Table 10
Organ weights of female F2 weanlings

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

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

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

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

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

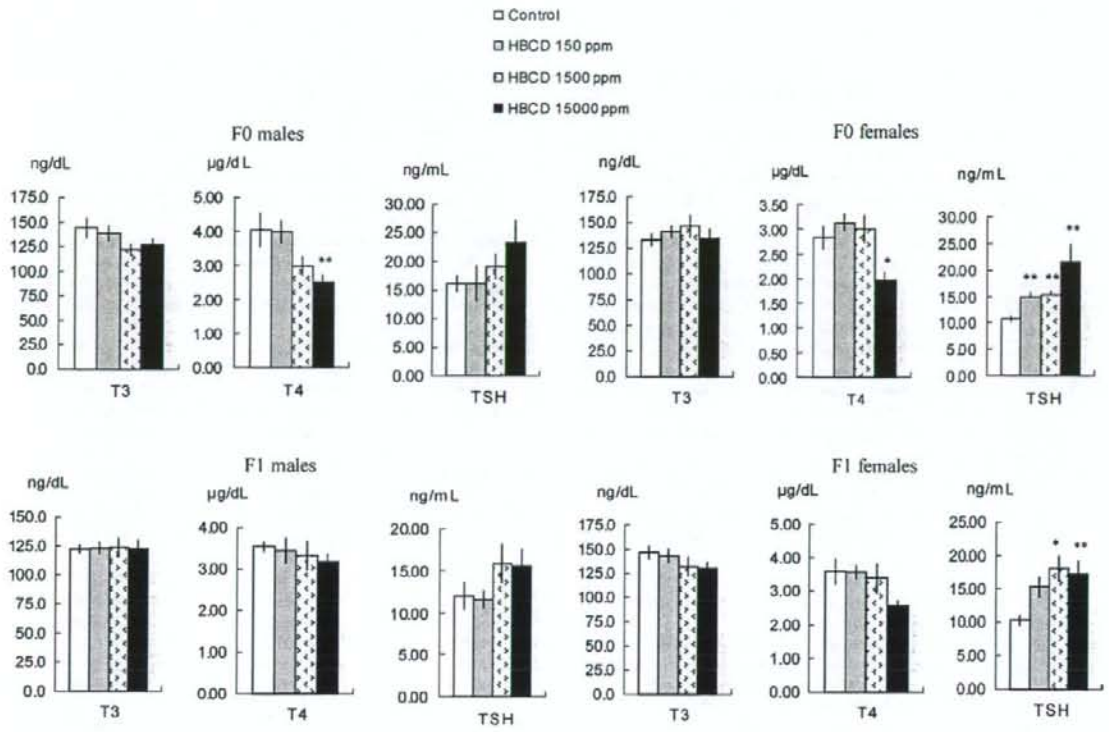

Fig. 4. Serum levels of T3, T4 and TSH in F0 and F1 rats. Values are given as the mean ± S.E.M. (*) Significantly different from the control, $P < 0.05$. (**) Significantly different from the control, $P < 0.01$.

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

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

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Repeated-Dose and Reproductive Toxicity of the Ultraviolet Absorber 2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole in Rats

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2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB) is widely used as an ultraviolet (UV) absorber. In this study, the repeated dose and reproductive toxicity of DBHCB was evaluated in rats. Crj:CD(SD)IGS rats were given DBHCB by gavage at 0, 2.5, 25, or 250 mg/kg/d. Male and female rats were dosed beginning 28 d before mating, and each female rat was mated with a male rat of the same dosage group. Males were dosed for a total of 56–57 d, and females were dosed for a total of 55–69 d up to Day 3 of lactation throughout the mating and pregnancy periods. Ten males from each group were killed on the next day of the last administration, and 10 females were killed on Days 4–6 after parturition. Five rats/sex treated at 0 and 250 mg/kg/d for 56 d were then kept without treatment for 14 d (recovery period). No deaths were found in any group. No effects of DBHCB on general condition, body weight, food consumption, or reproductive/developmental parameters were observed. Significant increases in serum albumin and an albumin/globulin ratio at 25 mg/kg/d and higher and alkaline phosphatase levels at 250 mg/kg/d were noted in males. The absolute and relative weights of the liver were significantly increased in males at 25 mg/kg/d and higher. Significantly increased serum albumin and absolute and relative liver weight were also found in males at 250 mg/kg/d after the recovery period. No changes in these parameters were observed in females of any DBHCB-treated groups. No significant changes in organ histopathology were found in males or females. These findings indicated a sex difference in the toxicity of DBHCB in rats.

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Keywords Repeated-dose toxicity, Reproductive and developmental toxicity, UV absorber, Benzotriazole, Rat.

