Table 8
Organ weights of female F1 adults

HBCD (ppm)	0 (control)	150	1500	15,000
No. of female F1 adults examined	22	22	20	13
Body weight (g)[a]	322.9 ± 25.9	327.0 ± 24.8	328.6 ± 20.2	307.8 ± 30.5
Brain (g)[a]	2.07 ± 0.09[b]	2.06 ± 0.07	2.06 ± 0.08	1.97 ± 0.06**
	0.645 ± 0.045[c]	0.634 ± 0.053	0.630 ± 0.045	0.646 ± 0.056
Pituitary gland (mg)[a]	14.7 ± 1.5[b]	15.8 ± 2.7	15.5 ± 1.8	14.3 ± 3.0
	4.56 ± 0.43[c]	4.83 ± 0.81	4.72 ± 0.59	4.62 ± 0.68
Thyroid (mg)[a,d]	19.3 ± 3.3[b]	19.8 ± 3.5	21.5 ± 4.6	23.9 ± 4.5**
	6.01 ± 1.01[c]	6.08 ± 1.05	6.54 ± 1.36	7.76 ± 1.36**
Thymus (mg)[a]	250 ± 62[b]	233 ± 62	276 ± 80	259 ± 76
	77.4 ± 17.4[c]	71.6 ± 19.9	83.8 ± 21.8	83.9 ± 22.2
Liver (g)[a]	13.49 ± 1.59[b]	14.30 ± 1.29	14.35 ± 1.41	15.58 ± 2.38**
	4.18 ± 0.42[c]	4.39 ± 0.44	4.38 ± 0.47	5.05 ± 0.50**
Kidney (g)[a,d]	2.36 ± 0.23[b]	2.31 ± 0.19	2.39 ± 0.18	2.23 ± 0.26
	0.732 ± 0.054[c]	0.710 ± 0.068	0.729 ± 0.070	0.726 ± 0.051
Spleen (mg)[a]	632 ± 124[b]	595 ± 68	624 ± 93	578 ± 70
	195 ± 33[c]	183 ± 24	190 ± 27	188 ± 16
Adrenal (mg)[a,d]	70.8 ± 10.4[b]	73.9 ± 10.5	74.8 ± 9.6	71.7 ± 13.4
	22.0 ± 3.1[c]	22.6 ± 3.1	22.8 ± 2.8	23.3 ± 3.5
Ovary (mg)[a,d]	102.4 ± 12.9[b]	106.4 ± 13.2	108.6 ± 18.0	104.9 ± 16.9
	31.8 ± 4.2[c]	32.6 ± 3.9	33.1 ± 5.3	34.1 ± 4.2
Uterus (mg)[a]	966 ± 216[b]	913 ± 188	955 ± 204	949 ± 156
	299 ± 64[c]	282 ± 65	291 ± 64	313 ± 69

[a] Values are given as the mean ± S.D.
[b] Absolute organ weight.
[c] Relative organ weight = organ weight (g or mg)/100 g body weight.
[d] Values are given as the total weights of the organs on both sides.
** Significantly different from the control, $P < 0.01$.

4. Discussion

In the present study, unscheduled deaths and euthanasia due to moribund condition were noted in a few animals. The deaths, euthanasia and clinical signs observed in the present study were not thought to be attributable to the administration of HBCD, because these incidences were very low and inconsistent across generations and sexes and these occurrences are not uncommon in toxicological studies. Lowered body weight and body weight gain accompanied by decreased food consumption were observed at 15,000 ppm in F1 males and females. These findings suggest that a dietary level of 15,000 ppm is generally toxic to rats.

Although a few F0 and F1 adults showed reproductive difficulties, necropsy and the histopathology of the reproductive organs revealed no compound-related changes in these rats. No adverse effects on spermatogenic endpoints observed in the present study are consistent with the previous results of sperm analysis [19].

Lowered body weight of pre-weaning pups was found at 15,000 ppm. More pronounced effects were noted on viability and body weight in F2 pups at this dose. These findings indicate that the dose levels of 15,000 ppm used in this study were potent enough to have adverse effects on the survival and growth of pups. Lochry [31] noted strong correlations between developmental landmark parameters and pup body weight data, which were consistently the more sensitive indicator of the developmental status of offspring. A higher completion rate of eye opening was noted in male and female F1 pups at 1500 ppm, but this rate was not dose-dependent and was not accompanied by changes in body weight. A lower completion rate of eye opening was found in female F2 pups at 1500 ppm and higher, and in male F2 pups at 15,000 ppm, and was associated with lowered body weight. This decreased rate in F2 pups seems to be due to lowered body weight. The lowered completion rate of mid-air righting reflex in female F2 at 15,000 ppm seemed to be due to decreased body weight, because reflex responses are also dependent on physical development [32]. These findings of pre-weaning developmental parameters suggest that high doses (>1500 ppm) of HBCD affect the growth of offspring and the resulting decreased body weight is associated with delays of pre-weaning developmental landmarks and reflex ontogeny.

In the present study, HBCD-related effects were not found on sex hormone-dependent events, such as estrous cyclicity, AGD [33], male preputial separation [34], female vaginal opening [35] or the weight of reproductive organs, or on sex hormone levels at scheduled necropsy. These findings suggest that HBCD has no effects on androgenic/estrogenic events or sexual differentiation.

