Body weight and the gain in each group are shown in Table 1. In the 500 mg/kg group, body weight was significantly reduced on day 7 and from day 21 to the end of the dosing period in males. In females, significant reduction of body weight was found on day 20 of gestation at 150 mg/kg and on days 14 and 20 of gestation at 500 mg/kg. Body weight gain during the whole period of administration in males and during the gestation period in females was significantly decreased in the 150 and 500 mg/kg groups.

Food consumption was significantly decreased on day 21 of the administration period at 50 mg/kg, on day 7 of the administration period at 150 mg/kg and on days 0, 7 and 21 of the administration period at 500 mg/kg in males, and on days 14 and 20 of the gestation period at 150 mg/kg and on day 0 of the premating period and days 0, 14 and 20 of the gestation period at 500 mg/kg in females (data not shown).

At necropsy, the incidence of small-sized thymus, testes and epididymides was significantly increased at 500 mg/kg in males. Significant increase in the incidence of a rough surface and white spots in the spleen was also found in both sexes of the 500 mg/kg group (data not shown).

Absolute and relative organ weight of scheduled-sacrifice animals in each group is shown in Table 2. Absolute pituitary weight was significantly decreased at 150 mg/kg and above in both sexes. Absolute and relative weight of the thymus, testes and epididymides were also significantly decreased in males of the 500 mg/kg group. In addition, significant decreases in absolute kidney weight at 500 mg/kg in males, and increases in the relative kidney weight at 150 mg/kg in females were detected.

On histopathology, test substance-related changes were observed in the thymus, spleen, testes and epididymides, as shown in Table 3. In the thymus, the incidence of atrophy was significantly increased at 500 mg/kg in males. In the spleen, the incidence of capsule inflammation was significantly increased at 500 mg/kg in both sexes, and the grade of extramedullary hematopoiesis was significantly decreased at 150 mg/kg and above in females. Significant increases in the incidence of seminiferous tubular atrophy and hyperplasia of interstitial cells in the testes, and cell debris and decreased sperm in the lumen of epididymides were also detected in males of the 500 mg/kg group.

3.2. Reproductive findings

The reproductive findings in rats given THFA are presented in Table 4. An estrous cycle of over 5 days was observed in only one female each in the control, 150 and 500 mg/kg groups, but the mean estrous cycle at 500 mg/kg was significantly prolonged. One pair at 15 mg/kg did not copulate and the male was found dead on day 7 of the mating period. One female each at 15 and 150 mg/kg did not become impregnated. The copulation index, precoital interval and fertility index were not significantly different between the control and THFA-treated groups. All pregnant females at 500 mg/kg and two of 11 pregnant females at 150 mg/kg did not deliver any pups. In these females, total early resorption (1/2 females at 150 mg/kg and 12/12 females at 500 mg/kg) or mummification of all fetuses (1/2 females at 150 mg/kg) were found in the uterus. In the 150 mg/kg group, the

Table 2
Organ weight of male and female rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
No. of males	12	11	12	12	12
Body weight (g)	550 ± 40	535 ± 30	538 ± 28	517 ± 22	489 ± 33**
Pituitary (mg)	15.6 ± 1.5	15.6 ± 2.0	14.2 ± 1.3	13.4 ± 1.5*	12.2 ± 1.2**
	(2.8 ± 0.3)	(2.9 ± 0.4)	(2.7 ± 0.3)	(2.6 ± 0.3)	(2.5 ± 0.2)
Kidneys (g)	3.10 ± 0.18	3.15 ± 0.32	3.09 ± 0.20	2.90 ± 0.20	2.71 ± 0.20**
	(0.57 ± 0.04)	(0.59 ± 0.07)	(0.58 ± 0.05)	(0.56 ± 0.03)	(0.55 ± 0.03)
Thymus (g)	0.36 ± 0.07	0.32 ± 0.06	0.35 ± 0.06	0.31 ± 0.07	0.19 ± 0.05**
	(0.07 ± 0.01)	(0.06 ± 0.01)	(0.07 ± 0.01)	(0.06 ± 0.01)	(0.04 ± 0.01**)
Testes (g)	3.41 ± 0.50	3.18 ± 0.83	3.52 ± 0.29	3.40 ± 0.45	1.77 ± 0.44**
	(0.63 ± 0.11)	(0.60 ± 0.15)	(0.66 ± 0.07)	(0.66 ± 0.10)	(0.36 ± 0.09**)
Epididymides (g)	1.40 ± 0.20	1.30 ± 0.30	1.38 ± 0.15	1.26 ± 0.17	0.87 ± 0.15**
	(0.26 ± 0.04)	(0.24 ± 0.05)	(0.26 ± 0.03)	(0.24 ± 0.04)	(0.18 ± 0.03**)
No. of females	12	10	12	9	0
Body weight (g)	363 ± 25	350 ± 35	339 ± 24	313 ± 27**	
Pituitary (mg)	20.1 ± 3.8	18.3 ± 1.7	17.6 ± 1.8	16.0 ± 1.9*	
	(5.5 ± 0.8)	(5.3 ± 0.3)	(5.2 ± 0.5)	(5.1 ± 0.2)	
Kidneys (g)	2.06 ± 0.19	2.00 ± 0.22	2.06 ± 0.23	1.98 ± 0.25	
	(0.57 ± 0.04)	(0.57 ± 0.06)	(0.61 ± 0.05)	(0.63 ± 0.05*)	
Thymus (g)	0.30 ± 0.08	0.28 ± 0.09	0.26 ± 0.07	0.22 ± 0.05	
	(0.08 ± 0.02)	(0.08 ± 0.03)	(0.08 ± 0.02)	(0.07 ± 0.01)	

