most enzymes have matured to adult activity levels by the first year of life, but cytochrome P450-mediated metabolism, glucuronidation, glutathione conjugation and acetylation are generally deficient in the neonate. Regarding renal clearance, although the adult function is also approached by 1 year of age, the faster development of filtering than absorptive or secretory functions results in a glomerulotubular imbalance. The lack of a balanced detoxication ability during the newborn period would be expected to affect toxicity of chemicals.

For the toxicity evaluation of various kinds of chemicals, repeated dose and reproductive/developmental toxicity studies have been generally conducted. However, the effects of direct exposure to chemicals during the newborn period have not been taken into account. Furthermore, there were no sufficient data on the differences between the newborn and young/adult in the susceptibility to the toxicity of chemicals. Therefore, for the purpose of understanding the sensitivity of the newborn and utilizing it in the toxicity evaluation, we conducted the repeated dose toxicity studies using newborn rats, and analyzed the differences of the sensitivity from that of young rats, which have been recently used to evaluate the chemical toxicity in general. These comparative studies were conducted as a part of an existing chemical testing program of Japan. As the candidate chemicals, phenolic and halogenated compounds were selected among chemicals in this program, considering the potential for endocrine disrupting action in the early development period. Because of no standard experimental protocol, repeated dose toxicity studies in newborn rats were conducted with our newly established protocol (Koizumi *et al.*, 2001), including a detailed examination of early development and a complete toxicity analysis after a sufficient recovery-maintenance period. The results were compared with those of a 28-day repeated dose toxicity study using young rats, which is generally conducted as a screening test in existing chemical testing program in Japan. For more precise comparison, in addition to the no observed adverse effect levels (NOAELs), we estimated unequivocally toxic levels, defined as doses inducing clear toxicity, including clinical toxic signs, death or critical histopathological damage. In order to estimate more appropriate NOAELs and unequivocally toxic levels than those depending on the dosages of main studies, the results of dose-finding studies for each case were incorporated. Earlier, we reported analytical results for five chemicals (4-nitrophenol, 2,4-dinitrophenol, 3-aminophenol, 3-methylphenol, tetra-

bromobisphenol A) (Koizumi *et al.*, 2001, 2002, 2003; Fukuda *et al.*, 2004). The susceptibility of newborn rats to the toxicity of the first four was 2 to 4 times higher than that of their young counterparts, although these chemicals had no impact on development in the newborn period and showed similar toxicity profiles in both age groups (mainly effects on the central nervous system). In the case of tetrabromobisphenol A, a specific rather than enhanced renal toxicity was observed in newborn rats.

In the present study, two halogenated alkanes, 1,3-dibromopropane (DBP) and 1,1,2,2-tetrabromoethane (TBE), were chosen as the sixth and seventh chemicals for comparative toxicity analysis, because these two chemicals have similar properties such as analogous chemical structures and hepatotoxicity after hepatic metabolism, and the lower susceptibility of the newborn to these chemicals was expected in preliminary analysis, contrary to all outcomes of previous analyses. There has hitherto been no sufficient information on toxicity of DBP, an intermediate in the production of pharmaceutical agents (Chemical Products' Handbook, 2004), except that the intraperitoneal lowest lethal dose is 750 mg/kg in mice (Sax, 1979). Applications of TBE are various as a fire retardant, in oils and fats, in solvents, for ore dressing, and as a reagent for microscopic examination and as a catalyst (Chemical Products' Handbook, 2004). Regarding its toxicity, inhalation exposure to TBE for 180-184 days (7 hr/day, 5 days/week) caused slight edema and congestion in lungs and slight centrilobular fatty degeneration in the livers of mice, rats, guinea pigs and rabbits at an average concentration of 4 ppm (Hollingsworth *et al.*, 1963). Gavage studies for 3 weeks using F344/N male rats have been conducted on many halogenated ethanes to examine renal toxicity, but all rats administered TBE (214 mg/kg/day and more) died or were killed on becoming moribund by dosing Day 11 (NTP, 1996). Cytoplasmic vacuolization of hepatocytes was observed in these rats. We have conducted the newborn rat studies on DBP and TBE and evaluated the results in comparison with published findings in young rats (MHLW, 2003a, 2003b), in the same manner as for the five chemicals already documented (Koizumi *et al.*, 2001, 2002, 2003).

MATERIALS AND METHODS

Materials

1,3-Dibromopropane (DBP, CAS No. 109-64-8, purity: 99.8%) and 1,1,2,2-tetrabromoethane (TBE,

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Susceptibility of newborn rats to 1,3-dibromopropane and 1,1,2,2-tetrabromoethane.

CAS No. 79-27-6, purity: 99.2%) were obtained from TOSOH CORPORATION (Tokyo, Japan), and dissolved in corn oil and olive oil, respectively. Test solutions were prepared at least once a week and kept cool and in the dark until dosing. The stability was confirmed to be at least 7 days under these conditions. All other reagents used in this study were specific purity grade.

Animals

Sprague-Dawley SPF rats [Crj:CD(SD)IGS] were purchased from Charles River Japan Inc. (Kanagawa, Japan) and maintained in an environmentally controlled room at 19-27°C with a relative humidity of 32-75%, a ventilation rate of more than 10 times per hour, and a 12:12 hr light/dark cycle. For 18-day newborn rat studies of DBP and TBE, 20 pregnant rats (gestation Day 14) were purchased for each and allowed to deliver spontaneously. All newborn were separated from dams at postnatal Day 3 (the date of birth was defined as postnatal Day 0), and those with good health without external abnormality were pooled according to sex. Groups of 12 males and 12 females were selected and assigned to each of the 4 dose groups, including the controls, by stratified random sampling based on the body weight. Twelve foster mothers were selected based on health and nursing conditions, and suckled the 4 males and 4 females assigned to each group up to weaning on postnatal Day 21 (termination of dosing). After weaning, the animals of the recovery-maintenance group (see Study design) were individually maintained for 9 weeks. In the 28-day study of young rats, 4 week-old rats were obtained and used at ages of 5-6 weeks after acclimation. All animals were allowed free access to basal diet (CRF-1: Oriental Yeast Co. Ltd., Tokyo, Japan, or LABO MR Stock: Nihon Nosan Kogyo Inc., Yokohama, Japan) and water (tap water or well water treated with sodium hypochlorite).