Transient changes were noted in performance in the water-filled T-maze in F1 males at 1500 ppm and higher, but HBCD

Please cite this article in press as: Ema M, et al., Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats, Reprod Toxicol (2008), doi:10.1016/j.reprotox.2007.12.004

Table 9
Organ weights of male F2 weanlings

HBCD (ppm)	0 (control)	150	1500	15,000
No. of male F2 weanlings examined	22	22	18	13
Body weight (g)[a]	82.2 ± 17.1	84.6 ± 8.7	81.3 ± 13.4	64.7 ± 11.2**
Brain (g)[a]	1.62 ± 0.13[b]	1.65 ± 0.08	1.60 ± 0.10	1.46 ± 0.09*
	2.08 ± 0.58[c]	1.96 ± 0.16	2.01 ± 0.29	2.31 ± 0.33**
Thymus (mg)[a]	343 ± 92[b]	336 ± 57	360 ± 88	282 ± 71
	414 ± 97[c]	397 ± 54	441 ± 69	434 ± 81
Liver (g)[a]	3.87 ± 0.90[b]	4.02 ± 0.55	4.12 ± 0.83	3.88 ± 0.68
	4.72 ± 0.59[c]	4.74 ± 0.35	5.04 ± 0.40*	6.00 ± 0.25**
Kidney (mg)[a,d]	965 ± 167[b]	958 ± 99	933 ± 135	749 ± 100*
	1201 ± 173[c]	1134 ± 56*	1155 ± 85	1170 ± 96
Spleen (mg)[a]	360 ± 83[b]	361 ± 54	346 ± 78	263 ± 50*
	443 ± 77[c]	429 ± 64	426 ± 69	411 ± 66
Adrenal (mg)[a,d]	23.4 ± 5.1[b]	25.1 ± 3.6	24.3 ± 5.2	19.6 ± 3.2*
	28.7 ± 4.4[c]	29.7 ± 3.2	29.9 ± 4.0	30.4 ± 2.0
Testis (mg)[a,d]	476 ± 138[b]	510 ± 81	475 ± 136	385 ± 92
	574 ± 123[c]	600 ± 55	572 ± 93	589 ± 54
Epididymis (mg)[a,d]	73.7 ± 16.8[b]	73.6 ± 10.7	71.8 ± 17.5	61.7 ± 9.5*
	90.7 ± 14.1[c]	87.2 ± 10.6	87.3 ± 9.6	96.2 ± 10.5
Ventral prostate (mg)[a]	40.6 ± 9.7[b]	42.3 ± 9.5	41.7 ± 12.1	29.5 ± 6.8*
	50.2 ± 9.3[c]	50.2 ± 10.7	50.8 ± 9.6	47.3 ± 15.8

[a] Values are given as the mean ± S.D.
[b] Absolute organ weight.
[c] Relative organ weight = organ weight (g or mg)/100 g body weight.
[d] Values are given as the total weights of the organs on both sides.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

did not cause any toxicological changes in spontaneous locomotor activity in F1 rats of both sexes. Previously, decreased locomotion at low and high doses and worse performance in the Morris water maze at high doses were reported in male mice given a single gavage dose with HBCD at 0.9 and 13.5 mg/kg bw on PND 10 [21]. The discrepancy in the behavior of offspring between the present and previous studies could be explained by the difference in the actual intake of HBCD in pups between the direct exposure of pups and maternal exposure, indirectly to pups via maternal milk, and by differences in the animal species used in these studies. Further studies are needed to clarify the transfer of HBCD to the nervous system in pre-weaning animals and species difference.

The changes in absolute and/or relative weight of the brain, pituitary, thymus, kidney, spleen, adrenal, testis, epididymis, seminal vesicle, ventral prostate, ovary and uterus observed in adults and/or weanlings of either sexes or generation are not thought to have toxicological significance, because these changes were not dose-dependent or were inconsistent across age, sex and generation. Increased absolute and/or relative weights of the liver were noted regardless of sex, age and generation in the present study. Previously, an increase in absolute and relative liver weight was reported in rat dams given dietary HBCD at 1.0% [23]. A dose-dependent weight increase of the liver was noted only in females given HBCD by gavage for 28 days [20]. Gavage dose of HBCD for 28 days caused increased absolute and relative weights of the liver, but not test article-related histopathological lesions, in male rats at 1000 mg/kg bw/day and in female rats at 350 mg/kg bw/day and higher [18]. In a rat 90-day repeated dose toxicity study of HBCD by gavage, increased absolute and relative weights of the liver were detected at 100 mg/kg bw/day and higher in males and females [19]. The liver change in males was characterized as minimal hepatocellular vacuolation, and a slight increase in the severity of this change was found in females at 300 mg/kg bw/day and higher. In females, minimal and mild centrilobular hepatocellular hypertrophy were also observed at 1000 mg/kg bw/day; however, the author concluded that these increases in liver weight were an adaptive, rather than a toxic response, and are not uncommon in rats, and are most likely the results of microsomal induction because of the absence of test article-related histopathological and serum chemistry changes [18,19]. It is known that hepatic enzyme induction produces increased liver weight without accompanied histopathological changes in rats [36]. In the present study, neither histopathological change in the liver in any sex, generation or age, nor gender difference in the effects of HBCD on the liver were noted; however, the increased levels of total protein and globulin, in F0 males and females and F1 males, observed in the present study were considered to result from the increased liver weight. The induction of CYP2B1 mRNA, CYP2B1/2B2 protein and 7-pentoxyresorufin *O*-depentylase activity, suggesting phenobarbital-type induction, was caused in juvenile/young rats given HBCD in feed for 28 days [37]. These findings suggest

Please cite this article in press as: Ema M, et al., Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats, Reprod Toxicol (2008), doi:10.1016/j.reprotox.2007.12.001

Table 10
Organ weights of female F2 weanlings

HBCD (ppm)	0 (control)	150	1500	15,000
No. of female F2 weanlings examined	21	22	20	13
Body weight (g)[a]	75.3 ± 12.5	75.8 ± 8.5	73.1 ± 12.8	57.9 ± 11.6**
Brain (g)[a]	1.57 ± 0.11[b]	1.58 ± 0.07	1.55 ± 0.12	1.41 ± 0.15**
	2.14 ± 0.37[c]	2.11 ± 0.20	2.17 ± 0.35	2.48 ± 0.34**
Thymus (mg)[a]	338 ± 85[b]	324 ± 50	331 ± 69	260 ± 80**
	447 ± 81[c]	429 ± 57	451 ± 51	445 ± 83
Liver (g)[a]	3.55 ± 0.64[b]	3.57 ± 0.48	3.63 ± 0.74	3.42 ± 0.77
	4.70 ± 0.27[c]	4.70 ± 0.28	4.94 ± 0.32	5.89 ± 0.44**
Kidney (mg)[a,d]	916 ± 131[b]	885 ± 98	868 ± 144	679 ± 138*
	1226 ± 93[c]	1169 ± 65	1194 ± 84	1177 ± 103
Spleen (mg)[a]	325 ± 59[b]	302 ± 42	299 ± 62	225 ± 45**
	436 ± 61[c]	399 ± 43	412 ± 61	392 ± 53
Adrenal (mg)[a,d]	22.1 ± 4.2[b]	21.5 ± 2.6	21.5 ± 4.3	17.6 ± 3.1*
	29.5 ± 4.1[c]	28.4 ± 3.4	29.4 ± 3.1	30.7 ± 2.6
Ovary (mg)[a,d]	20.0 ± 3.9[b]	22.9 ± 2.6*	20.9 ± 3.9	18.2 ± 4.0
	26.9 ± 5.1[c]	30.5 ± 3.9*	28.8 ± 4.2	32.1 ± 7.5*
Uterus (mg)[a]	60.8 ± 16.1[b]	63.6 ± 15.1	57.0 ± 15.7	47.6 ± 11.4*
	80.9 ± 16.3[c]	84.4 ± 21.0	78.7 ± 21.7	83.7 ± 20.3

