

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

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

Values represent the number of animals with the finding.

±, very slight; +, slight; ++, moderate.

*Significantly different from the control ($p < 0.05$).

**Significantly different from the control ($p < 0.01$).

DISCUSSION

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

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

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

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

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

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

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

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

higher sensitivity of male rats to hematotoxicity of dapsone, which is a major component of the multidrug regimen for the treatment of leprosy, was due to the greater capacity for the N-hydroxylation. Another example was an amino acid antitumor agent, acivicin, of which the LD₅₀ was much higher in male mice than that in females. McGovren et al. (1981) showed that the plasma half-time was much longer in female mice and speculated that the sexual variation may be related to differences in the renal excretion.

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

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

CONCLUSION

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

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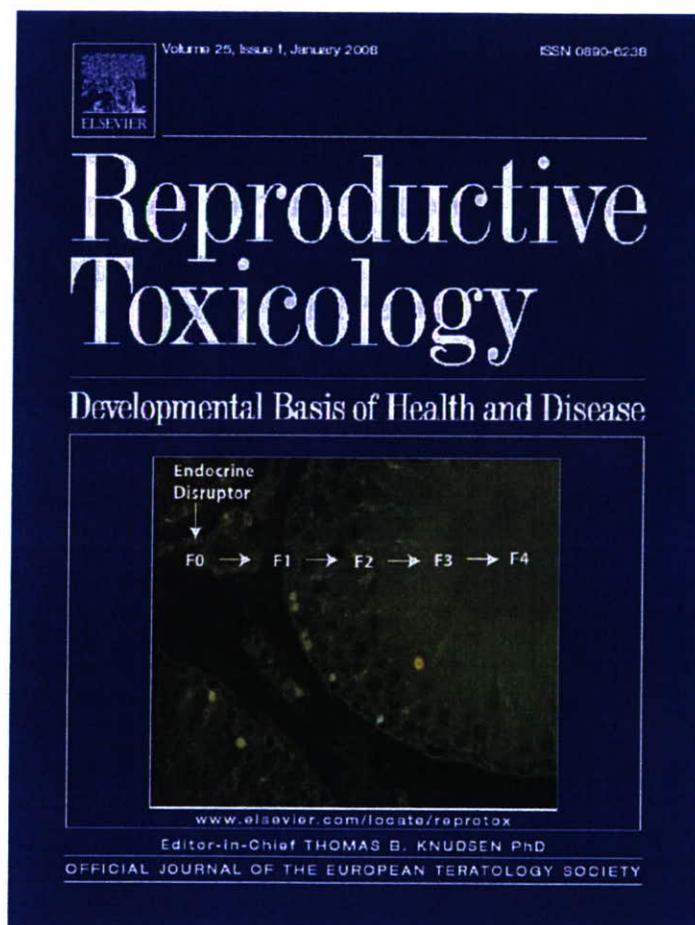
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Evaluation of developmental neurotoxicity of polysorbate 80 in rats

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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 dispersing 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

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

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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

CrI: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 dissolved 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 recorded, 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

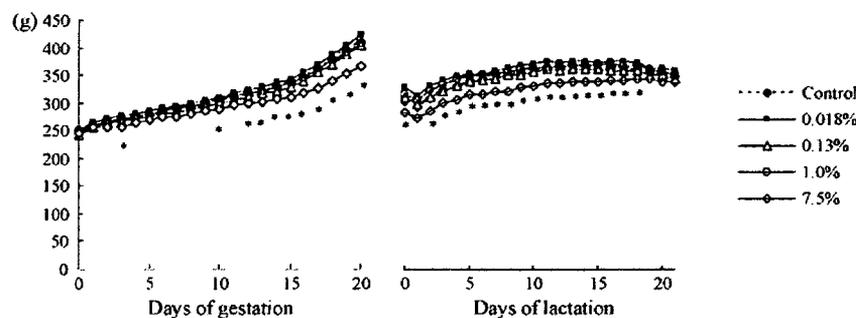


Fig. 1. Body weight of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

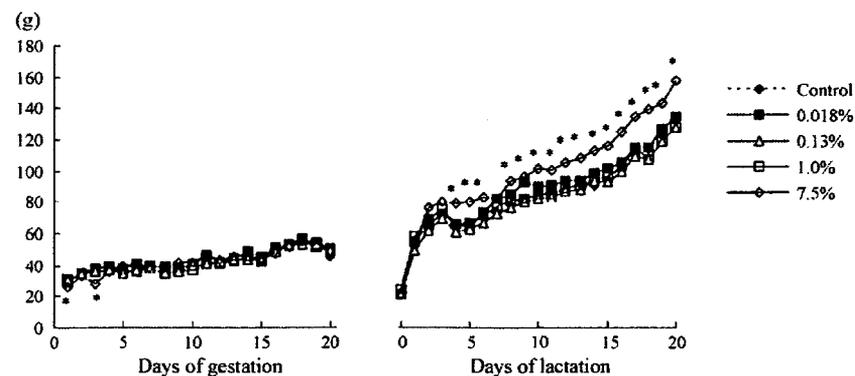


Fig. 2. Water consumption of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