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

Table 3
Histopathological findings in male and female rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Grade	Dose (mg/kg/day)				
		0	15	50	150	500
Males						
Thymus		(12)	(5)	(5)	(5)	(12)
Atrophy	+	0	0	0	1	8] **
	++	0	0	0	0	1] **
Spleen		(5)	(5)	(5)	(5)	(12)
Extramedullary hematopoiesis	+	2	3	3	4	10
	++	3	2	2	0	2
Capsule inflammation	+	0	0	0	3	5] **
	++	0	0	0	0	4] **
	+++	0	0	0	0	2] **
Testes		(12)	(5)	(5)	(5)	(12)
Atrophy of seminiferous tubule	+	0	0	0	1	4] **
	++	1	0	0	0	7] **
	+++	0	0	0	0	1] **
Hyperplasia of interstitial cells	+	1	0	0	0	9] **
	++	0	0	0	0	1] **
Epididymides		(12)	(5)	(5)	(5)	(12)
Decrease in sperm	+	0	0	0	1	3] **
	++	1	0	0	0	8] **
	+++	0	0	0	0	1] **
Cell debris in lumen	+	1	0	0	1	3] **
	++	0	0	0	0	9] **
Females						
Thymus		(12)	(5)	(5)	(5)	(12)
Atrophy	+	1	0	1	2	4
Spleen		(5)	(5)	(5)	(5)	(12)
Extramedullary hematopoiesis	+	0	0	1	5	11
	++	4	4	4	0] **	1] **
	+++	1	1	0	0] **	0] **
Capsule inflammation	+	0	0	0	1	5] **
	++	0	0	0	1	4] **
	+++	0	0	0	0	3] **

Values represent the number of animals with findings. Values in parentheses are the number of animals examined. +, slight; ++, moderate; +++, severe.
**Significantly different from the control ($P < 0.01$).

remaining nine pregnant females began to deliver on days 24–25 of gestation, but five did not have any pups the next morning. The gestation length in the 150 mg/kg group was significantly prolonged. The gestation index was significantly decreased at 150 mg/kg and above.

3.3. Developmental findings

The developmental findings in rats given THFA are shown in Table 5. No effects of THFA were observed in the number of corpora lutea and implantations, and the implantation index. At 500 mg/kg, no pups were obtained. A significantly decreased total number of pups born, number of live pups on PNDs 0 and 4, and delivery and live birth index, and an increased number of dead pups on PND 0 were found at 150 mg/kg. There was no significant difference in the sex ratio of live pups, the viability index on PND 4, and body weight of male and female pups on PNDs 0 and 4 between the control and THFA-treated groups. Although one pup with general edema was observed at 150 mg/kg, no significant difference in the incidence of pups with malformation was found. Pups with internal variations, such as thymic remnants in the neck and/or left umbilical artery, were observed in all groups, including the control group; however, the total numbers of pups with internal and individual variations were not significantly increased in any THFA-treated groups.