Study design (Time schedule as reported previously (Koizumi et al., 2001))

1. 18-Day repeated dose study in newborn rats

In a dose-finding study, DBP was administered by gastric intubation to newborn rats (5/sex/dose) from postnatal Days 4 to 21 and TBE from postnatal Days 4 to 20. The dosages were set at 0, 10, 30, 100 or 200 mg/kg/day for DBP and at 0, 12, 50 or 200 mg/kg/day for TBE, based on the results of young rat study, mentioned below. They were examined for general behavior and body weights during the dosing period, and

sacrificed at postnatal Day 21 or 22 for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

In the main study, newborn rats (12/sex/dose) were administered test substances by gastric intubation from postnatal Days 4 to 21. Based on results of the dose-finding study, the dosage was set at 10, 50 or 150 mg/kg/day for DBP and 3, 12 or 50 mg/kg/day for TBE. On postnatal Day 22, 6 males and 6 females in each treated group were sacrificed (the scheduled-sacrifice group) and the rest of animals in all groups (6/sex/dose) were maintained for 9 weeks without chemical treatment and then sacrificed at 12 weeks of age (the recovery-maintenance group). During the study, general behavior, body weight and food consumption (only the recovery-maintenance period) were examined at least once a day. In addition, some developmental parameters were assessed, such as surface righting and visual placing reflex for reflex ontogeny, fur appearance, incisor eruption and eye opening for external development, and preputial separation, vaginal opening and estrous cycle for sexual development. Urinalysis (color, pH, occult blood, protein, glucose, ketone bodies, bilirubin, urobilinogen, sediment, volume of the urine, osmotic pressure) was conducted in the late recovery-maintenance period.

At weaning age of postnatal Day 22 after the last treatment, blood was collected under anesthesia from the abdomen of all animals in the scheduled-sacrifice group. In the recovery-maintenance group, it was conducted at 85 days of age after overnight starvation. One portion of the blood was treated with EDTA-2K and examined for hematological parameters such as the red blood cell count (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, white blood cell count, platelet count, reticulocyte count and differential leukocyte count. In the recovery-maintenance group, blood was also treated with 3.8% sodium citrate and blood clotting parameters such as prothrombin time and activated thromboplastin time were examined. Serum or plasma from the remaining portions of blood were analyzed for blood biochemistry (total protein, albumin, albumin-globulin (A/G) ratio, glucose, total cholesterol, triglycerides, phospholipid, total bilirubin, urea nitrogen, creatinine, glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), alkaline phosphatase, γ-glutamyl transpeptidase (γ-GTP), calcium, inorganic phosphorus, sodium, potassium, chlorine). Following collection of blood, all animals were

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sacrificed by exsanguination, and organs and tissues of the entire body were macroscopically observed. The brain, pituitary gland, thymus, thyroids, heart, lungs, liver, spleen, kidneys, adrenals, testes, epididymides, ovaries and uterus were weighed, and fixed in 10% buffered formalin-phosphate (following Bouin's fixation for testes and epididymides). Paraffin sections were routinely prepared and stained with hematoxylin-eosin for microscopic examination. All studies were conducted in compliance with the Good Laboratory Practice Act of the Japanese Government.

2. 28-Day repeated dose study in young rats

In a dose-finding study, DBP and TBE were administered by gastric intubation to five-week old rats (5 or 4/sex/dose) for 14 days. The dosages were determined at 0, 20, 60, 200 or 600 mg/kg/day for DBP, and at 0, 10, 20, 50, 100 or 200 mg/kg/day for TBE, based on the results of the preliminary single-dose study. The general behavior, body weight and food consumption were examined, and the animals were sacrificed the day after the last treatment for assessment of hematology, blood biochemistry, macroscopic findings and organ weights.

Referring to the results of the dose-finding study, doses in a main study were set at 10, 50 and 250 mg/kg/day for DBP and at 6, 20, 60 and 200 mg/kg/day for TBP. In the main study, 5-6 week old rats were given the test substances by gastric intubation daily for 28 days and sacrificed after overnight starvation following the last treatment (scheduled-sacrifice group). Recovery groups (0, 50, 250 mg/kg/day for DBP and 0, 200 mg/kg/day for TBE) were maintained for 2 weeks without chemical treatment and sacrificed at 11 or 12 weeks of age. The number of animals for each sex/dose for both scheduled-sacrifice and recovery cases was 6 for DBP and 5 for TBE. Rats were examined for general behavior, body weight, food consumption, urinalysis, hematology and blood biochemistry, necropsy findings, organ weights and histopathological findings in compliance with the Test Guideline in the Japanese Chemical Control Act (Official Name: Law Concerning the Examination and Regulation of Manufacture, etc. of Chemical Substances) under Good Laboratory Practice conditions.

Statistical analysis

Parametric data such as body weights, food consumption, urinalysis findings (except for the results of qualitative analysis), 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 (p < 0.01 or 0.05). If not homogenous, the data were analyzed using Steel's multiple comparison test (Steel, 1959) or the mean rank test of the Dunnett type (Hollander and Wolfe, 1973) (p < 0.01 or 0.05). If the number of groups was two, parametric data were analyzed by the F test (Snedecor and Cochran, 1967). When homogeneity was recognized, the Student's *t*-test (Steel and Torrie, 1980) was conducted and if not, the Aspinn-Welch's *t* test (Snedecor and Cochran, 1967) (p < 0.01 or 0.05). For histopathological findings, the Mann-Whitney's U test (Mann and Whitney, 1947) or the Fisher's exact test (Fisher, 1973) were performed (p < 0.01 or 0.05). In the newborn study, the chi square test (Fisher, 1922) was conducted for physical and sexual development and reflex ontogeny (p < 0.01 or 0.05).