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) ^b					
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, 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

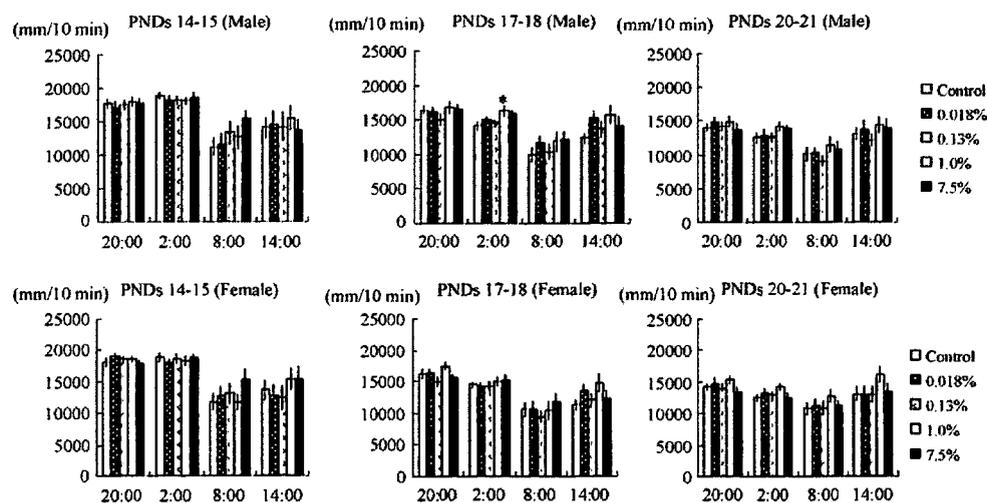


Fig. 3. Locomotor activity in pre-weaning offspring of rats given polysorbate 80 during pregnancy and lactation. Values are given as the mean ± S.E.M. *Significantly different from the control, $p < 0.05$.

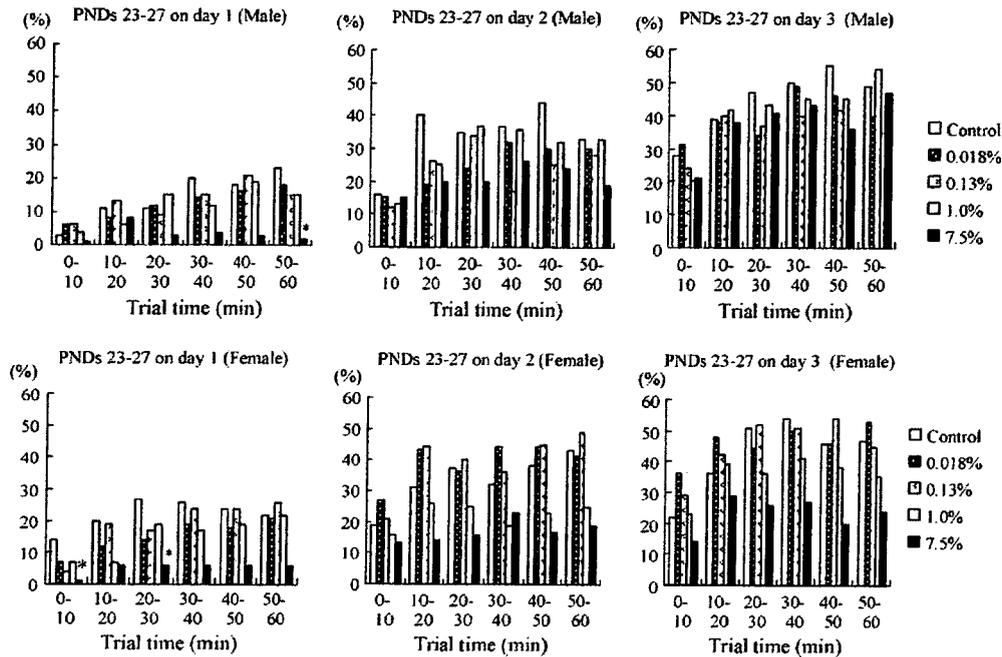


Fig. 4. The rate of successful responses in conditioned avoidance test on postnatal days 23–27 in offspring of rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

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 polyoxyethylene 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