[a] Value are given as the mean ± S.D.
[b] Absolute organ weight.
[c] Relative organ weight = organ weight (g or mg)/100 g body weight.
[d] Values are given as the total weights of the organs of both sides.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.

that the increased liver weight and blood biochemistry changes observed in the present study may be attributable to enzyme induction.

In the previous 90-day repeated dose toxicity study, HBCD caused increases in the absolute and relative weights of the thyroid/parathyroid in females and thyroid follicular cell hypertrophy in males and females at 300 mg/kg bw/day and higher, and depressed serum T4 levels in males at 100 mg/kg bw/day and higher and in females at 300 mg/kg bw/day and higher [19]. van der Ven et al. [20] described that the most striking effect of HBCD was on the thyroid hormone axis, including lowered T4 levels, increased immunostaining for TSH in the pituitary, increased weight/activation of the pituitary and thyroid, induction of hepatic T4-glucuronyl transferase, and decreased thyroid follicles size, and these effects were restricted to females. They also noted that higher sensitivity in females may be due to higher liver concentrations of HBCD than in males [20]. In the present study, reduced levels of serum T4 in males and females at 15,000 ppm and increased levels of serum TSH at 1500 ppm and higher in females were observed. It seems likely that the lowered T4 levels may be related to enhanced elimination of T4 due to the induction of hepatic drug metabolizing enzymes and that increased TSH levels may be due to feedback resulting from decreased T4 levels. The increased TSH levels in F0 females at 150 ppm were not considered to have toxicological meaning, because these changes were not accompanied by histopathological changes in the thyroid or decreased T4 levels, or were inconsistent across generations at this dose. Increased thyroid weight at 15,000 ppm and decreased thyroid follicle size and hypertrophy of thyroid follicular cells at 1500 ppm and higher were also noted in male and female F0 and F1 generations. These present findings are essentially consistent with the previous findings [19,20].

Primordial follicles preserve oocytes during the reproductive life span and constitute a stockpile of nongrowing follicles in mammalian ovaries. The primordial follicle population represents a female's total reproductive potential, because primordial follicles do not proliferate or grow [38]. It is reported that busulfan destroyed primordial germ cells, rendering the individual deficient in primordial follicles [39,40]. A reduced primordial stockpile was observed in female offspring of SD rats given busulfan on day 13–15 of pregnancy [41]. In a continuous breeding study in which female Long-Evans hooded rat offspring, after maternal intraperitoneal injection of busulfan on day 14 of pregnancy, were bred with control males for eight breeding cycles, the number of pups delivered was reduced at 2.5 and 5.0 mg/kg bw and no pups were delivered at 10 mg/kg bw [42]. Gray et al. [43] mentioned that continuous breeding of females exposed to reproductive toxicants during critical developmental periods is more useful than a single breeding trial in the detection of subfertility. In the present study, histopathological examinations of the ovary of F1 females revealed a decreased number of primordial follicles at 1500 and 15,000 ppm. Variation exists in primordial follicle counts dependent upon the methodology used [44], but follicle counts provide a more sensitive indicator of potential toxicity than did measures of fertility [45]. Parker

Please cite this article in press as: Ema M, et al., Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats, Reprod Toxicol (2008), doi:10.1016/j.reprotox.2007.12.004