Judgment criteria for NOAEL and the unequivocally toxic level

NOAEL is the greatest dose at which no adverse effects are observed. In the case of hepatotoxicity, increased liver weights or changes in biochemical parameters alone are not considered to be adverse effects. The unequivocally toxic level has been used only for our comparative toxicity analysis as a clear toxic dose. However, it is generally not readily definable because it depends on the type of toxicity. In this study, centrilobular hypertrophy of hepatocytes was observed as a major histopathological change with both chemicals. Appearance of hypertrophic hepatocytes may not be considered to be a sign of clear toxicity because it is not usually accompanied by increase in GOT and GPT, typically found with hepatotoxic agents. Therefore, for the special purposes of this study, the unequivocally toxic level was estimated on the basis of concomitant changes in organ weights, histopathology, biochemical parameters and body weights.

RESULTS

1,3-Dibromopropane (DBP)
1. 18-Day study in newborn rats (including the dose-finding study)

In the dose-finding study at doses of 10, 30, 100 and 200 mg/kg, 2 of 5 males and 2 of 5 females of the highest group died on dosing Days 2 to 3, but no

change in general behavior was observed in the others. In the 200 mg/kg group, body weights were also lower by 15-25% than the control values from dosing Day 4 in males and from dosing Day 8 in females. Blood biochemical examination showed a slight increase in total cholesterol in females given 200 mg/kg. For organ weight, increases in relative liver weights were demonstrated in both sexes at 100 mg/kg and more with absolute liver weights in males at 100 mg/kg. Decrease in absolute and relative testis weights were also observed in males of 200 mg/kg group. At autopsy, there were no gross abnormalities except hepatomegaly in all animals, including the dead rats at 200 mg/kg. Based on these results, 10, 50 and 150 mg/kg were selected as the doses for the main study in newborn rats.

In the main study, no change in general behavior was noted during the dosing period in any dose group. Body weights of both sexes given 150 mg/kg were lowered during the dosing period (Fig.1) and gain was also decreased by approx. 10%. No definitive changes in parameters for external and sexual development and reflex ontogeny were detected in any dose group. At the scheduled sacrifice, blood biochemical examination of the 150 mg/kg group showed increases in γ-GTP in males and total bilirubin in females. There were no dose-related changes in hematological parameters. Significant increase of absolute and relative liver weights was noted in males given 50 mg/kg and in both

sexes given 150 mg/kg. The relative liver weights were also increased in females at 10 and 50 mg/kg. Absolute brain weights were lower in both sexes given 150 mg/kg, this being considered due to the lowered body weights. On histopathological examination, hypertrophy of centrilobular hepatocytes was noted in all animals given 150 mg/kg, being mild in 3/6 males and 4/6 females (Table 1). In four of each sex, the endoplasmic reticulum in hypertrophic hepatocytes showed a ground glass appearance. In addition, single cell necrosis was also noted in 3/6 males and 1/6 females at 150 mg/kg. During and at the end of the recovery-maintenance period, the changes observed in scheduled sacrificed group had disappeared.

The results of the dose-finding study and main study of DBP in newborn rats are summarized in Table 2. The NOAEL was concluded to be 50 mg/kg/day because increase in liver weight without biochemical and histopathological changes in this dose of the main study was not considered as an adverse effect. The unequivocally toxic level was concluded to be 150 mg/kg/day, based on increase of liver weight, mild centrilobular hypertrophy of hepatocytes, increase of γ-GTP and total bilirubin, and lowering of body weights at this dose in the main study, taking additional account of the 40% mortality rate at 200 mg/kg in the dose-finding study.
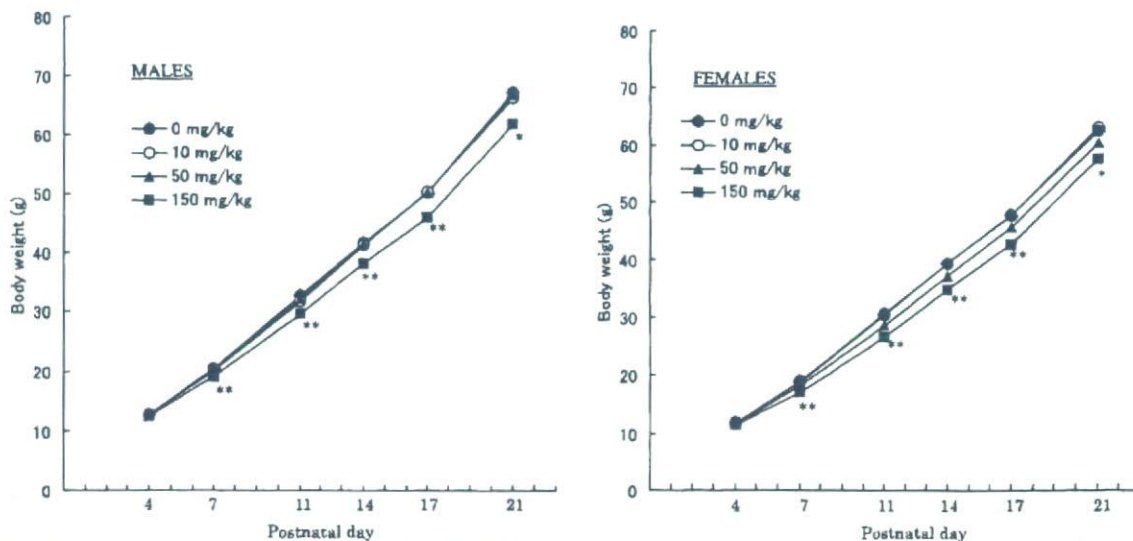

Fig. 1. Body weight curves for the 18-day study of 1,3-dibromopropane in newborn rats.
*: Significantly different from the controls (p < 0.05), **: Significantly different from the controls (p < 0.01).

