Table 1) but no significant prolongation of PT or APTT.

Blood biochemical examination revealed significant increases in the A/G ratio at 0.5 mg/kg and more, and levels of glucose at 2.5 mg/kg and more, albumin, ALT, and ALP at 12.5 mg/kg and more, and BUN and AST at 62.5 mg/kg in males (Table 2). On the other hand, for females, a significant increase in the levels of glucose, A/G ratio, total cholesterol, triglyceride, and ALT was noted only at 62.5 mg/kg.

At necropsy, absolute liver weight was significantly increased at 2.5 mg/kg and more in males and at 12.5 mg/kg and more in females with a significant increase in the relative weight at all doses in males and at 12.5 mg/kg and more in females (Table 3). In the highest dose group, there was also a significant increase in absolute and relative kidney weight in males and in absolute heart weight in females. No test-substance-related significant change was detected in other organs. Macroscopically, enlargement of the liver was observed at all doses in males and at 12.5 mg/kg and more in females. In the liver, a white patch/zone was found at 2.5 mg/kg and more in males and at 62.5 mg/kg in females.

Table 1: Principal hematologic values in male and female rats given HDBB by gavage for 28 days.

	At the completion of the administration period					At the completion of the recovery period	
	0 mg kg⁻¹ day⁻¹	0.5 mg kg⁻¹ day⁻¹	2.5 mg kg⁻¹ day⁻¹	12.5 mg kg⁻¹ day⁻¹	62.5 mg kg⁻¹ day⁻¹	0 mg kg⁻¹ day⁻¹	62.5 mg kg⁻¹ day⁻¹
Male							
No. of animals	5	5	5	5	5	5	5
Red blood cells (10^6/mm³)	7.89 ± 0.18	7.65 ± 0.32	7.23 ± 0.33*	7.18 ± 0.27**	7.16 ± 0.46**	8.26 ± 0.16	7.65 ± 0.38*
Hemoglobin (g/dL)	15.2 ± 0.4	14.8 ± 0.5	13.9 ± 0.8**	13.6 ± 0.3**	13.2 ± 0.3**	15.3 ± 0.3	13.2 ± 0.9**
Hematocrit (%)	45.6 ± 1.8	44.6 ± 1.5	42.5 ± 2.4*	41.9 ± 1.2**	40.7 ± 0.9**	44.6 ± 1.0	40.1 ± 2.7**
MCV (μm³)	57.8 ± 1.9	58.3 ± 1.5	58.7 ± 1.2	58.3 ± 1.7	57.0 ± 2.7	54.0 ± 1.6	52.5 ± 2.6
MCH (pg)	19.3 ± 0.7	19.4 ± 0.6	19.3 ± 0.4	19.0 ± 0.9	18.4 ± 0.9	18.5 ± 0.5	17.3 ± 0.9*
MCHC (%)	33.4 ± 0.7	33.2 ± 0.5	32.8 ± 0.2	32.5 ± 0.7*	32.3 ± 0.3*	34.2 ± 0.3	32.9 ± 0.6**
Reticulocyte (%)	2.8 ± 0.3	3.3 ± 0.4	3.2 ± 0.3	3.9 ± 0.5*	3.2 ± 1.0	2.5 ± 0.4	4.4 ± 0.2**
Platelet count (10^3/mm³)	1202 ± 75	1265 ± 107	1280 ± 116	1572 ± 430	1639 ± 227*	1196 ± 145	1502 ± 134*
Fibrinogen (mg/dL)	249 ± 13	224 ± 8	189 ± 15**	198 ± 21**	193 ± 20**	240 ± 24	214 ± 13
Female							
No. of animals	5	5	5	5	5	5	5
Red blood cells (10^6/mm³)	7.81 ± 0.38	7.62 ± 0.61	7.79 ± 0.22	7.46 ± 0.30	7.49 ± 0.30	7.80 ± 0.27	7.64 ± 0.38
Hemoglobin (g/dL)	15.1 ± 0.9	14.9 ± 1.3	15.2 ± 0.4	14.8 ± 0.7	14.1 ± 0.6	14.9 ± 0.5	14.2 ± 0.6
Hematocrit (%)	43.7 ± 1.7	43.5 ± 3.1	44.0 ± 1.3	43.1 ± 1.8	41.6 ± 1.6	42.2 ± 1.0	40.6 ± 1.6
MCV (μm³)	56.0 ± 1.1	57.1 ± 1.6	56.4 ± 0.8	57.7 ± 1.4	55.6 ± 1.0	54.2 ± 0.8	53.2 ± 1.8
MCH (pg)	19.3 ± 0.4	19.6 ± 0.6	19.5 ± 0.4	19.8 ± 0.5	18.9 ± 0.4	19.1 ± 0.3	18.6 ± 0.6
MCHC (%)	34.5 ± 0.8	34.3 ± 0.8	34.5 ± 0.4	34.4 ± 0.3	34.0 ± 0.4	35.2 ± 0.3	35.1 ± 0.4
Reticulocyte (%)	2.1 ± 0.4	3.5 ± 1.7	2.6 ± 0.4	2.5 ± 0.2	2.4 ± 0.3	2.7 ± 0.4	2.6 ± 0.3
Platelet count (10^3/mm³)	1295 ± 118	1360 ± 155	1367 ± 79	1368 ± 138	1350 ± 194	1166 ± 64	1410 ± 95**
Fibrinogen (mg/dL)	193 ± 11	222 ± 46	186 ± 9	184 ± 29	155 ± 10*	210 ± 7	241 ± 7**

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