4. Discussion

The current study was conducted to examine the possible effects of THFA on reproduction and development in rats. The dosage of THFA used in this study was sufficiently high to be expected to induce general toxic effects in parental animals. As

Table 4
Reproductive findings in rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
No. of pairs	12	12	12	12	12
Estrous cycles (day)[a]	4.3 ± 0.6	4.0 ± 0.1	4.1 ± 0.3	4.5 ± 0.6	4.8 ± 0.5*
Copulation index (male/female)[b]	100/100	91.7/91.7	100/100	100/100	100/100
No. of pairs with successful copulation	12	11	12	12	12
Precoital interval (day)[a]	2.7 ± 1.2	2.5 ± 1.4	2.9 ± 1.2	2.3 ± 1.4	3.7 ± 2.7
Fertility index [c]	100	90.9	100	91.7	100
No. of pregnant females	12	10	12	11	12
No. of pregnant females with parturition	12	10	12	9	0
Gestation length (day)[a]	22.6 ± 0.5	22.7 ± 0.5	22.9 ± 0.3	24.7 ± 0.7*	
Gestation index[d]	100	100	100	36.4**	0**
No. of dams delivering live pups	12	10	12	4	0

[a] Values are given as the mean ± S.D.
[b] Copulation index (%) = no. of copulated rats/no. of pairs × 100.
[c] Fertility index (%) = no. of pregnant females/no. of pairs with successful copulation × 100.
[d] Gestation index (%) = no. of dams with live pups/no. of pregnant females × 100.
* Significantly different from the control group ($P < 0.05$).
** Significantly different from the control group ($P < 0.01$).

expected, changes in locomotor activity, lowered body weight, and/or histopathological changes in the thymus, spleen, testes and epididymides were observed at 150 mg/kg and above.

Death at 15 mg/kg was considered to be incidental because death occurred in only one male and showed no dose dependency. Also, the decrease in food consumption found in males of the 50 mg/kg group was considered to be toxicologically insignificant because the decrease was transient and was not accompanied with changes in body weight.

In males, body weight gain during the whole administration period was suppressed at 150 and 500 mg/kg, but decreased food consumption was found only during the early administration period at 500 mg/kg and was transient at 150 mg/kg; therefore, factors other than reduced food consumption must be involved in the inhibitive effect of THFA on body weight. In females, the inhibition of body weight gain during the late gestation period at 150 mg/kg and above is considered to be mainly due to the lack of embryos/fetuses because the total number of pups born was markedly decreased in these groups. Similarly, decreased food consumption during the late gestation period is due to decreased nutritional requirement accompanied with embryonic/fetal loss.

Atrophy of the thymus detected at 500 mg/kg in males was accompanied with a marked decrease in organ weight (about 50% of the control value). In addition to these findings, capsule inflammation and/or decreased extramedullary hematopoiesis detected in the spleen of males at 500 mg/kg and of females at 150 mg/kg and above suggests that THFA affects hematological and immunological parameters. Actually, decreased levels of hemoglobin and/or platelet counts were reported in an unpublished 90-day inhalation and feeding study of THFA using rats [11].

Seminiferous tubular atrophy in the testes could be recognized as direct action on the germinal epithelium or secondary change through decreased secretion of gonadotrophic hormone from the pituitary [17]. In the present study, seminiferous tubular atrophy was associated with hyperplasia of interstitial cells, which develops with increased levels of luteinizing hormone (LH) in rats [17]; therefore, THFA is considered to exert effects directly on the testes and to impair spermatogenesis. THFA might impair testosterone synthesis, leading to increased LH levels via negative feedback. The reduced pituitary weight found in males in the 150 and 500 mg/kg groups might be related to such disruption of the hypothalamus–pituitary–gonadal axis.

Despite such histopathological changes in the testes with decreased sperm number in the epididymides, no effects of THFA on reproductive parameters, such as precoital interval, copulation and fertility index, were observed in the present study. These findings are supported by the following descriptions by Parker [18]. Rodent males produce sperm in numbers that greatly exceed the minimum requirements for fertility, particularly as evaluated in reproductive studies that allow multiple mating. It is also reported that sperm production can be drastically reduced (by up to 90% more) without affecting fertility in Sprague–Dawley and Wistar rats [19,20].

The prolonged estrous cycle at 500 mg/kg and decreased pituitary weight at 150 mg/kg in females might also suggest disruption of the hypothalamus–pituitary–gonadal axis; however, because the degree of change in the estrous cycle was slight and most females showed 4- to 5-day estrous cycles, this change is considered to be toxicologically insignificant. Parker [18] noted that estrous cyclicity can be impaired at doses below those that alter fertility, and such changes without associated changes in reproductive or hormonal endpoints would not be considered adverse.