INTRODUCTION

Benzotriazole ultraviolet (UV) absorbers, which have a phenolic group attached to the benzotriazole structure, are known to have the most excellent absorption capacity within the full spectrum of UV absorption (Tenkazai.com, 2007) and are, therefore, used in a variety of polymers. 2-(3′,5′-Di-*tert*-butyl–2′-hydroxyphenyl)-5-chlorobenzotriazole (CAS No. 3864–99–1; DBHCB), one of the benzotriazole UV absorbers, is a slightly yellowish powder that is stable under ordinary conditions and insoluble in water. The annual production and import from April 2005 to March 2006 was 532 tons in Japan (METI, 2006). This chemical provides effective light stabilization and prevents the yellowing and degradation of polymers such as polypropylene, high-density polyethylene, unsaturated polyesters, styrene-based thermoplastic elastomers, polyamides, and impact polystyrenes (Chemical Land21, 2005). The finished polymers, which contain DBHCB less than 0.5% by weight of polyethylene phthalate polymers in compliance with 21 CFR 177.1630 (FDA, 2005a), may be used in contact with foods and used under certain conditions, as described in 21 CFR 176.170 (FDA, 2000; 2005b). UV absorbers are used in food packaging to prevent polymer degradation and/or a change in the quality of the packed food due to UV light.

There is growing concern that humans have been exposed to these chemicals from environmental contamination and from the contamination of packaged food. Exposure could lead to adverse effects due to the potential toxicity of the chemicals. Important information can be gained by studying the biological effects of environmental chemicals in laboratory animals.

Only limited information on the toxicity of DBHCB is available. DBHCB was not estrogenic in a recombinant yeast assay (Miller et al., 2001) or a yeast two-hybrid assay (Kawamura et al., 2003). It has been found that the oral LD50 for DBHCB is greater than 5,000 mg/kg in rats, that DBHCB causes slight skin and eye irritation in rabbits, and that DBHCB treatment resulted in dose-dependent increases in the liver weight and signs of liver toxicity at 22–800 mg/kg/day, but not at 3.7 mg/kg/day, in rats (Everlight Chemical Industrial Corporation, 2002). We previously reported that the maternal administration of DBHCB on Days 5–19 of pregnancy caused no adverse effects in dams and fetuses at doses up to 1,000 mg/kg/day (Ema et al., 2006).

Although testing for reproductive toxicity has become an important part of the overall toxicology profile for chemicals, no report is available for the reproductive toxicity of DBHCB. The present study was, therefore, conducted by using a study design similar to the OECD Guideline 422 Combined Repeated

Dose Toxicity Study with Reproduction/Developmental Toxicity Screening Study in rats (OECD, 1996).

MATERIALS AND METHODS

Animals

International Genetic Standard (Crj: CD (SD) IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in reproductive and developmental toxicity studies and historical control data are available. Males at 11 weeks of age and females at 10 weeks of age were purchased from Hino Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The rats were acclimatized to the laboratory for one week prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Animals were reared on a basal diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water *ad libitum*. Rats were maintained in an air-conditioned room at 21.5–22.1°C, with a relative humidity of 47–67%, a 12-h light/dark cycle, and ventilation with 15 air changes/h. Rats were housed individually, except during the acclimation, mating, and nursing periods. From Day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared by using wooden chips as bedding (White Flake; Charles River Laboratories Japan, Inc.). This experiment was approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd. (SNBL; Kagoshima, Japan) and performed in accordance with the ethics criteria contained in the bylaws of the committee of SNBL.

Chemicals and Dosing

DBHCB was obtained from Musashino Chemical Laboratory, Ltd. (Kitaibaraki, Japan). The DBHCB (Lot no. 05004IX3) used in this study was 99.9% pure based on high-performance liquid chromatography (HPLC) analysis, and it was kept in a dark, cool place at room temperature under airtight conditions. The purity and stability of the chemical were verified by analysis before the study.

DBHCB was suspended in 5% gum arabic solution. The volume of each dose was adjusted to 10 mL/kg body weight based on the latest body weight. The control rats were given only 5% gum arabic solution. Stability of the formulations kept in a dark, cool place under airtight conditions had been confirmed for up to 14 d. During use, the formulations were maintained under these conditions for no more than seven days and were 97.3–100.1% of the target concentration.

The initial numbers of the rats were 15/sex at 0 (control) and 250 mg/kg/d, and 10/sex at 2.5 and 25 mg/kg/d. Male and female rats were dosed once-daily

beginning 28 d before mating, and each female rat was mated with a male rat of the same dosage group. Males were dosed for a total of 56–57 d, and females were dosed for a total of 55–69 days to Day 3 of lactation throughout the mating and pregnancy periods. Ten males from each group were killed after 56–57 d of administration, and ten females were killed on Days 4–6 after parturition. The remaining five rats/sex treated at 0 and 250 mg/kg/d for 56 d were kept without treatment for 14 d (recovery period). Dosage levels were determined based on the results of our dose-finding study, in which significantly increased liver weight occurred in males at 250 mg/kg/d and higher, but not in females, even at 1,000 mg/kg/day, after the administration of DBHCB for 14 d in rats.

Observations

All rats were observed twice a day for clinical signs of toxicity during the administration period and once a day during the nonadministration period. The body weight was recorded twice a week in males, and twice a week during the premating period, on Days 0, 7, 14, and 20 of pregnancy and on Days 0, 3, and 4 of lactation in females. Food consumption was recorded twice a week for males, and twice a week during the premating period, on Days 1, 4, 7, 11, 15, 17, and 20 of pregnancy and on Days 1 and 3 of lactation for females.