2. 28-Day study in young rats (including the dose-finding study)

In the 14-day dose-finding study at doses of 20, 60, 200 and 600 mg/kg, all animals died within 6 days after the first treatment in the highest group. They showed various toxic signs such as decrease in spontaneous movement, oligopnea and adoption of a prone/lateral position. Blood biochemical examination showed increase in total protein in males and in total cholesterol in females at 200 mg/kg. Increase in absolute and relative liver weights was observed in both sexes of the 60 and 200 mg/kg groups and relative liver weights in males of 10 mg/kg. In addition, increase

was found in relative kidney weights in males and in absolute and relative kidney and heart weights in females at 200 mg/kg. There were no other dose-related changes evident. Based on the results, 250 mg/kg, at which it was predicted that clear toxic signs would appear, was selected as the top dose for the main study, and by one-fifth division 50 and 10 mg/kg were derived.

In the main study, salivation was observed from dosing Day 12 in 5 to 10 of each sex given 250 mg/kg. In males at this dose, body weights were significantly lowered by approx. 10% from dosing Day 18, in spite of no dose-related change in food consumption. On

Table 1. Histological findings for the liver after 18-day repeat dosing of 1,3-dibromopropane in newborn rats (main study).

	Grade	Dose (mg/kg)			
		0	10	50	150
Males					
No. of animals examined		6	6	6	6
Liver					
– Single cell necrosis	±	0	0	0	3
– Centrilobular hypertrophy of hepatocytes	±	0	0	0	3
	+	0	0	0	3
				*	
Females					
No. of animals examined		6	6	6	6
Liver					
– Single cell necrosis	±	0	0	0	1
– Centrilobular hypertrophy of hepatocytes	±	0	0	0	2
	+	0	0	0	4
				*	

±: Slight, +: Mild, *: Significantly different from the control group (p<0.01).

Table 2. Summary of the results of the repeated dose studies of 1,3-dibromopropane in newborn rats.

	Dose-finding Study (5 rats/sex/dose)				Main Study (6 rats/sex/dose)		
Dose (mg/kg/day)	10	30	100	200	10	50	150
Toxic Effects							
– Death (No. of dead animals)	0	0	0	2M, 2F	0	0	0
– Body weight	–	–	–	15-25%↓	–	–	10%↓
– Blood biochemical parameters	–	–	–	F: Cho (↑)	–	–	M: GTP↑ F: TB↑
– Relative liver weight	–	–	↑	↑	F: ↑	↑	↑
– Histopathological changes ±	n.d.	n.d.	n.d.	n.d.	0	0	3M, 2F
(No of animals with the findings*) +	n.d.	n.d.	n.d.	n.d.	0	0	3M, 4F

±: Slight change, +: Mild change, M: Males, F: Females, ↑: Increase, ↓: Decrease, (↑): Slight increase, –: No change, Cho: Total cholesterol, GTP: γ-GTP, TP: Total protein, n.d.: No available data, *Centrilobular hypertrophy of hepatocytes.

Susceptibility of newborn rats to 1,3-dibromopropane and 1,1,2,2-tetrabromoethane.

hematological examination at the scheduled sacrifice, slight anemic changes with decrease in Hb and Ht, and an increased reticulocyte ratio were observed in females receiving 250 mg/kg. At 250 mg/kg, many blood biochemical parameters, including total protein, albumin, total cholesterol, triglycerides, phospholipids and total bilirubin, were also increased with an upward trend of GOT and GPT. With 50 mg/kg, slight increase in total protein was only observed in males. Significant increases were found in absolute and relative liver weights of both sexes at 250 mg/kg and in relative liver weights of females at 50 mg/kg. There was also increase in relative heart weights and relative kidney weights in both sexes of the 250 mg/kg group. On histopathological examination, slight to mild centrilobular hypertrophy of hepatocytes was observed at 50 mg/kg and more (Table 3). Perilobular vacuolation of hepatocytes tended to decrease with the dose. Most of the above changes became less prevalent or disappeared during the recovery period. However, body weights remain lower throughout this period in males and the relative liver and heart weights continued to be

Table 3. Histological findings in the repeated dose study of 1,3-dibromopropane in young rats (main study).

	Grade	Scheduled-sacrifice group (mg/kg)				Recovery group (mg/kg)		
		0	10	50	250	0	50	250
Males								
No. of animals examined		6	6	6	6	6	6	6
Liver								
– Centrilobular hypertrophy of hepatocytes	±	0	0	4	2	0	–	0
	+	0	0	0	4	0	–	0
				*				
				**				
– Perilobular vacuolation of hepatocytes	±	0	1	2	5	5	–	6
	+	6	5	4	1	1	–	0
				**				
Spleen								
– Extramedullary hematopoiesis	±	5	–	–	5	6	3	0
	+	0	–	–	1	0	3	6
	++	1	–	–	0	0	0	0
							**	
– Deposits of brown pigment	±	6	–	–	6	6	6	1
	+	0	–	–	0	0	0	5
							**	
Females								
No. of animals examined		6	6	6	6	6	6	6
Liver								
– Centrilobular hypertrophy of hepatocytes	±	0	0	3	2	0	–	0
	+	0	0	0	4	0	–	0
				**				
– Perilobular vacuolation of hepatocytes	±	1	1	4	5	4	–	5
	+	5	5	2	1	2	–	1
				*				
Spleen								
– Extramedullary hematopoiesis	±	6	–	–	5	6	6	4
	+	0	–	–	1	0	0	2
– Deposits of brown pigment	±	6	–	–	5	4	5	1
	+	0	–	–	1	2	1	5

±: Slight, +: Mild, ++: Moderate, *: Significantly different from the control group (p<0.05),
**: Significantly different from the control group (p<0.01).

high in females at 250 mg/kg. At the same time, decreases in RBC, Hb, Ht and increase in the reticulocyte ratio appeared in males given 250 mg/kg with an increased incidence of extramedullary hematopoiesis and deposits of brown pigment in the spleen (Table 3).