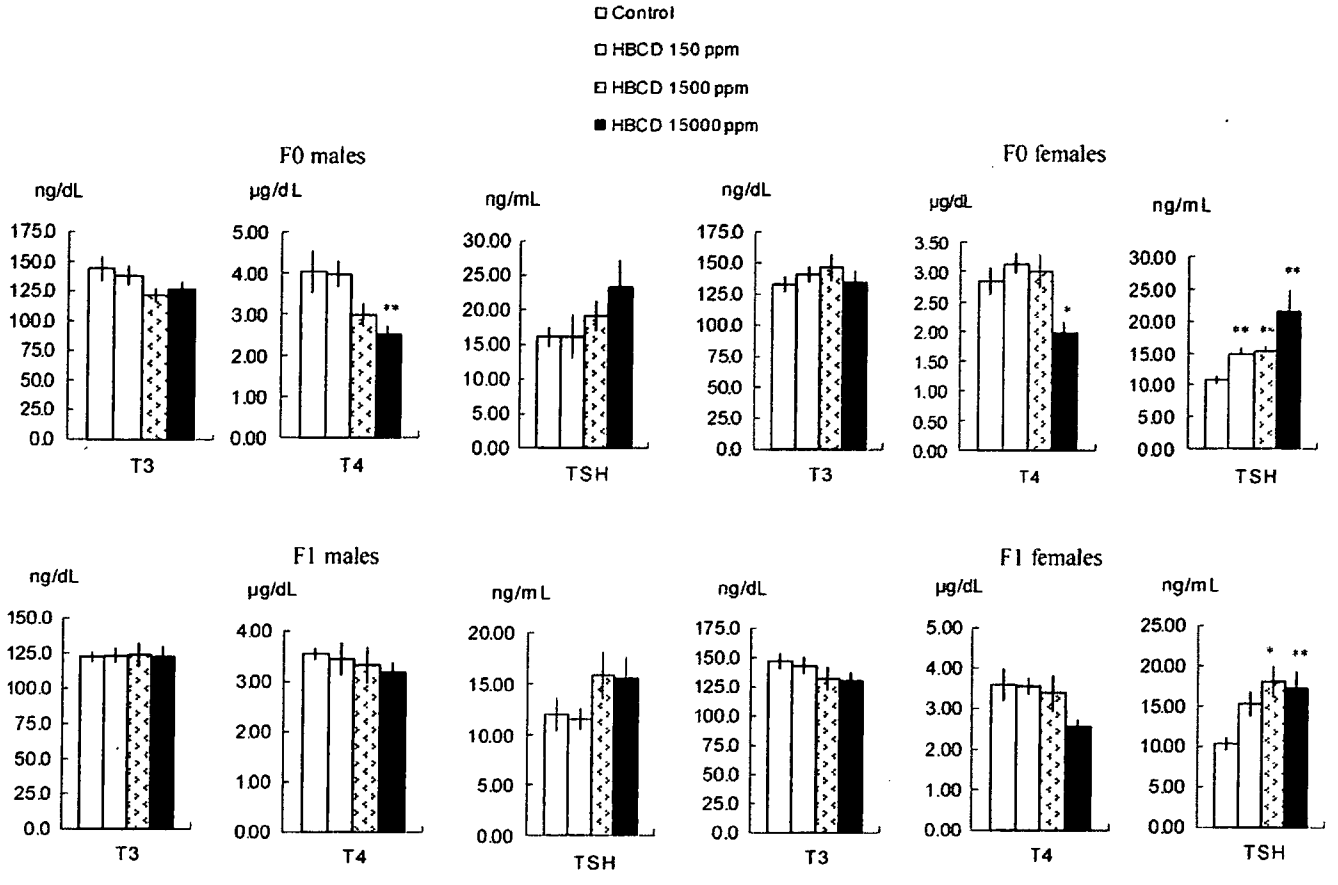

Fig. 4. Serum levels of T3, T4 and TSH in F0 and F1 rats. Values are given as the mean ± S.E.M. (*) Significantly different from the control, $P<0.05$. (**) Significantly different from the control, $P<0.01$.

[46] noted that a decrease in primordial follicle count is usually considered a biomarker of an adverse reproductive effect because no recovery is possible. Although these findings suggest that HBCD is potentially reproductively toxic, no adverse effects on reproductive parameters in F1 dams, or on the numbers of implantations or F2 pups delivered were noted in the present study. In the present study, F1 parent rats were subjected to a single breeding trial. A continuous breeding study of HBCD may be needed to clarify the reproductive toxicity of HBCD, especially the adverse effects of HBCD on the reproductive life span.

In conclusion, the results of the two-generation reproductive toxicity study described here provide a more comprehensive toxicity profile of HBCD than has been previously reported, and the NOAEL of HBCD in this study was considered to be 150 ppm (10.2 mg/kg bw/day) in rats. NCR [4] estimated that the average oral dose rate was 0.026 mg/kg bw/day. The estimated human intake of HBCD is well below the NOAEL in the present study.

Acknowledgement

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

References

[1] Reistad T, Fonnum F, Mariussen E. Neurotoxicity of the pentabrominated diphenyl ether mixture, DE-71, and hexabromocyclododecane (HBCD) in rat cerebellar granule cells in vitro. Arch Toxicol 2006;80:785–96.
[2] BSEF (Bromine Science and Environmental Forum). Major brominated flame retardants volume estimates: total market demand by region. Brussels; 2003 [cited 2007, Aug 28]. Available from: www.bsef.com.
[3] American Chemical Council. HPV data summary and test plan for hexabromocyclododecane (HBCD); 2001.
[4] NRC (National Research Council). Hexabromocyclododecane, eds. Toxicological risks of selected flame-retardant chemicals. Washington, DC: National Academy Press; 2000. p. 53–71.
[5] Law RJ, Kohler M, Heeb NV, Gerecke AC, Schmid P, Voorspoels S, et al. Hexabromocyclododecane challenges scientists and regulators. Environ Sci Technol 2005;39:281A–7A.
[6] Sellstrom U, Kierkegaard A, de Wit C, Jansson B. Polybrominated diphenyl ethers and hexabromocyclododecane in sediment and fish from a Swedish river. Environ Toxicol Chem 1998;17:1065–72.
[7] Eljarrat E, de la Cal A, Raldua D, Duran C, Barcelo D. Occurrence and bioavailability of polybrominated diphenyl ethers and hexabromocyclododecane in sediment and fish from the Cinca River, a tributary of the Ebro River (Spain). Environ Sci Technol 2004;38:2603–8.
[8] Veith GD, De Feo DL, Bergstedt BV. Measuring and estimating the bioconcentration factor of chemicals in fish. J Fish Res Board Can 1979;36:1040–8.

Please cite this article in press as: Ema M, et al., Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats, Reprod Toxicol (2008), doi:10.1016/j.reprotox.2007.12.004