In the current study, total embryonic loss was noted in pregnant females in the higher dose groups. These findings were consistent with the previous developmental toxicity study, in which total embryonic loss was found at 500 mg/kg and above [11]. At 150 mg/kg in the present study, most females showed parturition behavior, but only about half of the dams had pups the next day and the total number of pups born markedly decreased. Cannibalism might have occurred in this group. Even animals

Table 5
Developmental findings in rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
No. of pregnant females	12	10	12	11	12
No. of corpora lutea[a]	17.7 ± 2.1	16.5 ± 2.7	17.8 ± 1.5	16.4 ± 2.0	17.0 ± 2.8
Implantation index[a,b]	88.8 ± 7.4	93.5 ± 7.4	90.7 ± 8.0	84.5 ± 13.1	87.9 ± 23.7
No. of implantation sites[a]	15.6 ± 1.3	15.3 ± 1.9	16.1 ± 1.8	13.7 ± 2.1	14.5 ± 3.7
No. of litters	12	10	12	4	0
Delivery index[a,c]	95.3 ± 7.1	94.7 ± 6.2	91.9 ± 5.9	46.4 ± 14.0*	
Total no. of pups born[a]	14.8 ± 1.6	14.5 ± 2.1	14.8 ± 1.7	7.0 ± 1.4**	
Live birth index[a,d]	100 ± 0	100 ± 0	98.8 ± 2.8	43.1 ± 29.3*	
No. of live pups on PND 0[a]	14.8 ± 1.6	14.5 ± 2.1	14.6 ± 1.8	3.0 ± 2.2**	
No. of dead pups on PND 0[a]	0	0	0.2 ± 0.4	4.0 ± 2.2**	
Sex ratio of live pups (male/female)	86/92	72/73	82/93	6/6	
Viability index on PND 4[a,e]	98.9 ± 2.6	99.3 ± 2.1	97.7 ± 3.5	26.7 ± 46.2	
No. of live pups on PND 4[a]	14.7 ± 1.6	14.4 ± 2.1	14.3 ± 2.0	1.3 ± 2.3**	
Body weight of live pups on PND 0 (g)[a]					
Male	7.3 ± 0.7	7.4 ± 0.5	7.1 ± 0.6	5.9 ± 0.6	
Female	7.0 ± 0.6	7.0 ± 0.5	6.9 ± 0.6	6.3 ± 0.1	
Body weight of live pups on PND 4 (g)[a]					
Male	11.8 ± 1.0	11.5 ± 0.7	11.0 ± 1.1	9.1	
Female	11.2 ± 1.0	10.9 ± 0.7	10.7 ± 0.9	8.4	
External examination of pups					
No. of pups (litters) examined	178 (12)	145 (10)	176 (12)	28 (4)	
No. of pups (litters) with malformations	0 (0)	0 (0)	0 (0)	1 (1)	
General edema	0 (0)	0 (0)	0 (0)	1 (1)	
Internal examination of pups					
No. of pups (litters) examined	178 (12)	144 (10)	175 (12)	27 (4)	
No. of pups (litters) with malformations	0 (0)	0 (0)	0 (0)	0 (0)	
No. of pups (litters) with variations	8 (6)	3 (2)	18 (7)	1 (1)	
Thymic remnants in the neck	6 (4)	3 (2)	14 (5)	1 (1)	
Left umbilical artery	2 (2)	0 (0)	4 (4)	0 (0)	

[a] Values are given as the mean ± S.D.
[b] Implantation index (%) = no. of implantation sites/no. of corpora lutea × 100.
[c] Delivery index (%) = total no. of pups born/no. of implantation sites × 100.
[d] Live birth index (%) = no. of liver pups on PND 0/total no. of pups born × 100.
[e] Viability index on PND 4 (%) = no. of liver pups on PND 4/no. of live pups on PND 0 × 100.
* Significantly different from the control group ($P < 0.05$).
** Significantly different from the control group ($P < 0.01$).

not ordinarily carnivorous, including nonhuman primates, are nevertheless likely to eat dead and moribund offspring, as well as those with malformations that involve skin lesions allowing the loss of body fluids or the exposure of viscera [21].

The malformations and variations found in the current study are those that occur spontaneously among control rats [22–24], and the incidence in the THFA-treated group was very low and not different from that of the control group. However, in the present study, only external and internal examination was performed for pups, and no skeletal examinations were performed. Furthermore, the effects of THFA on the morphological development of offspring could not be evaluated at higher doses because a sufficient number of offspring was not obtained. To accurately evaluate prenatal developmental toxicity, including teratogenicity, it is necessary to interrupt pregnancy a few hours or days before the expected term, either by hysterectomy or the necropsy of maternal animals [21,25]. Such a prenatal developmental toxicity study of THFA is only available as a dose range-finding study using a small number of animals [11]. In this study, an insufficient number of fetuses were morphologically examined due to high embryonic loss at 500 mg/kg and above. This prenatal study adopted a wide dose range, and the next lowest dose was 100 mg/kg. Prenatal developmental effects of THFA at the higher dose should be examined with a sufficient number of dams and fetuses.