Summary of the results of the dose-finding and main study of DBP in young rats are shown in Table 4. The NOAEL was concluded to be 10 mg/kg/day from the main study, as the 20 mg/kg in dose-finding study was not appropriate because of the lack of histopathological examination. The unequivocally toxic level was concluded to be 250 mg/kg/day, at which increase of liver weight, mild centrilobular hypertrophy of hepatocytes, increase of many biochemical parameters with an upward trend of GOT and GPT, slight anemic effects and lowering body weight were observed in the main study.

1,1,2,2-Tetrabromoethane (TBE)
1. 18-Day study in newborn rats (including the dose-finding study)

In the dose-finding study, when newborn rats were given TBE at 12, 50 and 200 mg/kg, hypoactivity and bradypnea were observed during the dosing period in all animals of the high dose group, the body weights being lowered by 10-20% in both sexes at dosing Days 8 to 17. On blood biochemical examination for this group, slight increase in total bilirubin was found in both sexes. In addition, absolute and relative liver weights were increased in females receiving the 50 mg/kg and both sexes of the 200 mg/kg group, and relative liver weights in females of the 12 mg/kg and males of the 50 mg/kg groups. There were also increases in relative kidney weights of females and decreases in abso-

lute spleen weights of both sexes and relative spleen weights of females at 200 mg/kg. No significant changes were observed on hematological and gross examination. Based on these results, it was predicted that some hepatotoxicity would be observed at 50 mg/kg, which was selected as the top dose in the main study, and 3 and 12 mg/kg were derived by approx. one-fourth divisions.

In the main study, no significant changes were noted in general behavior and body weight (Fig.2). There were also no definitive changes in the parameters for external and sexual development and reflex ontogeny at any dose. At scheduled sacrifice, blood biochemical examination in the 50 mg/kg group showed only a slight increase in total protein in males. There were also increases in absolute and relative liver weights in both sexes, relative kidney weights in males and relative heart weights in females of the 50 mg/kg group. After the recovery-maintenance period, no significant changes were observed in blood biochemical findings and in kidney and heart weights, but the relative liver weights still remained high in males at 50 mg/kg. There were no dose-related changes in food consumption, urinalysis, hematology and histopathology throughout the study, including the recovery-maintenance period.

As shown in summary of the results in Table 5, in the 50 mg/kg group, relative liver weights were increased in both dose-finding and main studies, and total protein was slightly increased only in males of the main study. These changes without histopathological alteration were not considered adverse effects. Therefore, the NOAEL was concluded to be 50 mg/kg/day. Unfortunately, no histopathological changes in the

Table 4. Summary of the results of the repeated dose studies of 1,3-dibromopropane in young rats.

Dose (mg/kg/day)	Dose-finding Study (5 rats/sex/dose)				Main Study(6 rats/sex/dose)		
	20	60	200	600	10	50	250
Toxic Effects							
−Death (No. of dead animals)	0	0	0	5M, 5F	0	0	0
−Body weight	−	−	−	n.d.	−	−	M: 10%↓
−Blood biochemical parameters	−	−	M: TP↑ F: Cho↑	n.d.	−	M: TP (↑)	Many↑
−Relative liver weight		M: ↑	↑	n.d.	−	F: ↑	↑
−Histopathological changes ±	n.d.	n.d.	n.d.	n.d.	0	4M, 3F	2M, 2F
(No of animals with the findings*) +	n.d.	n.d.	n.d.	n.d.	0	0	4M, 4F

±: Slight change, +: Mild change, M: Males, F: Females, ↑: Increase, ↓: Decrease, (↑): Slight increase, −: No change,
Cho: Total cholesterol, TP: Total protein, Many: Many parameters including Cho, TP, albumin, triglycerides, phospholipids and total bilirubin, n.d.: No available data, * Centrilobular hypertrophy of hepatocytes.

liver were observed at the highest dose of 50 mg/kg in the main study, meaning that the dose setting was not appropriate. Therefore, an unequivocally toxic level could not be estimated. The dose of 200 mg/kg in the dose-finding study was clearly toxic because of effects on the central nervous system (hypoactivity and bradypnea) and lowering of body weight (10-20% reduction), although no histopathological examination was conducted.

2. 28-Day study in young rats (including the dose-finding study)