Prior to scheduled terminal necropsy, blood samples for hematological and biochemical evaluation were collected from the abdominal aorta of five fasted male and female rats per group under anesthesia by an intraperitoneal injection of sodium pentobarbital. Blood samples were analyzed for the following hematological parameters by using K_2-EDTA as an anticoagulant: red blood cell count (RBC), white blood cell count (WBC), hematocrit value, hemoglobin concentration, platelet count, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), reticulocyte ratio, and differential white blood cell ratio (Hematology System ADVIA 120; Bayer Diagnostics Manufacturing Ltd., Dublin, Ireland), using sodium citrate as an anticoagulant: prothrombin time (PT) and activated partial thromboplastin time (APTT) (Automated Blood Coagulation Measuring Apparatus CA-5000; Sysmex Corp., Kobe, Japan).

Serum samples obtained from centrifuged whole blood were analyzed for the following biochemistry parameters: aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase, total bilirubin, total protein, albumin, total cholesterol, triglyceride, glucose, blood urea nitrogen (BUN), creatinine, inorganic phosphorus, calcium, sodium, potassium, chloride (Automatic analyzer JCA-BM8; JOEL Ltd., Tokyo, Japan), total bile acid (Spectrophotometer U-3200; Hitachi Ltd., Tokyo, Japan), protein fraction (Automatic Electrophoresis Apparatus, AES-4000; Olympus Corp., Tokyo, Japan), and albumin/globulin (A/G) ratio.

At the scheduled terminal necropsy, all rats were euthanized by exsanguination under anesthesia. All rats were subjected to gross necropsy, which included an external examination of all body orifices and surfaces, and examinations of all cranial, thoracic, and abdominal organs. The brain, heart, liver, kidney, spleen, thymus, and adrenal gland in males and females, the testis, epididymis, seminal vesicle, and prostate in males, and the ovary in females were removed and weighed. Relative organ weights (mg or g/100 g of body weight) were calculated on the basis of the terminal body weight. In females, the numbers of corpora lutea and implantation sites were recorded. Samples of tissues and organs were preserved in neural phosphate-buffered 10% formaldehyde solution. The testis and epididymis were fixed in Bouin's solution. Histopathological evaluations for five rats/sex /group were performed on the tissues specified below after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin; the brain, heart, thymus, kidney, spleen, adrenal gland, small and large intestine, lung, trachea, thyroid, submandibular and mesenteric lymph node, femur bone marrow, spinal cord, sciatic nerve, tibial nerve, urinary blabber, testis, epididymis, seminal vesicle, prostate, ovary, and uterus in the control and highest dose groups, and the liver in all groups.

Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered evidence of successful mating. The day of successful mating was designated as Day 0 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal days (PNDs) 4–6. The day on which parturition was completed was designated as PND 0. Litter size and numbers of live and dead pups were recorded, and the live pups were sexed and individually weighed on PNDs 0 and 4. Dead pups were examined grossly. On PND 4, the pups were euthanized by exsanguination under anesthesia, and gross external and internal examinations were performed.

Data Analysis

The statistical analysis of pups was carried out by using the litter as the experimental unit. The body weight, body-weight gain and food consumption, precoital interval, length of gestation, numbers of implantations and live pups per litter and pup weight, delivery index, viability index, hematological and blood biochemical parameters, and organ weight were analyzed with Bartlett's test for homogeneity of variance at the 5% level of significance. When the variance was homogeneous, Dunnett's test was performed to compare the mean value in the control group with that in each DBHCB group. When the variance was heterogeneous, a Dunnett-type test was performed to

compare the mean value in the control group with that in each DBHCB group after rank conversion. Recovery in the control and highest dose groups was analyzed in the following way. Variance ratio was analyzed by an F test. If the variance ratio was equivalent, the groups were compared by a Student's t-test. If the variance was not equivalent, the Wilcoxon test was performed.

RESULTS

No deaths or DBHCB-related clinical signs of toxicity were found in male or female rats of any groups. There was no significant difference in the body weight and body-weight gain between the control and DBHCB-treated groups in males and females, including during pregnancy and lactation. No significant changes in the food consumption were found, except for a significant decrease on Days 28–29 in males and an increase on Days 31–32 in females at 250 mg/kg.

The reproductive and developmental findings in rats given DBHCB are presented in Table 1. Although one pair did not copulate in the control group, all pairs copulated and all copulated females were impregnated and delivered their pups in all DBHCB-treated groups. There was no significant difference in the copulation index, fertility index, gestation index, precoital interval, or gestation length between the control and DBHCB-treated groups. No effects of DBHCB were observed on the numbers of corpora lutea or implantations, preimplantation loss, numbers of pups delivered, live pups, or stillborn or sex ratio of live pups. There was no significant difference in the viability or body weight of pups on PNDs 0 or 4 between the control and DBHCB-treated groups. External and internal examinations revealed no morphological anomalies in the pups of any group.

Table 2 shows the hematological findings in rats given DBHCB at the end of the administration period. A significantly decreased RBC at 250 mg/kg/d and shorter APTT at 25 and 250 mg/kg/d were observed in males. The number of neutrophils was significantly increased, at 250 mg/kg/d, in males. In females, the only significant change was a lowered number of eosinophils, at 25 and 250 mg/kg/d. At the end of the recovery period, significantly increased numbers of platelets and neutrophils, as well as an increased neutrophil ratio, were observed in males at 250 mg/kg/d, in addition to a decreased lymphocyte ratio.