The present study was performed in compliance with the OECD guideline 421 "Reproduction/Developmental Toxicity Screening Test" [13]. This screening test guideline 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 endpoints, and, therefore, had reduced power in detecting any small effects. Although the results of the current study clearly showed the adverse effects of THFA on the reproduction and development of rats, information on the effects of THFA on reproduction and development is not sufficient at this time. The present results showed that a full reproductive and developmental toxicity study of THFA is required.

In conclusion, the results of this reproductive and developmental toxicity study provide a more comprehensive toxicity profile of THFA than has been previously reported, and the NOAELs for parental and reproductive/developmental toxicity were concluded to be 50 mg/kg/day.

Acknowledgement

This study was supported by the Ministry of Health, Labour and Welfare, Japan.

References

[1] OECD (Organisation for Economic Co-operation and Development). 2-Furanmethanol, tetrahydro-. SIDS documents for SIAM 20, April 19–21, 2005. Available at: http://cs3-hq.oecd.org/scripts/hpv/, accessed on August 8, 2007.

[2] METI (Ministry of Economy, Trade and Industry, Japan). Research results on the actual production and import volume of chemicals in 2004 (in Japanese). Available at: http://www.meti.go.jp/policy/chemical_management/kasinhou/jittaichousa/kakuhouchi18.pdf, accessed on August 8, 2007.

[3] Penn Specialty Chemicals, Inc. Technical Bulletin of Tetrahydrofurfuryl alcohol. 2005. Available at: http://www.pschem.com/pdfs/thfabulletin4.pdf, accessed on August 8, 2007.

[4] Allen LVJr. Compounding topical dosage forms: ointments, creams, pastes and lotions. Secundum Artem—current & practical compounding information for the pharmacist, Vol. 3, No. 2. Minnesota: Paddock Laboratories, Inc. Available at: http://www.paddocklabs.com/forms/secundum/volume_3_2.pdf, accessed on July 30, 2007.

[5] MHLW (Ministry of Health, Labour and Welfare, Japan). Flavoring agents as food additives. 2003. Available at: http://www.jetro.go.jp/jpn/regulations/guidebook/pdf/free/flavor2003aug-e.pdf#search='Tetrahydrofurfuryl%20alcohol%209799-4', accessed on July 30, 2007.

[6] FDA (Food and Drug Administration, USA). Synthetic flavoring substances and adjuvants. Code of Federal Regulations. Title 21, Vol.3, 21 CFR 172.515, lastly amended at 69 FR 24511, May 4, 2004.

[7] EC (European Commission). Commission decision of 23 February 1999 adopting a register of flavouring substances used in or on foodstuffs drawn up in application of Regulation (EC) No. 2232/96 of the European Parliament and of the Council of 28 October 1996 (1999/217/EC). Official Journal of the European Communities. L 84:March 27;1999.

[8] Deichmann WB, Heyroth FW, Rowe VK, et al. Tetrahydrofurfuryl alcohol, $C_4H_7OCH_2OH$. In: Fassett DW, Irish DD, editors. Industrial hygiene and toxicology, Vol. 2. Sydney: Interscience Publishers; 1963. p. 1491–2.

[9] Coquet PH, Durand G, Laillier J, Plazonnet B. Evaluation of ocular irritation in the rabbit: objective versus subjective assessment. Toxicol Appl Pharmacol 1977;39:129–39.

[10] Lashmar UT, Hadgraft J, Thomas N. Topical application of penetration enhancers to the skin of nude mice: a histopathological study. J Pharm Pharmacol 1989;41:118–22.

[11] TSCA. A dose range-finding developmental toxicity study in rats with THFA. 8EHQ-1092-8576S; October 1992.

[12] MHLW (Ministry of Health, Labour, Welfare, Japan). Tetrahydrofurfuryl alcohol. Toxicity testing reports of environmental chemicals, Vol. 11. Tokyo: Chemicals Investigation Promoting Council; 2004. p. 155–194.

[13] OECD (Organisation for Economic Co-operation and Development). Guideline 421, Reproduction/Developmental Toxicity Screening Test (adopted on July 27, 1995), OECD Guidelines for the Testing of Chemicals, section 5.

[14] EPA (Environmental Protection Agency, USA). Hazard assessment for the tolerance reassessment of tetrahydrofurfuryl alcohol (THFA). Office of Prevention, Pesticides and Toxic Substances; February, 21; 2006.