[9] Morris S, Allchin CR, Zegers BN, Haftka JJ, Boon JP, Belpaire C, et al. Distribution and fate of HBCD and TBBPA brominated flame retardants in North Sea estuaries and aquatic food webs. Environ Sci Technol 2004;38:5497–504.
[10] Covaci A, Gerecke AC, Law RJ, Voorspoels S, Kohler M, Heeb NV, et al. Hexabromocyclododecanes (HBCDs) in the environment and humans: a review. Environ Sci Technol 2006;40:3679–88.
[11] Darnerud PO. Toxic effects of brominated flame retardants in man and in wildlife. Environ Int 2003;29:841–53.
[12] Thomsen C, Frøshaug M, Broadwell SL, Becher G, Eggesbø. Levels of brominated flame retardants in milk from the Norwegian human milk study: HUMIS. Organohalog Comp 2005;67:509–12.
[13] Fängström B, Athanassiadis I, Strid A, Odsjö T, Guvenius D, Norén K, et al. Temporal trends of PBDES and HBCDD in milk from Stockholm mothers, 1980–2004. Organohalog Comp 2006;68:774–7.
[14] Ryan JJ, Wainman BC, Schecter A, Moisey J, Sun WF. Trends of the brominated flame retardants, PBDES and HBCD, in human milks from North America. Organohalog Comp 2006;68:778–81.
[15] Clayson DB, Krewski DR. Objectives of toxicity testing. In: Arnold DL, Grice HC, Krewski DR, editors. Handbook of in vivo toxicity testing. San Diego: Academic Press; 1990. p. 3–18.
[16] de Wit CA. An overview of brominated flame retardants in the environment. Chemosphere 2002;46:583–624.
[17] Birnbaum LS, Staskal DF. Brominated flame retardants: cause for concern? Environ Health Perspect 2004;112:9–17.
[18] Chengelis CP. A 28-day oral (gavage) toxicity study of HBCD in rats. WIL-186004. Arlington, VA: Brominated Flame Retardant Industry Panel. Chemical Manufacturers Association; 1997.
[19] Chengelis CP. A 90-day oral (gavage) toxicity study of HBCD in rats. WIL-186012. Arlington, VA: Brominated Flame Retardant Industry Panel. Chemical Manufacturers Association; 2001.
[20] van der Ven LT, Verhoef A, van de Kuil T, Slob W, Leonards PE, Visser TJ, et al. A 28-day oral dose toxicity study enhanced to detect endocrine effects of hexabromocyclododecane in Wistar rats. Toxicol Sci 2006;94:281–92.
[21] Eriksson P, Fischer C, Wallin M, Jakobsson E, Fredriksson A. Impaired behaviour, learning and memory, in adult mice neonatally exposed to hexabromocyclododecane (HBCDD). Environ Toxicol Pharmacol 2006;21:317–22.
[22] Stump D. A prenatal developmental toxicity study of hexabromocyclododecane (HBCD) in rats. WIL-186009. Arlington, VA: Brominated Flame Retardant Industry Panel. Chemical Manufacturers Association; 1999.
[23] Murai T, Kawasaki H, Kanoh S. Studies on the toxicity of insecticides and food additives in pregnant rats: fetal toxicity of hexabromocyclododecane. Pharmacometrics (Japan) 1985;29:981–6 [Japanese].
[24] OECD. OECD Test Guideline for Testing of Chemicals. Proposal for Updating Guideline 416, Two-generation Reproduction Toxicity Study (Organization for Economic Co-operation and Development); 2001.
[25] ME, MHLW and METI (Ministry of the Environment, Ministry of Health, Labour and Welfare, and Ministry of Economy, Trade and Industry, Japan). On standard of Testing Facility Conducting Studies Concerning New Chemical Substances, November 21, 2003, revised April 1, 2005.
[26] Altman J, Sudarshan K. Postnatal development of locomotion in the laboratory rat. Anim Behav 1975;23:896–920.
[27] Gallavan Jr RH, Holson JF, Stump DG, Knapp JF, Reynolds VL. Interpreting the toxicologic significance of alterations in anogenital distance: potential for confounding effects of progeny body weights. Reprod Toxicol 1999;13:383–90.
[28] Biel W. Early age differences in maze performance in the albino rats. J Genet Psychol 1940;56:439–45.
[29] Heindel JJ. Oocyte quantitation and ovarian histology. In: Daston G, Kimmel C, editors. An evaluation and interpretation of reproductive endpoints for human health risk assessment. Washington DC: International Life Sciences Institute Press; 1999. p. 57–74.
[30] Gad SC. Statistics for toxicologist. In: Hayes AW, editor. Principles and methods of toxicology. 4th ed. Philadelphia: Taylor and Francis Group; 2001. p. 285–364.
[31] Lochry EA. Concurrent use of behavioral/functional testing in existing reproductive and developmental toxicity screens: practical considerations. J Am Coll Toxicol 1987:433–9.
[32] ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use). ICH Harmonized Tripartite Guideline: Detection of Toxicity to Reproduction for Medicinal Products and Toxicity to Male Fertility, S5 (R2), Current Step 4 version; 2005.
[33] Heinrichs WL. Current laboratory approaches for assessing female reproductive toxicity. In: Dixon RL, editor. Reproductive toxicology. New York: Raven Press; 1985. p. 95–108.
[34] Korenbrot CC, Huhtaniemi IT, Weiner RI. Preputial separation as an external sign of pubertal development in the male rat. Biol Reprod 1977;17:298–303.
[35] Parker Jr CR, Mahesh VB. Hormonal events surrounding the natural onset of puberty in female rats. Biol Reprod 1976;14:347–53.
[36] Amacher DE, Schomaker SJ, Burkhardt JE. The relationship among microsomal enzyme induction, liver weight and histological change in rat toxicology studies. Food Chem Toxicol 1998;36:831–9.
[37] Germer S, Piersma AH, van der Ven L, Kamyschnikow A, Fery Y, Schmitz HJ, et al. Subacute effects of the brominated flame retardants hexabromocyclododecane and tetrabromobisphenol A on hepatic cytochrome P450 levels in rats. Toxicology 2006;218:229–36.
[38] Kezele P, Skinner MK. Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. Endocrinology 2003;144:3329–37.
[39] Forsberg JG, Olivecrona H. The effect of prenatally administered Busulphan on rat gonads. Biol Neonat 1966;10:180–92.
[40] Merchant H. Rat gonadal and ovarioan organogenesis with and without germ cells. An ultrastructural study. Dev Biol 1975;44:1–21.
[41] Hirshfield AN. Relationship between the supply of primordial follicles and the onset of follicular growth in rats. Biol Reprod 1994;50:421–8.
[42] Gray Jr LE. Chemically induced alterations of reproductive development in female mammals. In: Boeklheide K, Chapin RE, Hoyer PB, editors. Comprehensive toxicology (reproductive and endocrine toxicology). Oxford: Elsevier Science; 1997. p. 329–38.
[43] Gray Jr LE, Ostby JS, Kavlock RJ, Marshall R. Gonadal effects of fetal exposure to the azo dye congo red in mice: infertility in female but not male offspring. Fundam Appl Toxicol 1992;19:411–22.
[44] Christian MS, Brown RW. Control observations regarding primordial follicle counts in EPA multigeneration studies in Crl Sprague-Dawley ("Gold Standard") rats. Reprod Toxicol 2002;16:408.
[45] Bolon B, Bucci TJ, Warbritton AR, Chen JJ, Mattison DR, Heindel JJ. Differential follicle counts as a screen for chemically induced ovarian toxicity in mice: results from continuous breeding bioassays. Fundam Appl Toxicol 1997;39:1–10.
[46] Parker RM. Testing for reproductive toxicity. In: Hood RD, editor. Developmental and reproductive toxicology. 2nd ed. Boca Raton: CRC Press (Taylor & Francis group); 2006. p. 425–87.