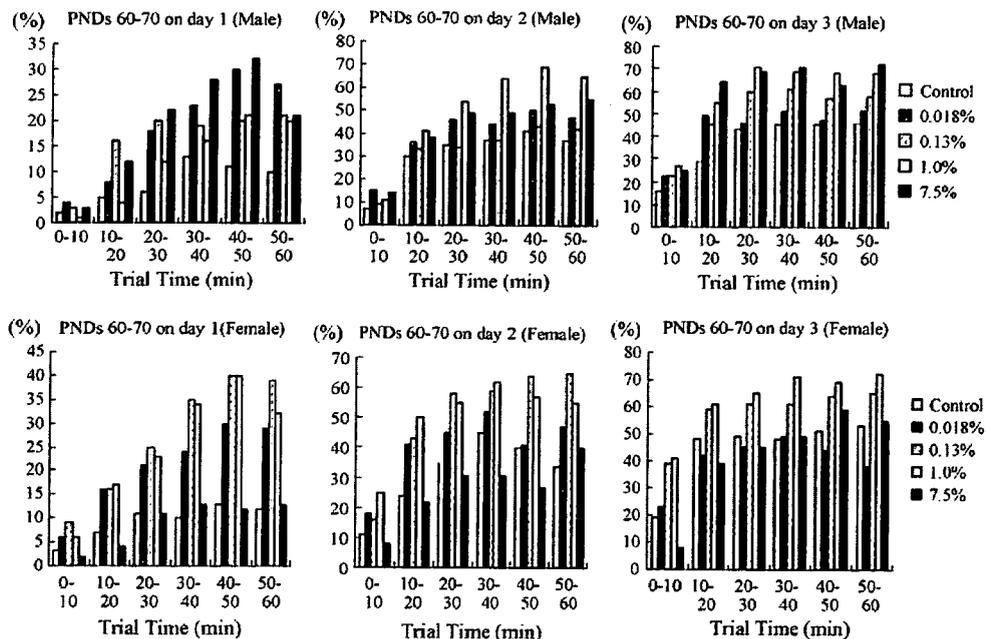


Fig. 5. The rate of successful responses in conditioned avoidance test on postnatal days 60–70 in offspring of rats given polysorbate 80 during pregnancy and lactation.

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 2.56 ± 0.29 ^c	1.55 ± 0.06 2.55 ± 0.18	1.53 ± 0.06 2.54 ± 0.18	1.53 ± 0.05 2.47 ± 0.17	1.49 ± 0.07 2.75 ± 0.33
Liver (g) ^a	2.45 ± 0.43 4.18 ± 0.77 ^c	2.38 ± 0.38 3.88 ± 0.37	2.25 ± 0.23 3.74 ± 0.29	2.44 ± 0.21 3.92 ± 0.23	2.21 ± 0.38 3.99 ± 0.33
Spleen (mg) ^a	266 ± 57 449 ± 59 ^d	308 ± 65 502 ± 91	302 ± 52 500 ± 66	290 ± 32 466 ± 35	274 ± 45 495 ± 37
Adrenal (mg) ^a	21 ± 4 35.5 ± 5.7 ^d	23 ± 3 38.0 ± 4.8	22 ± 5 36.8 ± 8.1	22 ± 4 35.2 ± 6.6	21 ± 4 38.9 ± 9.5
Kidney (mg) ^a	720 ± 97 1222 ± 84 ^d	746 ± 80 1219 ± 70	725 ± 61 1202 ± 45	738 ± 49 1187 ± 76	666 ± 53 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 2.65 ± 0.28 ^c	1.47 ± 0.06 2.63 ± 0.29	1.45 ± 0.06 2.61 ± 0.21	1.46 ± 0.04 2.54 ± 0.16	1.43 ± 0.08 2.75 ± 0.25
Liver (g) ^a	2.09 ± 0.38 3.77 ± 0.35 ^c	2.15 ± 0.34 3.78 ± 0.26	2.21 ± 0.43 3.99 ± 0.86	2.23 ± 0.21 3.87 ± 0.14	2.07 ± 0.27 3.95 ± 0.25
Spleen (mg) ^a	263 ± 44 479 ± 80 ^d	295 ± 56 519 ± 75	266 ± 30 477 ± 40	265 ± 51 458 ± 64	262 ± 36 501 ± 50
Adrenal (mg) ^a	23 ± 4 41.5 ± 6.6 ^d	21 ± 4 37.7 ± 7.2	22 ± 5 39.0 ± 8.8	22 ± 6 38.6 ± 10.1	19 ± 2 36.4 ± 5.5
Kidney (mg) ^a	717 ± 90 1299 ± 92 ^d	724 ± 72 1282 ± 57	711 ± 65 1277 ± 99	726 ± 66 1262 ± 82	676 ± 52 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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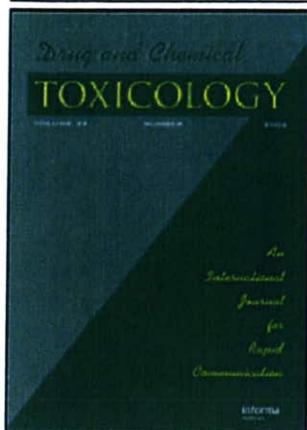
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A 52-Week Repeated Dose Toxicity Study of Ultraviolet Absorber

2-(2'-Hydroxy-3',5'-di-tert-butylphenyl)benzotriazole in Rats

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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

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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.

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