[15] OECD (Organization for Economic Co-operation and Development). Principles on Good Laboratory Practice (revised in 1997). OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring. No. 1.

[16] EA, MHW, MITI (Environment Agency, Ministry of Health and Welfare, Ministry of International Trade and Industry, Japan). Testing Facility Provided in the Article 4 in the Ordinance Prescribing Test Relating to New Chemical Substances and Toxicity Research of Designated Chemical Substances. Joint notification by Planning and Coordination Bureau, Environment Agency (Kanpogyo No. 39), Pharmaceutical Affairs Bureau, Ministry of Health and Welfare (Yakuhatsu No. 229) and Basic Industries Bureaus, Ministry of International Trade and Industry (Kikyoku No. 85), March 31, 1984, lastly amended on January 23–24, 2001.

[17] Kandori H, Chatani F, Miyajima H. Male reproductive organs. In: The Japanese society of toxicologic pathology editor Toxicologic Histopathology (in Japanese). Tokyo: International Press Editing Centre Incorporation; 2000. p. 283–314.

[18] Parker RM. Testing for reproductive toxicity. In: Hood RD, editor. Developmental and reproductive toxicology—a practical approach. Florida: CRC Press, Taylor & Fransis Group; 2006. p. 425–87.

[19] Robaire B, Smith S, Hales BF. Suppression of spermatogenesis by testosterone in adult male rats: effect on fertility, pregnancy outcome and progeny. Biol Reprod 1984;31:221–30.

[20] Aafjes JH, Vels JM, Schenck E. Fertility of rats with artificial oligozoospermia. J Reprod Fertil 1980;58:345–51.

[21] Wilson JG. Methods for administering agents and detecting malformations in experimental animals. In: Wilson JG, Warkany J, editors. Teratology: principles and techniques. Chicago: The University of Chicago Press; 1965. p. 262–77.

[22] Kameyama Y, Tanimura T, Yasuda M, editors. Spontaneous malformations in laboratory animals-photographic atlas and reference data. Cong Anom 1980;20:25–106.

[23] Morita H, Ariyuki F, Inomata N, Nishimura K, Hasegawa Y, Miyamoto M, et al. Spontaneous malformations in laboratory animals: frequency of external, internal and skeletal malformations in rats, rabbits and mice. Cong Anom 1987;27:147–206.

[24] Nakatsuka T, Horimoto M, Ito M, Matsubara Y, Akaike M, Ariyuki F. Japan Pharmaceutical Manufacturers Association (JPMA) survey on background control data of developmental and reproductive toxicity studies in rats, rabbits and mice. Cong Anom 1997;37:47–138.

[25] Wilson JG. Collection and interpretation of results. In: Wilson JG, editor. Environment and birth defects, vol. 1. New York: Academic Press; 1973. p. 173–93.

Drug and Chemical Toxicology, 31:275–287, 2008
Copyright © Informa Healthcare USA, Inc.
ISSN: 0148-0545 print / 1525-6014 online
DOI: 10.1080/01480540701873368

informa
healthcare

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

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

[1]Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan
[2]Drug Safety Research Laboratories, Shin Nippon Biomedical Laboratories, Ltd. (SNBL DSR), Kagoshima, Japan