Please cite this article in press as: Ema M, et al., Two-generation reproductive toxicity study of the flame retardant hexabromocyclododecane in rats, Reprod Toxicol (2008), doi:10.1016/j.reprotox.2007.12.004

Available online at www.sciencedirect.com

ScienceDirect

Reproductive Toxicology 25 (2008) 231–238

Reproductive Toxicology
Developmental Basis of Health and Disease
www.elsevier.com/locate/reprotox

Reproductive and developmental toxicity screening test of tetrahydrofurfuryl alcohol in rats

Mutsuko Hirata-Koizumi [a], Atsushi Noda [b], Akihiko Hirose [a], Eiichi Kamata [a], Makoto Ema [a,*]

[a] *Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Department of Toxicology, Research Institute for Animal Science in Biochemistry & Toxicology, Sagamihara, Japan*

Received 24 August 2007; received in revised form 7 November 2007; accepted 15 November 2007
Available online 22 November 2007

Abstract

Twelve male and female rats per group were given tetrahydrofurfuryl alcohol (THFA) by gavage at 0, 15, 50, 150 or 500 mg/kg/day. Males were dosed for 47 days, beginning 14 days before mating, and females were dosed for 42–52 days beginning 14 days before mating to day 4 of lactation throughout the mating and gestation period. Changes in locomotor activity, inhibition of body weight gain, and/or histopathological changes in the thymus, spleen, testes and/or epididymides were observed in males and females at 150 mg/kg and above. No effects of THFA were found on the copulation index, fertility index, or the number of corpora lutea and implantations in pregnant females. At 500 mg/kg, no pregnant females delivered any pups. At 150 mg/kg, gestation length was prolonged, and the total number of pups born and the number of live pups on postnatal days 0 and 4 was markedly decreased. No effects of THFA were found on the sex ratio and body weight of live pups, or the incidence of pups with malformations or variations. Based on these findings, the NOAELs for parental and reproductive/developmental toxicity of THFA were concluded to be 50 mg/kg/day in rats.

Keywords: Tetrahydrofurfuryl alcohol; Reproductive and developmental toxicity; Postimplantation loss; Postnatal loss; Testicular toxicity; Rat

1. Introduction

Tetrahydrofurfuryl alcohol (THFA; CAS No. 97-99-4) is a colorless and flammable liquid with a slight ether odor [1]. In Japan, the annual production and import volume of THFA was reported to be from 100 to 1000 tonnes in 2004 [2], but there is no data available on that in other countries. The major uses of this chemical are as a solvent for various products (fats, waxes, resins, dyes and others) and as an intermediate in industrial applications [1]. While the extensive use of THFA by industry creates significant potential for occupational exposure, there is also the possibility of exposure of the general population to THFA because some of the applications include consumer uses, such as floor polish removers, graffiti removers and oven cleaners [3]. In particular, THFA application as a solvent for nail-cleaning agents [1] and absorption enhancer in various lotions and transdermal medications [4] would cause relatively high levels of exposure due to direct use on the skin. Such occupational and consumer exposure could occur through inhalation and dermal routes. On the other hand, THFA is directly added to food as a flavoring agent in Japan [5], and its use as a food additive for flavoring is also permitted in the US [6] and EU [7]. Furthermore, this chemical is known as the "solvent of choice" for a variety of agricultural applications, including pest control, weed control and growth regulation [3]. These uses suggest possible exposure of the general population to THFA via food. For each application, there are no data available on the actual use volume and exposure levels at this time. The possibility of human exposure to THFA has aroused concern regarding its toxicological potential.

Only limited information is available about the toxicity of THFA. It was reported that oral LD_{50} was 1.6–3.2 g/kg in rats and 0.8–1.6 g/kg in guinea pigs, and inhalation exposure for 6 h caused 2/3 deaths of rats at 12,650 ppm [8]. THFA showed eye irritation in rabbits [9] but did not irritate mouse skin [10].

* 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.2007.11.006

Unpublished repeated dose toxicity data are briefly summarized in OECD SIDS (Screening Information Data Set) documents [1]. In a 90-day feeding study using rats, body weight gain was depressed at 1000 ppm and above, the relative weight of epididymides decreased at 5000 ppm and above, and relative testis weight decreased with moderate testicular degeneration accompanied with complete loss of spermatogenic activity observed at 10,000 ppm. Adverse effects on body weight gain and male reproductive organs were also found in a 90-day inhalation and dermal study of THFA using rats. As for reproductive and developmental toxicity, only a dose range-finding developmental toxicity study is available [11]. In rats given THFA by gavage on days 6–15 of pregnancy, total embryonic loss occurred in all females at 500 mg/kg and above, at which inhibition of maternal body weight gain was also observed. Fetuses with a filamentous tail (5/124 fetuses) and lowering of fetal weight were found at 100 mg/kg without maternal toxicity.

Since there is insufficient information on toxicity, this chemical was selected as an object substance in an existing chemical testing program by the Japanese government [12]. In this program, a reproduction/developmental toxicity screening test was performed according to OECD test guideline 421 [13], because the evaluation of reproductive and developmental toxicity is essential in the risk assessment of chemicals. The results are summarized in OECD SIDS documents [1] and an assessment report prepared by US EPA, "Hazard assessment for the tolerance reassessment of tetrahydrofurfuryl alcohol (THFA)" [14]; however, detailed data have not been published in scientific journals. In this paper, therefore, we reported the data of a reproduction/developmental toxicity screening test of THFA.

2. Materials and methods

This study was performed in compliance with OECD guideline 421 "Reproduction/Developmental Toxicity Screening Test" [13], and in accordance with the principles for Good Laboratory Practice [15,16] at the Research Institute for Animal Science in Biochemistry & Toxicology (Sagamihara, Japan). The experiment was approved by the Animal Care and Use Committee of the Research Institute for Animal Science in Biochemistry & Toxicology, and was performed in accordance with the ethics criteria contained in the bylaws of the Committee.