In the dose-finding study with 14-day exposure at 0, 10, 20, 50, 100 or 200 mg/kg, there were no significant changes in body weight, food consumption and urinalysis at any dose. Hematological examination showed increase in reticulocytes of both sexes at 200 mg/kg, and decrease in Hb in both sexes at 200 mg/kg and in males at 100 mg/kg, as well as Ht in males at 100 and 200 mg/kg and RBC in females at 200 mg/kg. On blood biochemical examination, increases in total
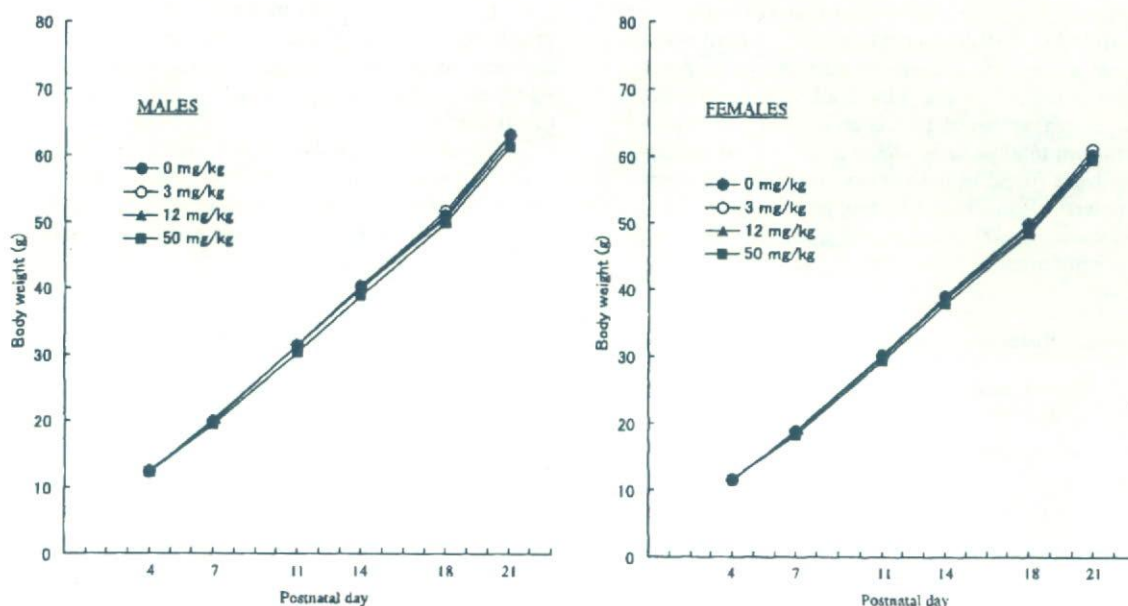

Fig. 2. Body weight curves in the 18-day study of 1,1,2,2-tetrabromoethane in newborn rats. Not significantly different from the controls.

Table 5. Summary of the results of the repeated dose studies of 1,1,2,2-tetrabromoethane in newborn rats.

Dose (mg/kg/day)	Dose-finding Study (4 rats/sex/dose)			Main Study (6 rats/sex/dose)		
	12	50	200	3	12	50
Toxic Effects						
−Death (No. of dead animals)	0	0	0*	0	0	0
−Body weight	−	−	10-20%↓	−	−	−
−Blood biochemical parameters	−	−	TB (↑)	−	−	M: TP (↑)
−Relative liver weight	F: ↑	↑	↑	−	−	↑
−Histopathological changes (No of animals with the findings)	n.d.	n.d.	n.d.	0	0	0

M: Males, F: Females, ↑: Increase, ↓: Decrease, (↑): Slight increase, −: No change, TB: Total bilirubin, TP: Total protein, n.d.: No available data, *Although there were no deaths in this group, hypoactivity and bradypnea were observed in all animals.

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cholesterol in both sexes, and total protein and triglycerides in females were noted at 200 mg/kg. In addition, increase in total cholesterol was found in females given 100 mg/kg. There were also increases in absolute liver weight in males at 100 and 200 mg/kg and in females at 200 mg/kg, relative liver weight in both sexes at 50 mg/kg and more, and kidney weights in females at 100 mg/kg and in both sexes at the highest dose. Because of the clear toxic effects, 200 mg/kg was selected as the top dose for the main study, and 60, 20 and 6 mg/kg were derived by one third division.

In the main study, there were no significant changes in body weight and food consumption. At scheduled sacrifice, hematological examination showed decrease in platelet counts in females of 200 mg/kg group. On blood biochemical examination, changes suggestive of effects on the liver, including increase in total protein, albumin, A/G, total cholesterol, were found in both sexes at the highest dose. There were also increases in total protein and albumin in females of the 20 and 60 mg/kg groups and increases in A/G in females of the 60 mg/kg groups. For organ weights, there were increases in absolute and relative liver weights of both sexes given 60 and 200 mg/kg and slight increase in relative liver weights in males given 20 mg/kg. In addition, relative kidney weights were higher in both sexes and absolute kidney weights in females of the 200 mg/kg group. On histopathological examination (Table 6), slight to mild centrilobular hypertrophy of hepatocytes was observed in both sexes given 20 mg/kg and more. In the thyroid, mild hypertrophy of follicular cells was found at 60 mg/kg and 200 mg/kg, and follicles were apt to be miniaturized and colloid to be decreased. At the end of the recovery period, changes observed in the scheduled-sacrifice group remained significant but with a tendency for recovery (total protein, total cholesterol, liver and thyroid weights, centrilobular hypertrophy of hepatocytes (Table 6)).

The results of the dose-finding and main study in young rats are summarized in Table 7. As slight hypertrophy of hepatocytes was observed at 20 mg/kg in the main study, the NOAEL was concluded to be 6 mg/kg/day. The unequivocally toxic level was considered to

Table 6. Histological findings in the repeated dose study of 1,1,2,2-tetrabromoethane in young rats (main study).

	Grade	Scheduled-sacrifice group					Recovery group	
		0	6	20	60	200	0	200
Males								
No. of animals examined		5	5	5	5	5	5	5
Liver								
– Centrilobular hepatocyte hypertrophy	±	0	0	3	4	0	0	3
	+	0	0	0	0	5	0	0
				*				
				**				
– Focal necrosis	±	2	1	3	1	5	1	0
Thyroid								
– Hypertrophy of follicular cells	±	0	0	0	1	4	0	0
Females								
No. of animals examined		5	5	5	5	5	5	5
Liver								
– Centrilobular hepatocyte hypertrophy	±	0	0	3	5	1	0	2
	+	0	0	0	0	4	0	0
				**				
				**				
– Focal necrosis	±	0	0	0	0	1	0	0
Thyroid								
– Hypertrophy of follicular cells	±	0	0	0	2	5	0	0
				**				

±: Slight, +: Mild, *: Significantly different from the control group (p<0.05), **: Significantly different from the control group (p<0.01).