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

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

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

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

Animals and Housing Conditions

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

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

Chemicals and Doses

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

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

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

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

Observations

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

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

Data Analysis

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

RESULTS

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

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

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

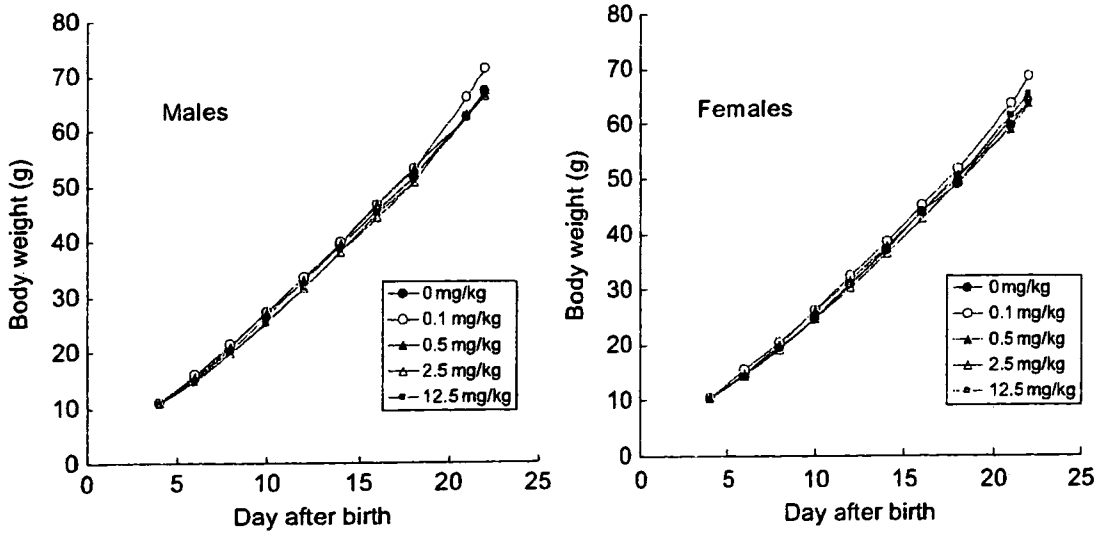

Figure 1: Body weight curves of male and female preweaning rats given HDBB by gavage.

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

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

CONCLUSION

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

ACKNOWLEDGMENTS

This study was supported by the Ministry of Health, Labour and Welfare, Tokyo, Japan.

REFERENCES

Agarwal, D. K., Misra, D., Agarwal, S., Seth, P. K., Kohli, J. D. (1982). Influence of sex hormones on parathion toxicity in rats: antiacetylcholinesterase activity of parathion and paraoxon in plasma, erythrocytes, and brain. *J. Toxicol. Environ. Health.* 9:451–459.

Alexander, B., Guzail, M. A., Foster, C. S. (1997). Morphological changes during hepatocellular maturity in neonatal rats. *Anat. Rec.* 248:104–109.

Bartlett, M. S. (1937). Properties of sufficiency and statistical tests. *Proc. R. Soc. Lond. Ser. A* 160:268–282.

Cattley, R. C., Popp, J. A. (2002). Liver: In: Haschek, W. M., Rousseaux, C. G., Wallig, M. A., eds. *Handbook of Toxicologic Pathology, 2nd ed.,* Vol. 2. San Diego, California, USA: Academic Press, pp. 187–225.

Coleman, M. D., Tingle, M. D., Winn, M. J., Park, B. K. (1990). Gonadal influence on the metabolism and haematological toxicity of dapsone in the rat. *J. Pharm. Pharmacol.* 42:698–703.

Coleman, M. D., Winn, M. J., Breckenridge, A. M., Park, B. K. (1990). Sex-dependent sensitivity to dapsone-induced methaemoglobinaemia in the rat. *Biochem. Pharmacol.* 39:805–809.

Dunnett, C. W. (1964). New tables for multiple comparisons with a control. *Biometrics* 20:482–491.

Gad, S. C. (2006). Metabolism. In: Gad, S. C., ed. *Animal Models in Toxicology, 2nd ed.* Boca Raton, Florida, USA: CRC Press, pp. 217–247.

Glaister, J. R. (1992). Histopathology of target organs—cardiovascular: In: *Principles of Toxicological Pathology (Japanese version supervised by Takahashi, M.).* Tokyo: Soft Science Inc., pp.135–142.

Harris, R. Z., Benet, L. Z., Schwartz, J. B. (1995). Gender effects in pharmacokinetics and pharmacodynamics. *Drugs* 50:222–239.

Hirata-Koizumi, M., Watari, N., Mukai, D., Imai, T., Hirose, A., Kamata, E., Ema, M. (2007). A 28-day repeated dose toxicity study of ultraviolet absorber 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole in rats. *Drug Chem. Toxicol.* 30: 327–341.

Hirata-Koizumi, M., Ogata, H., Imai, T., Hirose, A., Kamata, E., Ema, M. (2008a). A 52-week repeated dose toxicity study of ultraviolet absorber 2-(2'-hydroxy-3', 5'-di-*tert*-butylphenyl)benzotriazole in rats. *Drug Chem. Toxicol.* 31:81–96.

Hirata-Koizumi, M., Matsuyama, T., Imai, T., Hirose, A., Kamata, E., Ema, M. (2008b). Gonadal influence on the toxicity of 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl) benzotriazole in rats. *Drug Chem. Toxicol.* 31:115–126.

Hollander, M., Wolfe, D. A. (1973). *Nonparametric Statistical Methods.* New York: John Wiley and Sons.

Knuckles, M. E., Inyang, F., Ramesh, A. (2004). Acute and subchronic oral toxicity of fluoranthene in F-344 rats. *Ecotoxicol. Environ. Saf.* 59:102–108.