Table 3 presents the blood biochemical findings in rats given DBHCB at the end of the administration period. In males, significantly increased levels of ALAT at 25 mg/kg/d, as well as decreased levels of creatinine at 25 mg/kg/d and higher, were observed. Additionally, males presented decreased levels of total bilirubin, and increased levels of ALP, at 250 mg/kg/d, were observed. The levels of total protein were significantly increased at 25 mg/kg/d. A significantly increased albumin percentage and A/G ratio and decreased

Table 1: Reproductive and developmental findings in rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of pairs	10	10	10	10
Copulation index (%)[b]	90	100	100	100
Fertility index (%)[c]	100	100	100	100
No. of pregnant females	9	10	10	10
Precoital interval (days)[a]	4.9 ± 4.4	3.4 ± 3.8	2.7 ± 1.3	2.8 ± 1.5
Gestation index (%)[d]	100	100	100	100
Gestation length (days)[a]	21.9 ± 0.4	21.9 ± 0.3	22.0 ± 0.4	22.0 ± 0.2
No. of litters	9	10	10	10
No. of corpora lutea[a]	16.1 ± 1.9	15.7 ± 1.8	15.3 ± 1.5	16.0 ± 1.9
No. of implantations[a]	15.3 ± 1.7	14.8 ± 1.5	14.1 ± 1.2	14.2 ± 3.2
Preimplantation loss (%)[a,e]	6.1 ± 4.3	6.6 ± 8.4	7.5 ± 7.0	10.9 ± 17.3
Delivery index (%)[a,f]	91.1 ± 7.2	93.8 ± 7.0	91.0 ± 13.8	96.5 ± 5.7
No. of pups delivered[a]	14.1 ± 2.2	14.0 ± 1.9	12.8 ± 2.0	14.0 ± 3.1
No. of live pups[a]	14.0 ± 2.2	13.9 ± 1.9	12.8 ± 2.0	13.9 ± 2.9
No. of stillborn[a]	0.1 ± 0.3	0.1 ± 0.3	0	0.1 ± 0.3
Sex ratio of live pups (female/total)[a]	0.53 ± 0.09	0.50 ± 0.15	0.53 ± 0.09	0.61 ± 0.16
Viability index during lactation (%)[a,g,h]				
Day 0	99.2 ± 2.4	99.3 ± 2.3	98.8 ± 3.7	98.8 ± 2.6
Day 4	100	98.8 ± 2.6	97.6 ± 4.0	97.7 ± 3.7
Male pup weight during lactation (g)[a]				
Day 0	6.5 ± 0.5	6.5 ± 0.5	6.8 ± 0.3	6.5 ± 0.4
Day 4	9.3 ± 1.1	9.4 ± 0.9	10.2 ± 0.7	9.6 ± 1.4
Female pup weight during lactation (g)[a]				
Day 0	6.0 ± 0.4	6.2 ± 0.5	6.3 ± 0.4	6.1 ± 0.4
Day 4	8.9 ± 1.0	9.0 ± 0.8	9.7 ± 0.7	9.1 ± 1.5

[a]Values are given as the mean ± SD.
[b]Copulation index (%) = (no. of females with successful copulation/no. of females paired) × 100.
[c]Fertility index (%) = (no. of females pregnant/no. of females with successful copulation) × 100.
[d]Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.
[e]Preimplantation loss (%) = ((no. of corpora lutea − no. of implantations)/no. of corpora lutea) × 100.
[f]Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.
[g]Viability index on postnatal day 0 (%) = (no. of live pups on postnatal day 0/no. of pups delivered) × 100.
[h]Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of live pups on postnatal day 0) × 100.

α2-globulin percentage were found in males at 25 and 250 mg/kg/d, as well as a decreased percentage of β-globulin at 2.5 mg/kg/d and higher. In females, the levels of total cholesterol were significantly decreased at 2.5 and 25 mg/kg/d. No significant changes in other blood biochemical parameters were noted in males and females in the DBHCB-treated groups. At the end of the recovery period, significantly increased levels of total protein, albumin, and total cholesterol and decreased creatinine levels and α2-globulin ratio were observed at 250 mg/kg/d in males. In females, parameters remained unchanged in all DBHCB-treated groups.