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be more than 200 mg/kg because of the lack of effects on body weights and parameters indicative of hepato-toxicity, such as GOT and GPT. Hypertrophy in the liver and thyroid, and increases in some biochemical parameters at this dose were not considered to be sufficient for a conclusion of toxicity.

DISCUSSION

As with human neonates, the metabolic ability of the newborn rat is known to be extremely immature, with a low cytochrome P450 content (Rich and Boobis, 1997) and a low capacity for glucuronidation (Gow et al., 2001). Therefore, it could be predicted that chemicals directly exerting adverse effects might show stronger toxicity in the newborn than in young/adult rats. As expected, our previous comparative studies demonstrated that the susceptibility to four chemicals (4-nitrophenol, 2,4-dinitorophenol, 3-aminophenol, 3-methylphenol), which may exert toxicity without metabolic activation, was 2 to 4 times greater in the newborn than in young rats (Koizumi et al., 2001, 2002, 2003).

In the present study, DBP and TBE, which differ from the earlier chemicals in requiring biotransformation differently from previous chemicals, were therefore examined. Although hitherto there has been no information on the repeated dose toxicity of DBP, hepatotoxicity with slight centrilobular fatty degeneration or cytoplasmic vacuolization has been already reported for TBE (Hollingsworth et al., 1963; NTP, 1996). The present study showed no effects of either chemical on early development in the newborn, but they caused hepatotoxicity, regardless of sex, in both newborn and young animals. The ratios for NOAELs and unequivocally toxic levels (young/newborn rats) for both chemicals are given in Table 8, the NOAELs for DBP and TBE being considerably higher in newborn than in young rats, so that the latter are clearly more susceptible. Unequivocally toxic levels could not be simply estimated for both chemicals because the hepatic influence observed was only hypertrophy of hepatocytes, usually without increase of GOT and GPT. Therefore, values were estimated on the basis of simultaneous changes of organ weights, histopathology, biochemical parameters and body weights. Based on our specified criteria, the unequivocally toxic level for DBP was in contrast lower in newborn than in young rats. Unfortunately an unequivocally toxic level of TBE could not be estimated for newborn or young rats. However, the dose of 200 mg/kg in the newborn dose-finding study was considered to be sufficiently toxic because of the 10 - 20% lowering of body weights observed, although no histopathology was conducted. The same dose in the young rat main study caused mild hypertrophy of hepatocytes but no change of body weights, was not considered a sufficient toxic level. These results suggest that the unequivocally toxic level of TBE in the newborn might be lower than that in young rats. The reasons for difference in susceptibility presumably lie with metabolic pathways and specific characteristics of newborn animals.

Three studies have demonstrated that DBP is conjugated with hepatic glutathione before or after oxidative biotransformation, leading to urinary excretion of cysteine or mercapturic acid derivatives and exhalation of CO_2 (James et al., 1981, Jones and Wells, 1981, Onkenhout et al., 1986). Activity of the conjugation

Table 7. Summary of the results of the repeated dose studies of 1,1,2,2-tetrabromoethane in young rats.

Dose (mg/kg/day)		Dose-finding Study (4 rats/sex/dose)					Main Study (5 rats/sex/dose)			
		10	20	50	100	200	6	20	60	200
Toxic Effects										
− Death (No. of dead animals)		0	0	0	0	0	0	0	0	0
− Body weight		−	−	−	−	−	−	−	−	−
− Blood biochemical parameters		−	−	−	F: TP↑	M: Cho↑ F: Cho, TG, TP↑	−	F: TP, Alb↑	F: TP, A/G, Alb↑	Many↑
− Relative liver weight		−	−	↑	↑	↑	−	M: (↑)	↑	↑
− Histopathological changes ±	n.d.	n.d.	n.d.	n.d.	n.d.	0	3M, 3F	4M, 5F	1F	
(No of animals with the findings*) +	n.d.	n.d.	n.d.	n.d.	n.d.	0	0	0	5M, 4F	

±: Slight, +: Mild, M: Males, F: Females, ↑: Increase, ↓: Decrease, (↑): Slight increase, −: No change, Alb: Albumin, Cho: Total cholesterol, TG: Triglycerides, TP: Total protein, Many: Many parameters including Alb, A/G, Cho and TP, n.d.: No available data, * Centrilobular hypertrophy of hepatocytes.

pathway is supported by a rapid drop in hepatic glutathione level after DBP administration (James *et al.*, 1981). Metabolism via conjugation with glutathione has in fact been indicated in common for dihaloalkanes or dihaloalkenes, such as 1,2-dibromopropane (Zoetemelk *et al.*, 1986), 1,2-dichloropropane (Trevisan *et al.*, 1989), 1,1-dichloroethylene (Jones and Hathway, 1978) and 1,3-dichloropropene (Climie *et al.*, 1979). In the case of 1,2-halogenated ethanes, it is considered that the oxidative metabolites might irreversibly bind to protein and that conjugate derivatives, episulphonium ions, might be responsible for the DNA adduct formation (Shih and Hill, 1981; Ozawa and Guengerich, 1983).

With TBE, Kennedy *et al.* (1993) identified various excretory metabolites after a single oral administration to rats, such as 1,2-dibromoethylene and tribromoethylene in exhaled air and dibromoacetic acid, glyoxylic acid, and oxalic acid in urine. They suggested that a number of metabolic intermediates produced by oxidative biotransformation may be involved in the mutagenicity, hepatotoxicity and nephrotoxicity of the compound. At least, dibromoacetic acid has unequivocal cytotoxicity and mutagenicity (Kargalioglu *et al.*, 2002).