2.1. Animals and housing conditions

Crj:CD(SD)IGS rats (SPF, 8 weeks old) were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). This strain was chosen because it is most commonly used in toxicity studies, including reproductive and developmental toxicity studies, and historical control data are available. The animals were acclimatized to the laboratory for 13 days and subjected to treatment at 10 weeks of age. They were carefully observed during the acclimation period, and male and female rats found to be in good health were selected for use. In addition, vaginal smears of each female were recorded, and only females showing a 4- to 5-day estrous cycle were used in the experiment. On the day before initial treatment, the rats were distributed into 5 groups of 12 males and 12 females each by stratified random sampling based on body weight.

Throughout the study, animals were maintained in an air-conditioned room at 21.9–22.4 °C, with a relative humidity of 49–57%, a 12-h light/dark cycle, and ventilation with more than 10 air changes/h. A basal diet (Labo MR Stock; Nosan Corporation, Yokohama, Japan) and sterile water were provided *ad libitum*. They were housed individually, except for mating and nursing periods. From day 0 of pregnancy to the day of sacrifice, individual dams and/or litters were reared using wood chips as bedding (White Flake; Charles River Japan, Inc., Yokohama, Japan).

2.2. Chemicals and doses

THFA was obtained from Koatsu Chemical Industries, Ltd. (Osaka, Japan) and kept in a cool (4 °C) and dark place. The THFA (Lot no. 2002–4) used in this study was 99.5% pure, and stability during the study was verified by gas chromatography. The test article was dissolved in purified water (Kyouei Pharmaceutical Co. Ltd., Takaoka, Japan), and administered to the animals by gastric intubation. Control rats received the vehicle alone. Dosing solutions were prepared at least once a week and kept in a cool (4 °C) and dark place until dosing, as stability under these conditions has been confirmed for up to 7 days. The concentrations of THFA in the formulations were confirmed to be 97.7–103.0% of the target by gas chromatography analysis.

Prior to the present reproductive and developmental toxicity screening study, a 14-day dose-finding study was performed. In the dose-finding study, male and female rats were given THFA by gavage at 50, 100, 200, 500 or 1000 mg/kg/day for 14 days. Changes in locomotor activity were observed at 100 mg/kg and above, decreases in absolute and relative weight of the pituitary and thymus were detected at 200 mg/kg and above, and piloerection, decrease in food consumption and dilatation of the cecum were found at 500 mg/kg and above (data not shown). Taking into account the results of this dose-finding study, the dose levels of THFA in the present study were set as 15, 50, 150 or 500 mg/kg/day. The daily application volume (5 ml/kg body weight) was calculated according to the latest body weight.

2.3. Study design

Male rats were dosed once daily for 47 days, beginning 14 days before mating and throughout the mating period. Female rats were also dosed once daily from 14 days prior to mating, and throughout the mating and gestation periods, to day 4 of lactation. The total administration period was 42–52 days. The day of the first dosing was designated as day 0 of the administration/premating period.

During the first 14-day administration period (premating period), vaginal lavage samples of each female were evaluated daily for estrous cyclicity. After this premating period, female rats were transferred to the home cage of a male of the same group, and cohabited on a 1:1 basis until successful copulation occurred or the mating period of 2 weeks had elapsed. During the mating period, vaginal smears were examined daily for the presence of sperm, and the presence of sperm in the vaginal smear and/or a vaginal plug were considered as evidence of successful mating. The day of successful mating was designated as day 0 of pregnancy. Pregnant females were allowed to deliver spontaneously and nurse their pups, and the day on which parturition was completed by 9:30 was designated as day 0 of lactation or postnatal day (PND) 0.

Throughout the study, all parental animals were observed for clinical signs of toxicity at least twice a day. The body weight was recorded on days 0, 7, 14, 21, 28, 35, 42 and 46 of the dosing period in males, and on days 0, 7 and 14 of the premating period, on days 0, 7, 14 and 20 of the gestation period and on days 0 and 4 of the lactation period in females. Food consumption was recorded on days 0, 7, 21, 28, 35, 42 and 45 of the dosing period in males, and on days 0 and 7 of the premating period, on days 0, 7, 14 and 20 of the gestation period and on days 0 and 3 of the lactation period in females.

All surviving male rats were euthanized by exsanguination under ether anesthesia on the day after the last administration. All female rats showing successful reproductive performance were euthanized in a similar way on day 5 of lactation. Females that did not copulate were euthanized on the day after the 52nd administration. Females that had not completed parturition were euthanized 5 days after the expected day of parturition (day 22 of gestation). When total litter loss was observed, the dams were euthanized within 4 days. For all parental animals, the external surfaces were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. For females, the numbers of corpora lutea and implantation sites were recorded. In males, the testes and epididymides were removed and weighed. The pituitary, thymus and kidneys were also weighed in both sexes.

Histopathological evaluations were performed on the pituitary, thymus, testes, epididymides and ovaries of all animals in the control and highest dose groups. In addition, the spleen of five animals in the control group and of all animals in the highest dose group was examined as test substance-related changes were macroscopically found in this organ. As a result of histopathological examination, test substance-related changes were found in the thymus,

spleen, testes and epididymides of the highest dose group; therefore, the organs of five animals in the other groups were also examined histopathologically. For females that showed reproductive failure, the pituitary, ovaries, uterus and/or mammary gland were examined histopathologically. For the histopathological examination, the target organs were fixed in 10% neutral-buffered formalin (following Bouin's fixation for the testes and epididymides), processed routinely for embedding in paraffin, and sections were prepared for staining with hematoxylin–eosin.

All live and dead pups were counted, and live pups were sexed, examined grossly and weighed on PND 0. They were daily observed for clinical signs of toxicity on PNDs 0–4. On PND 4, the number and body weight of live pups was recorded. The pups were then euthanized by exsanguination under ether anesthesia, and gross internal examinations were performed.