Table 2: Hematological findings in male and female rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of male rats	5	5	5	5
RBC (10^6/mL)	8.18 ± 0.32[a]	7.95 ± 0.31	8.07 ± 0.30	7.63 ± 0.36*
WBC (10^3/mL)	9.41 ± 1.06	8.22 ± 2.94	8.10 ± 2.37	8.94 ± 1.13
Hematocrit value (%)	45.6 ± 1.9	44.3 ± 0.9	44.7 ± 2.2	42.7 ± 1.7
Hemoglobin concentration (g/dL)	15.2 ± 0.4	14.9 ± 0.5	15.1 ± 0.9	14.2 ± 0.7
Platelet count (10^3/mL)	1063 ± 110	1145 ± 134	1202 ± 119	1205 ± 108
MCV (fL)	55.7 ± 2.3	55.8 ± 1.5	55.4 ± 0.9	55.9 ± 0.7
MCH (pg)	18.7 ± 0.7	18.7 ± 0.8	18.7 ± 0.4	18.6 ± 0.3
MCHC (g/dL)	33.5 ± 0.7	33.5 ± 0.7	33.8 ± 0.4	33.3 ± 0.4
Reticulocyte ratio (%)	2.60 ± 0.34	2.74 ± 0.57	3.00 ± 0.40	3.02 ± 0.44
PT (sec)	8.52 ± 0.42	9.50 ± 0.97	9.20 ± 0.57	8.50 ± 0.58
APTT (sec)	20.1 ± 0.8	20.9 ± 0.7	18.3 ± 1.0**	18.2 ± 0.7**
No. of female rats	5	5	5	5
RBC (10^6/mL)	6.81 ± 0.49[a]	6.90 ± 0.36	6.82 ± 0.14	6.50 ± 0.24
WBC (10^3/mL)	5.95 ± 0.96	6.19 ± 1.38	6.34 ± 1.46	5.05 ± 0.71
Hematocrit value (%)	40.2 ± 2.1	41.1 ± 1.7	39.4 ± 1.2	39.6 ± 2.3
Hemoglobin concentration (g/dL)	13.4 ± 0.7	14.0 ± 0.8	13.1 ± 0.4	13.4 ± 0.8
Platelet count (10^3/mL)	1468 ± 237	1518 ± 44	1496 ± 208	1503 ± 157
MCV (fL)	59.1 ± 2.4	59.6 ± 1.8	57.8 ± 2.1	60.9 ± 1.5
MCH (pg)	19.7 ± 0.8	20.3 ± 0.5	19.3 ± 0.7	20.5 ± 0.5
MCHC (g/dL)	33.3 ± 0.2	34.0 ± 0.6	33.4 ± 0.7	33.7 ± 0.4
Reticulocyte ratio (%)	6.48 ± 2.55	4.88 ± 1.04	4.48 ± 1.28	6.28 ± 2.55
PT (sec)	7.38 ± 0.29	7.28 ± 0.19	7.42 ± 0.27	6.94 ± 0.32
APTT (sec)	18.6 ± 1.2	19.1 ± 1.9	18.8 ± 0.3	14.7 ± 3.4

[a]Values are given as the mean ± SD.
*Significantly different from the control, $p < 0.05$.
**Significantly different from the control, $p < 0.01$.

The organ weights of male rats given DBHCB at the end of the administration period are presented in Table 4. The absolute and relative weights of the liver were significantly higher at 25 mg/kg/d and higher. No significant changes in the weight of the reproductive organs were found. At the end of recovery period the absolute and relative weights of the liver at 250 mg/kg/d, were still significantly increased.

Table 5 shows the organ weight of female rats given DBHCB at the end of the administration period. There were no significant changes in the absolute and relative weights of organs, including the reproductive organs. At the end of the recovery period, no significant changes in the absolute or relative weight of organs were observed at 250 mg/kg/d.

No changes related to the administration of DBHCB were found in the necropsy findings. Histopathological examinations revealed no test compound–related toxicological changes in the liver of males and females in all the DBHCB-treated groups. There were also no changes in the other organs, including the male and female reproductive organs, in the 250 mg/kg/d group.

Table 3: Blood biochemical findings in male and female rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of male rats	5	5	5	5
ASAT (IU/L)	116 ± 26[a]	92 ± 18	136 ± 28	121 ± 23
ALAT (IU/L)	38.8 ± 3.7	39.2 ± 2.9	58.2 ± 25.5*	48.8 ± 7.5
ALP (IU/L)	539 ± 57	476 ± 78	617 ± 178	943 ± 150**
Total bilirubin (mg/dL)	0.052 ± 0.008	0.048 ± 0.016	0.046 ± 0.013	0.024 ± 0.009**
BUN (mg/dL)	20.7 ± 1.2	19.7 ± 2.6	21.8 ± 1.9	21.3 ± 3.8
Creatinine (mg/dL)	0.312 ± 0.053	0.274 ± 0.022	0.226 ± 0.037**	0.248 ± 0.022**
Total cholesterol (mg/dL)	68.0 ± 6.9	58.4 ± 12.8	64.0 ± 7.3	61.2 ± 16.5
Glucose (mg/dL)	186 ± 14	173 ± 14	190 ± 15	198 ± 27
Total protein (g/dL)	5.60 ± 0.10	6.04 ± 0.27	6.26 ± 0.41**	5.92 ± 0.034
Albumin (%)	51.5 ± 2.3	53.3 ± 1.8	58.6 ± 2.5**	61.0 ± 1.7**
A/G ratio	1.07 ± 0.10	1.14 ± 0.09	1.42 ± 0.14**	1.57 ± 0.11**
α1-Globulin (%)	20.4 ± 2.7	20.7 ± 2.5	19.1 ± 2.9	18.1 ± 1.2
α2-Globulin (%)	9.4 ± 0.5	9.0 ± 0.3	7.8 ± 0.2**	7.6 ± 0.4**
β-Globulin (%)	14.5 ± 0.9	12.9 ± 1.0**	10.6 ± 0.6**	9.0 ± 0.3**
γ-Globulin (%)	4.2 ± 1.0	4.2 ± 0.3	4.0 ± 0.8	4.2 ± 0.8
No. of female rats	5	5	5	5
ASAT (IU/L)	130 ± 11[a]	113 ± 37	106 ± 15	104 ± 23
ALAT (IU/L)	59.0 ± 9.1	42.8 ± 7.8	49.4 ± 9.9	60.2 ± 15.3
ALP (IU/L)	215 ± 29	185 ± 71	184 ± 56	194 ± 59
Total bilirubin (mg/dL)	0.058 ± 0.016	0.074 ± 0.030	0.044 ± 0.011	0.056 ± 0.013
BUN (mg/dL)	26.1 ± 8.2	17.3 ± 5.3	19.8 ± 4.1	18.9 ± 5.0
Creatinine (mg/dL)	0.308 ± 0.044	0.290 ± 0.040	0.330 ± 0.029	0.282 ± 0.028
Total cholesterol (mg/dL)	79.6 ± 16.8	58.4 ± 3.2*	57.6 ± 13.3*	64.2 ± 12.9
Glucose (mg/dL)	109 ± 16	109 ± 13	120 ± 7	115 ± 24
Total protein (g/dL)	5.74 ± 0.31	5.60 ± 0.27	5.54 ± 0.36	5.50 ± 0.22
Albumin (%)	55.0 ± 1.8	54.2 ± 2.1	55.5 ± 0.8	55.4 ± 1.8
A/G ratio	1.23 ± 0.09	1.19 ± 0.10	1.25 ± 0.04	1.25 ± 0.09
α1-Globulin (%)	17.8 ± 2.1	19.2 ± 1.4	17.8 ± 2.2	17.6 ± 1.3
α2-Globulin (%)	8.8 ± 1.2	8.8 ± 0.9	7.9 ± 0.8	8.3 ± 0.3
β-Globulin (%)	13.5 ± 0.9	13.3 ± 0.9	13.7 ± 0.8	13.4 ± 1.0
γ-Globulin (%)	4.9 ± 1.2	4.4 ± 0.4	5.1 ± 0.5	5.3 ± 0.4