Based on the available information, oxidative biotransformation mediated by cytochrome P450 might be a critical step for the initial hepatotoxic effects of both chemicals. The rate of production of active metabolites, including free radical intermediates, would be expected to be significantly less or negligible in newborn animals at least around 50 mg/kg, at which clearly hepatic changes were observed in young rats for both chemicals, because of their lower content

of cytochrome P450 (Rich and Boobis, 1997). This metabolic character for both chemicals as well as the lower blood flow to the liver during the newborn period (Gow *et al.*, 2001) would make a major contribution to the much higher NOAEL in the newborn than in young rats. Similar results have already been demonstrated for aflatoxin B1 (Behroozikha *et al.*, 1992), acetaminophen, bromobenzene and carbon tetrachloride (Gergus and Klaassen, 1998). On the other hand, unequivocally toxic levels for both chemicals appeared to be only 3 to 4 times higher than the NOAELs in newborn rats, in contrast to 25 to >33 times higher in their young counterparts (Table 8). One possible explanation for these differences might be a low capacity for protection against deleterious oxidative stress in the newborn when the toxic chemical burden crosses a threshold in the liver. It has been reported that the content of glutathione and glutathione-*S*-transferase activity in rat liver drops in the early days after birth (Tee *et al.*, 1992).

In our series of comparative studies, the results of the repeated dose toxicity study using newborn rats have been compared with those of routine repeated dose toxicity studies. The routine repeated dose studies have value in identifying target sites for toxicity and providing dose-response information that may be useful for human safety assessment, irrespective of life stage, but the developing period, which could be most vulnerablev to chemical toxicity during life, is not directly evaluated by the studies (Dourson *et al.*, 2002). To compensate for this period, reproductive/developmental toxicity studies that exposed the developing animals via placenta or maternal milk have been conducted. However, the direct exposure to chemicals dur-

Table 8. Comparison of NOAELs and unequivocally toxic levels in newborn and young rats.

	Level (mg/kg/day)		Ratio (young/newborn)
1,3-Dibromopropane			
NOAEL (newborn)	50	× 3	0.2
NOAEL (young)	10		
Unequivocally toxic level (newborn)	150	× 25	1.67
Unequivocally toxic level (young)	250		
1,1,2,2,-Tetrabromoethane			
NOAEL (newborn)	50	× 4*	0.12
NOAEL (young)	6		
Unequivocally toxic level (newborn)	200*	× >33*	>1.0*
Unequivocally toxic level (young)	> 200*		

*: Tentative levels or ratios, due to lack of histology alteration in the newborn and no change in body weight in young rats.

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ing the newborn period is not included in these studies, despite the significant possibility that the newborn are exposed to chemicals directly via mouthing toys and household materials, or having chemical-contaminated milk and baby food, and so on. In the routine repeated dose toxicity study, rats at approximately 5-6 weeks of age have generally been used, and this start period is largely a matter of practical convenience and feasibility. Rats much younger than this age, especially newborn rats, are so difficult to handle such as grouping, direct dosing and other testing or observation. Economic issues and lack of the human resource with this technical difficulty make it impossible to subject the newborn rat study to the routine one. Our series of comparative studies are the first systematic study to look into the direct effects of chemicals in newborn animals, and the comparative analysis on the susceptibility of the newborn rats to the toxicity of chemicals with that of young rats would give important information for considering the effects by chemical exposure during the newborn period in risk assessment.

In conclusion, the target organ of DBP and TBE was here found to be the liver in both newborn and young rats, but the doses at which the toxic signs began to appear were higher in newborn rats. In contrast, the doses at which clear toxicity was observed appeared to be lower in the newborn case. However, no special concern with regard to newborn risk is necessary in cases of chemicals which induce toxicity after biotransformation via hepatic cytochrome P450, because the tolerable daily intake (TDI) used for regulation is generally derived from NOAEL in toxicity studies in young/adult animals.

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ELSEVIER

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.

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].

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	$489 \pm 33^{**}$
Pituitary (mg)	15.6 ± 1.5	15.6 ± 2.0	14.2 ± 1.3	$13.4 \pm 1.5^{*}$	$12.2 \pm 1.2^{**}$
	(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	$2.71 \pm 0.20^{**}$
	(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	$0.19 \pm 0.05^{**}$
	(0.07 ± 0.01)	(0.06 ± 0.01)	(0.07 ± 0.01)	(0.06 ± 0.01)	$(0.04 \pm 0.01^{**})$
Testes (g)	3.41 ± 0.50	3.18 ± 0.83	3.52 ± 0.29	3.40 ± 0.45	$1.77 \pm 0.44^{**}$
	(0.63 ± 0.11)	(0.60 ± 0.15)	(0.66 ± 0.07)	(0.66 ± 0.10)	$(0.36 \pm 0.09^{**})$
Epididymides (g)	1.40 ± 0.20	1.30 ± 0.30	1.38 ± 0.15	1.26 ± 0.17	$0.87 \pm 0.15^{**}$
	(0.26 ± 0.04)	(0.24 ± 0.05)	(0.26 ± 0.03)	(0.24 ± 0.04)	$(0.18 \pm 0.03^{**})$
No. of females	12	10	12	9	0
Body weight (g)	363 ± 25	350 ± 35	339 ± 24	$313 \pm 27^{**}$	
Pituitary (mg)	20.1 ± 3.8	18.3 ± 1.7	17.6 ± 1.8	$16.0 \pm 1.9^{*}$	
	(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)	$(0.63 \pm 0.05^{*})$	
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 $\pm$ 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	$46.4 \pm 14.0^*$	
Total no. of pups born[a]	14.8 ± 1.6	14.5 ± 2.1	14.8 ± 1.7	$7.0 \pm 1.4^{**}$	
Live birth index[a,d]	100 ± 0	100 ± 0	98.8 ± 2.8	$43.1 \pm 29.3^*$	
No. of live pups on PND 0[a]	14.8 ± 1.6	14.5 ± 2.1	14.6 ± 1.8	$3.0 \pm 2.2^{**}$	
No. of dead pups on PND 0[a]	0	0	0.2 ± 0.4	$4.0 \pm 2.2^{**}$	
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	$1.3 \pm 2.3^{**}$	
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.