2.4. Data analysis

Parametric data, such as body weight, food consumption, organ weight, gestation length and the number of corpora lutea, implantations and pups born, were analyzed by Bartlett's test for homogeneity of distribution. When homogeneity was recognized, one-way analysis of variance was performed. If a significant difference was detected, Sheffé's test was conducted for comparisons between control and individual treatment groups. Data without homogeneity or some non-parametric data (implantation index, live birth index, delivery index, variability index, the incidence of pups with malformations or variations) were analyzed using the Kruskal–Wallis's rank sum test. If significant differences were found, the mean rank test of Scheffé's type was conducted for comparison between the control and each dosage group.

For toxicological signs, autopsy results and histopathological findings, Fisher's exact test was conducted for comparison of the incidences in each group. The sex ratio of live pups was also compared by Fisher's exact test. The copulation index, fertility index and gestation index were compared using the χ^2-test.

Pups were statistically analyzed using the litter as the experimental unit. The 5% level of probability was used as the criterion for significance.

3. Results

3.1. Parental toxicity

One male of the 15 mg/kg group was found dead after the 22nd administration. No substance-related clinical signs of toxicity were detected at 15 and 50 mg/kg. Increase and decrease in locomotor activity was observed in 10/12 males and 11/12 females in the 150 mg/kg group and in all animals of the 500 mg/kg group. This change was found mainly in the first half of the administration period in both sexes at 150 mg/kg and in females at 500 mg/kg, and also in the second half of the administration period in males at 500 mg/kg. Vaginal hemorrhage was observed during the late gestation period in 1/11 pregnant female at 150 mg/kg and 2/12 pregnant females at 500 mg/kg, which did not deliver their pups or experienced total litter loss.

Table 1
Body weight of male and female rats given tetrahydrofurfuryl alcohol (THFA) by gavage

	Dose (mg/kg/day)				
	0	15	50	150	500
Males (no. = 12)					
Body weight during administration (g)					
Day 0	393 ± 17	394 ± 17	393 ± 14	392 ± 17	392 ± 16
Day 7	422 ± 23	420 ± 18	421 ± 16	419 ± 22	400 ± 18*
Day 14	448 ± 28	441 ± 21	445 ± 18	444 ± 24	424 ± 21
Day 21	470 ± 28	459 ± 29	469 ± 19	466 ± 24	443 ± 19*
Day 28	492 ± 31	482 ± 22	488 ± 21	482 ± 21	458 ± 22*
Day 35	516 ± 34	506 ± 24	510 ± 25	491 ± 22	472 ± 28*
Day 42	536 ± 38	524 ± 29	523 ± 28	505 ± 21	482 ± 31*
Day 46	550 ± 40	532 ± 29	533 ± ± 27	513 ± 21	489 ± 32**
Gain	157 ± 29	136 ± 19	140 ± 25	122 ± 16*	98 ± 23**
Females (no. = 12)					
Body weight during premating (g)					
Day 0	236 ± 15	234 ± 13	232 ± 14	235 ± 16	234 ± 14
Day 7	249 ± 14	244 ± 13	241 ± 14	243 ± 20	242 ± 15
Day 14	265 ± 18	255 ± 15	252 ± 18	260 ± 21	256 ± 16
Gain	29 ± 10	21 ± 7	20 ± 10	25 ± 9	22 ± 10
Body weight during gestation (g)					
Day 0	275 ± 23	266 ± 19	261 ± 18	259 ± 20	262 ± 20
Day 7	317 ± 24	304 ± 25	300 ± 23	301 ± 21	297 ± 18
Day 14	357 ± 23	339 ± 26	335 ± 27	332 ± 21	322 ± 20*
Day 20	438 ± 23	422 ± 31	411 ± 34	373 ± 27*	320 ± 20**
Gain	164 ± 9	156 ± 15	150 ± 18	114 ± 20*	58 ± 8**
Body weight during lactation (g)					
Day 0	343 ± 19	327 ± 28	321 ± 26	308 ± 17	
Day 4	361 ± 22	351 ± 34	341 ± 28	306	
Gain	18 ± 12	24 ± 13	20 ± 9	3	

Values are given as the mean ± S.D.
* Significantly different from the control group ($P < 0.05$).
** Significantly different from the control group ($P < 0.01$).

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 [1].

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

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.

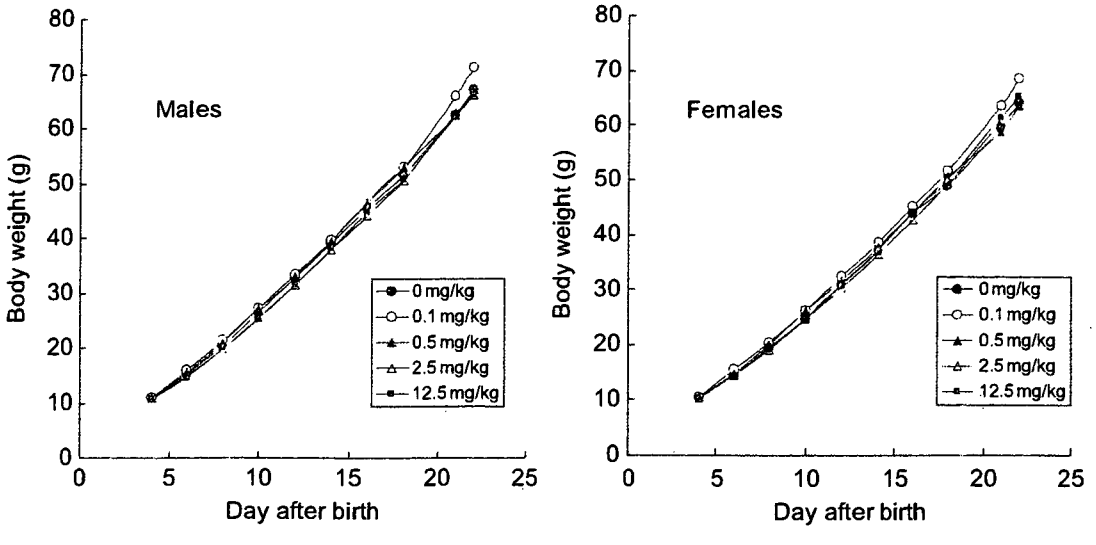

Figure 1: Body weight curves of male and female preweaning rats given HDBB by gavage.

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 ($p < 0.01$).