[a]Values are given as the mean ± SD.
*Significantly different from the control, $p < 0.05$.
**Significantly different from the control, $p < 0.01$.

DISCUSSION

The present study was conducted to determine the repeated-dose and reproductive toxicity of DBHCB. The data show that the repeated oral dosing of DBHCB caused changes in the liver in males, but not in females, and no changes in the reproductive function of male and female rats.

In the present study, there were no changes in the reproductive parameters regarding copulation, fertility, parturition, and nursing of their pups in rats given DBHCB beginning 28 d before mating, during pregnancy, and shortly after parturition. No changes in weight or histopathology were found in male and female reproductive organs. Moreover, the prenatal and postnatal

Table 4: Organ weights of male rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of male rats	5	5	5	5
Body weight (g)	451 ± 35[a]	463 ± 26	454 ± 37	437 ± 11
Brain (g)	2.06 ± 0.07[b]	2.09 ± 0.06	2.06 ± 0.11	2.00 ± 0.09
	0.461 ± 0.034[c]	0.453 ± 0.034	0.457 ± 0.061	0.459 ± 0.030
Heart (g)	1.41 ± 0.07[b]	1.52 ± 0.11	1.44 ± 0.16	1.42 ± 0.11
	0.314 ± 0.011[c]	0.329 ± 0.018	0.312 ± 0.018	0.325 ± 0.024
Thymus (mg)	391 ± 90[b]	401 ± 104	412 ± 174	396 ± 88
	87.4 ± 22.7[c]	86.2 ± 18.6	89.4 ± 31.5	90.8 ± 20.5
Liver (g)	14.81 ± 1.43[b]	16.46 ± 1.70	20.11 ± 3.76*	24.11 ± 2.60**
	3.28 ± 0.13[c]	3.54 ± 0.20	4.41 ± 0.55*	5.52 ± 0.66**
Kidney (g)	3.17 ± 0.25[b]	3.49 ± 0.31	3.50 ± 0.40	3.34 ± 0.18
	0.706 ± 0.082[c]	0.753 ± 0.051	0.769 ± 0.045	0.763 ± 0.045
Spleen (mg)	853 ± 82[b]	957 ± 205	908 ± 218	790 ± 62
	190 ± 19[c]	206 ± 38	199 ± 35	181 ± 17
Adrenal (mg)	61.2 ± 9.5[b]	62.1 ± 9.2	61.5 ± 6.9	50.8 ± 3.2
	13.6 ± 2.3[c]	13.4 ± 2.1	13.6 ± 2.2	11.6 ± 0.6
Testis (g)	3.23 ± 0.14[b]	3.39 ± 0.17	3.01 ± 0. 28	3.00 ± 0.25
	0.720 ± 0.062[c]	0.732 ± 0.051	0.666 ± 0.073	0.686 ± 0.058
Epididymis (g)	1.26 ± 0.07[b]	1.27 ± 0.06	1.23± 0.14	1.24 ± 0.13
	0.281 ± 0.027[c]	0.274 ± 0.010	0.271 ± 0.018	0.284 ± 0.028
Seminal vesicle (g)	1.71 ± 0.18[b]	1.69 ± 0.14	1.70 ± 0.21	1.60 ± 0.12
	0.383 ± 0.060[c]	0.365 ± 0.040	0.376 ± 0.040	0.366 ± 0.027
Prostate (g)	1.37 ± 0.099[b]	1.25 ± 0.10	1.42 ± 0.34	1.39 ± 0.19
	0.305 ± 0.035[c]	0.270 ± 0.025	0.309 ± 0.057	0.319 ± 0.038