McGovren, J. P., Neil, G. L., Chan, P. J., Stewart, J. C. (1981). Sex- and age-related mouse toxicity and disposition of the amino acid antitumor agent, acivicin. *J. Pharmacol. Exp. Ther.* 216:433–440.

METI (Ministry of Economy, Trade and Industry of Japan). (2006). 2-(2H-1,2,3-benzotriazole-2-yl)-4,6-di-*tert*-butylphenol (In Japanese), document distributed in Committee on Safety of Chemical Substances, Chemical

Substances Council, 30 June 2006. Available at: http://www.meti.go.jp/committee/materials/g60705aj.html Accessed on September 19, 2007.

Mode, A., Gustafsson, J. A. (2006). Sex and the liver—a journey through five decades. *Drug Metab. Rev.* 38:197–207.

Morris, M. E., Lee, H. J., Predko, L. M. (2003). Gender differences in the membrane transport of endogenous and exogenous compounds. *Pharmacol. Rev.* 55:229–240.

Muraoka, Y., Itoh, F. (1980). Sex difference of mercuric chloride-induced renal tubular necrosis in rats—from the aspect of sex differences in renal mercury concentration and sulfhydryl levels. *J. Toxicol. Sci.* 5:203–214.

Nishino, H., Nakajima, K., Kumazaki, M., Fukuda, A., Muramatsu, K., Deshpande, S. B., Inubushi, T., Morikawa, S., Borlongan, C. V., Sanberg, P. R. (1998). Estrogen protects against while testosterone exacerbates vulnerability of the lateral striatal artery to chemical hypoxia by 3-nitropropionic acid. *Neurosci. Res.* 30:303–312.

Ogirima, T., Tano, K., Kanehara, M., Gao, M., Wang, X., Guo, Y., Zhang, Y., Guo, L., Ishida, T. (2006). Sex difference of adenine effects in rats: renal function, bone mineral density, and sex steroidogenesis. *Endocr. J.* 53:407–413.

Raheja, K. L., Linscheer, W. G., Cho, C. (1983). Hepatotoxicity and metabolism of acetaminophen in male and female rats. *J. Toxicol. Environ. Health.* 12:143–158.

Vidair, C. A. (2004). Age dependence of organophosphate and carbamate neurotoxicity in the postnatal rat: extrapolation to the human. *Toxicol. Appl. Pharmacol.* 196:287–302.

Walthall, K., Cappon, G. D., Hurtt, M. E., Zoetis, T. (2005). Postnatal development of the gastrointestinal system: a species comparison. *Birth Defects Res. B Dev. Reprod. Toxicol.* 74:132–156.

Wang, R. H., Schorer-Apelbaum, D., Weinstock, M. (2001). Testosterone mediates sex difference in hypothermia and cholinesterase inhibition by rivastigmine. *Eur. J. Pharmacol.* 433:73–79.

Waxman, D. J., Chang, T. K. (2005). Hormonal regulation of liver cytochrome P450 enzymes. In: Ortiz de Montellano, P. R., ed. *Cytochrome P450—Structure, Mechanism, and Biochmistry, 3rd ed.*New York: Kluwer Academic/ Plenum Publishers, pp. 347–376

Wilcoxon, F. (1945). Individual comparisons by ranking methods. *Biometrics Bull.* 1:80–83.

Zoetis, T., Hurtt, M. E. (2005a) Species comparison of anatomical and functional renal development. *Birth Defects Res. B Dev. Reprod. Toxicol.* 68:111–120.

Zoetis, T., Hurtt, M. E. (2005b) Species comparison of lung development. *Birth Defects Res. B Dev. Reprod. Toxicol.* 68:121–124.

Available online at www.sciencedirect.com
ScienceDirect
Reproductive Toxicology 25 (2008) 89–99
Reproductive Toxicology
Developmental Basis of Health and Disease
www.elsevier.com/locate/reprotox

Evaluation of developmental neurotoxicity of polysorbate 80 in rats

Makoto Ema [a,*], Hiroaki Hara [b], Mariko Matsumoto [a], Mutsuko Hirata-Koizumi [a], Akihiko Hirose [a], Eiichi Kamata [a]

[a] *Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan*
[b] *Ina Research Inc., Ina, Japan*

Received 15 May 2007; received in revised form 19 July 2007; accepted 21 August 2007
Available online 25 August 2007

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).

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 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

* Corresponding author. Tel.: +81 3 3700 9878; fax: +81 3 3700 1408.
E-mail address: ema@nihs.go.jp (M. Ema).

0890-6238/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.reprotox.2007.08.003

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 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