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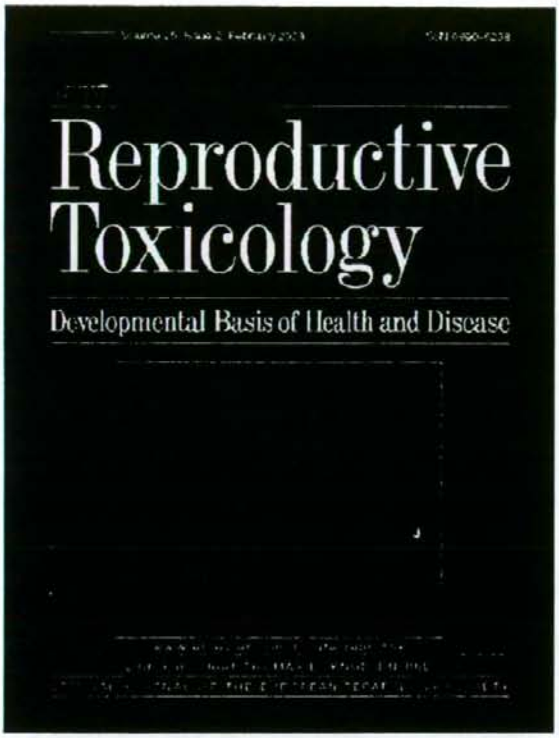
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Reproductive and developmental toxicity screening test of tetrahydrofurfuryl alcohol in rats

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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].

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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 (2.8 ± 0.3)	15.6 ± 2.0 (2.9 ± 0.4)	14.2 ± 1.3 (2.7 ± 0.3)	13.4 ± 1.5* (2.6 ± 0.3)	12.2 ± 1.2** (2.5 ± 0.2)
Kidneys (g)	3.10 ± 0.18 (0.57 ± 0.04)	3.15 ± 0.32 (0.59 ± 0.07)	3.09 ± 0.20 (0.58 ± 0.05)	2.90 ± 0.20 (0.56 ± 0.03)	2.71 ± 0.20** (0.55 ± 0.03)
Thymus (g)	0.36 ± 0.07 (0.07 ± 0.01)	0.32 ± 0.06 (0.06 ± 0.01)	0.35 ± 0.06 (0.07 ± 0.01)	0.31 ± 0.07 (0.06 ± 0.01)	0.19 ± 0.05** (0.04 ± 0.01**)
Testes (g)	3.41 ± 0.50 (0.63 ± 0.11)	3.18 ± 0.83 (0.60 ± 0.15)	3.52 ± 0.29 (0.66 ± 0.07)	3.40 ± 0.45 (0.66 ± 0.10)	1.77 ± 0.44** (0.36 ± 0.09**)
Epididymides (g)	1.40 ± 0.20 (0.26 ± 0.04)	1.30 ± 0.30 (0.24 ± 0.05)	1.38 ± 0.15 (0.26 ± 0.03)	1.26 ± 0.17 (0.24 ± 0.04)	0.87 ± 0.15** (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 (5.5 ± 0.8)	18.3 ± 1.7 (5.3 ± 0.3)	17.6 ± 1.8 (5.2 ± 0.5)	16.0 ± 1.9* (5.1 ± 0.2)	
Kidneys (g)	2.06 ± 0.19 (0.57 ± 0.04)	2.00 ± 0.22 (0.57 ± 0.06)	2.06 ± 0.23 (0.61 ± 0.05)	1.98 ± 0.25 (0.63 ± 0.05*)	
Thymus (g)	0.30 ± 0.08 (0.08 ± 0.02)	0.28 ± 0.09 (0.08 ± 0.03)	0.26 ± 0.07 (0.08 ± 0.02)	0.22 ± 0.05 (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.

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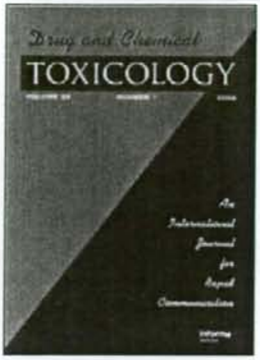

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A 52-Week Repeated Dose Toxicity Study of Ultraviolet Absorber 2-(2'-Hydroxy-3',5'-di-**tert**-butylphenyl)benzotriazole in Rats

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

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

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

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INTRODUCTION

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

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

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

MATERIALS AND METHODS

This study was performed in compliance with the OECD Guideline 452 "Chronic Toxicity Studies" (OECD, 1981) and in accordance with the principles for Good Laboratory Practice (OECD, 1998; EA, MHW and MITI, 2000) at the Safety Assessment Laboratory, Panapharm Laboratories Co., Ltd. (Kumamoto, Japan).

Chemicals

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

Animals

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

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

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

Experimental Design

Male and female rats were given HDBB once-daily by gavage for 52 weeks at 0 (vehicle control), 0.1, 0.5, or 2.5 mg/kg/day and at 0, 0.5, 2.5, or 12.5 mg/kg/day, respectively. The dosage levels were determined based on the results of our previous 28-day repeated dose toxicity study in rats given HDBB by gavage at 0.5, 2.5, 12.5, or 62.5 mg/kg/day, in which adverse effects, mainly on the liver and hearts, were found at all doses in males, and at 12.5 mg/kg and more in females (Hirata-Koizumi et al., 2007). The volume of each dose was adjusted to 5 mL/kg of body weight, based on the latest body weight. At the end of the 13-week administration period, 10 males and 10 females from each group were euthanized for the assessment of hematology, blood biochemistry, organ weights, and macroscopic and microscopic findings. The remaining animals in all groups (10 rats/sex/dose) were fully examined at the completion of the 52-week administration period.

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

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

Following the collection of blood, all animals were sacrificed by exsanguination, and organs and tissues of the entire body were macroscopically observed. The brain, pituitary, thymus, thyroids (including parathyroids), heart, lungs (including bronchus), liver, spleen, kidneys, adrenals, testes, epididymides, ovaries, and uterus were then excised and weighed. The trachea, pancreas, lymph nodes (mandibular and mesenteric), tongue, sublingual gland, submandibular gland, parotid gland, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, eyeballs, optic nerve, Harderian gland, spinal cord (pectoral and lumbar part), sciatic nerve, seminal vesicles, prostates, vagina, mammary gland, aorta (thoracic), bone (sternum and femur including bone marrow), skeletal muscle (biceps femoris muscle), and skin (hypogastric) as well as the above organs were fixed in 10% neutral-buffered formalin solution (following Bouin's fixation for testes and epididymides, and 2.5% glutaraldehyde fixation for eyeballs, optic nerve, and Harderian gland). Histopathological examination of these organs was conducted for all animals found dead or moribund, and for scheduled-sacrifice animals in the control and highest dose groups. In addition, the livers of males in the lowest dose group and of both sexes in the middle-dose group were examined, since test substance–related changes were found in the higher group. Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin and eosin.

Data Analysis

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

RESULTS

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

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

Examination at Completion of the 13-Week Administration Period

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

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

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

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

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

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

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

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

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

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

Examination at Completion of the 52-Week Administration Period

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

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

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

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

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

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

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