[a]Values are given as the mean ± SD.
[b]Absolute organ weight.
[c]Relative organ weight = organ weight (g or mg)/100 g body weight.
*Significantly different from the control, $p < 0.05$.
**Significantly different from the control, $p < 0.01$.

developmental parameters regarding embryonic/fetal/neonatal survival and growth and morphological development of offspring were not affected by the administration of DBHCB. These results are consistent with the results of our previous study, in which no maternal or prenatal developmental toxicity was noted in rats given DBHCB by gavage on Days 5–19 of pregnancy at 1,000 mg/kg/d (Ema et al., 2006). These findings indicate that DBHCB has no potential for reproductive or developmental toxicity in rats.

On the hematological examination, changes in some parameters were noted in both male and female rats at higher doses. However, these changes are not considered to indicate toxicological significance because they were relatively small and were dose independent. The lowered RBC, for example, in males at 250 mg/kg/d is unlikely to represent anemia because the degree of decrease is slight and other anemic parameters, such as hematocrit, hemoglobin, MCV, MCH, MCHC, and reticulocyte count, were not affected by the administration of DBHCB. Anemia is defined clinically as the condition characterized by a hemoglobin concentration below the lower reference limit (Hall, 2007). Regarding renal function, it has been described that serum

Table 5: Organ weights of female rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of female rats	5	5	5	5
Body weight (g)	282 ± 33[a]	290 ± 14	276 ± 15	283 ± 21
Brain (g)	1.96 ± 0.04[b]	1.96 ± 0.06	1.97 ± 0.09	1.94 ± 0.06
	0.706 ± 0.100[c]	0.677 ± 0.044	0.716 ± 0.068	0.688 ± 0.058
Heart (g)	1.06 ± 0.14[b]	1.00 ± 0.04	0.98 ± 0.08	1.01 ± 0.10
	0.376 ± 0.019[c]	0.346 ± 0.012	0.357 ± 0.022	0.358 ± 0.035
Thymus (mg)	219 ± 40[b]	272 ± 60	247 ± 87	253 ± 64
	77.6 ± 7.8[c]	93.6 ± 18.6	90.2 ± 32.9	90.2 ± 26.5
Liver (g)	9.89 ± 1.64[b]	8.99 ± 0.67	9.16 ± 0.69	9.69 ± 0.54
	3.51 ± 0.37[c]	3.10 ± 0.19*	3.32 ± 0.10	3.43 ± 0.20
Kidney (g)	2.16 ± 0.28[b]	2.07 ± 0.14	1.98 ± 0.21	2.03 ± 0.03
	0.770 ± 0.067[c]	0.713 ± 0.033	0.715 ± 0.057	0.721 ± 0.049
Spleen (mg)	716 ± 178[b]	713 ± 125	666 ± 172	749 ± 62
	252 ± 44[c]	246 ± 47	240 ± 52	265 ± 16
Adrenal (mg)	95.7 ± 15.2[b]	85.0 ± 10.3	85.3 ± 10.3	89.5 ± 4.0
	34.1 ± 5.23[c]	29.3 ± 3.6	30.9 ± 3.3	31.7 ± 1.6
Ovary (mg)	95.9 ± 10.4[b]	96.4 ± 6.2	95.6 ± 11.6	104.9 ± 18.8
	34.5 ± 5.9[c]	33.2 ± 1.9	34.7 ± 4.2	36.9 ± 4.5

[a]Values are given as the mean ± SD.
[b]Absolute organ weight.
[c]Relative organ weight = organ weight (g or mg)/100 g body weight.
*Significantly different from the control, $p < 0.05$.

creatinine levels parallel changes in BUN caused by alterations in renal blood flow, renal function, or urinary outflow (Hall, 2007). The changes in creatinine levels in male rats at 25 mg/kg/d and higher are not thought to have toxicological significance because there were no changes in BUN or histopathological alterations of the kidney in the DBHCB-treated groups. In male rats, changes in some blood biochemical parameters suggestive of liver toxicity were observed at higher doses. The increased levels of total protein and albumin suggest an acceleration of protein synthesis in the liver, and these phenomena are supported by the increased weight of the liver at higher doses. These changes were noted only in males, indicating a sex difference in the toxicity of DBHCB.

The no observed adverse effect level (NOAEL) for repeated-dose toxicity of DBHCB is considered to be 2.5 mg/kg/d in male rats, based on the increased levels of albumin and weight of the liver, and 250 mg/kg/d, the highest dose used in the present study, in female rats. Our findings indicate that male rats have more than a 100-fold greater susceptibility to DBHCB toxicity than female rats. Previously, we showed sex differences in toxicity in the 28-d and 52-week repeated-dose toxicity studies of a structurally similar compound, 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), which is also used as a UV absorber (Hirata-Koizumi et al., 2007, 2008a). In the 28-d repeated-dose toxicity study, using rats given HDBB by gavage at 0, 0.5, 2.5,

12.5, or 62.5 mg/kg/d, adverse effects on the liver and heart were noted at all doses in males and at 12.5 mg/kg and higher in females (Hirata-Koizumi et al., 2007). In the 52-week repeated-dose toxicity study with rats given HDBB by gavage at 0, 0.1, 0.5, or 2.5 mg/kg/d in males and 0, 0.5, 2.5, or 12.5 mg/kg/d in females, toxic effects were observed in the liver at 0.5 mg/kg/d and higher in males and 12.5 mg/kg/d in females (Hirata-Koizumi et al., 2008a).

It has been recognized that there are sex differences in the toxicity of chemical compounds in rats. A recent subchronic toxicity study showed that fluoranthene, a polycyclic aromatic hydrocarbon, had greater effects on males than females, especially in the kidney, in F344 rats (Knuckles et al., 2004). On the other hand, female rats exhibited a higher susceptibility to hypothermic effects and inhibition of hypothalamic cholinesterase by the carbamate cholinesterase inhibitor, rivastigmine (Wang et al., 2001). These findings suggest that sexual hormones may play an important role in sex differences in toxicity. It has already been shown that orchidectomy resulted in the complete ablation of the above-mentioned sex differences in hypothalamic cholinesterase inhibition induced by rivastigmine (Wang et al., 2001). Testosterone is likely to interfere with the effects of rivastigmine, because testosterone decreases cholinesterase inhibition in gonadectomized males and females. More recently, we showed that castration markedly reduced sex differences in the toxicity of HDBB in male and female rats (Hirata-Koizumi et al., 2008b). We also reported no sex differences in susceptibility to the toxic effects of HDBB in preweaning rats (Hirata-Koizumi et al., 2008c). It is important to investigate the role of sex steroids in the mediation of sex differences in susceptibility to DBHCB toxicity and to determine the toxic effects of DBHCB in preweaning rats. A repeated-dose toxicity study of DBHCB is currently in progress, using castrated and preweaning male and female rats.

To date, there has been no available data for human exposure to this chemical. Actual human exposure to DBHCB may be very low because it was not detected in polyethylenterephthalate bottles in Brazil (Monteiro et al., 1998) or polyethylene products in Japan (Kawamura et al., 1997). Consideration of these findings and the results of the present study together suggest that the human risk of adverse effects from DBHCB exposure is very low.

CONCLUSIONS

In conclusion, the administration of DBHCB during premating, mating, and pregnancy, as well as shortly after parturition, caused no changes in the reproductive function of male and female rats. DBHCB produced increases in the liver weight, albumin levels, and A/G ratio at 25 mg/kg/d and higher, as well as ALP levels at 250 mg/kg/d in males, but no change in females. These findings indicate a sex difference in the toxicity of DBHCB in rats.

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Reproductive and Developmental Toxicity Screening Study of 4-Aminophenol in Rats

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Twelve male and female rats per group were given 4-aminophenol (PAP) by gavage at 0, 20, 100, or 500 mg/kg/day. Males were dosed for a total of 49 days, beginning 14 days before mating. Females were dosed for a total of 40–60 days, from 14 days before mating to Day 3 of lactation throughout the mating and gestation periods. Four males and 2 females died at 500 mg/kg/day, and all surviving males and females showed brown urine at 100 mg/kg/day and above. Body-weight gain was lower in males and females at 500 mg/kg/day, and food consumption was decreased in males at 500 mg/kg/day and in females at 100 and 500 mg/kg/day. Absolute and relative weights of the testes and epididymides were decreased at 500 mg/kg/day. Histopathological examinations revealed decreased spermatocyte and spermatid levels in the testis, debris of germ cell in the epididymis lumen, basophilic tubules in the kidney, and deposits of hemosiderin in the red pulp and extramedullary hematopoiesis in the spleen in males at 500 mg/kg/day. Longer gestation period, decreased delivery index, and lower body weight of pups on postnatal day (PND) 0 and increased number of stillborns at 500 mg/kg/day were also observed. At this dose, the viability of pups on PND 4 was decreased markedly. No adverse effects on reproduction or development were detected at 20 and 100 mg/kg/day. These findings indicate that PAP is general and reproductive/developmental toxic, but is unlikely to be teratogenic, in rats.

Keywords Reproductive and developmental toxicity, 4-Aminophenol, Dye, Testicular toxicity, Neonatal death, Rat.

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INTRODUCTION

4-aminophenol (CAS No. 123-30-8; PAP) has numerous applications, and more than 500 tons a year is produced in the United States (Scorecard, 2005) and a further 100 tons is produced in Japan (NITE, 2004). PAP is used industrially as a dye for textiles, fur, and feathers as a photographic developer (Haz-Map, 2007) and is generally used in hair dyes with other aminophenols. In pharmaceutics, PAP is known as a breakdown product of acetaminophen (paracetamol), which is widely used as an antipyretic and analgesic over-the-counter drug (Gemborys and Mudge, 1981; Newton et al., 1982). It has caused some anxiety that humans have been exposed to PAP in occupational surroundings, from environmental contamination and from consumer products.

The possibility of chemical compounds entering the biological system has aroused great concern about their toxic potential. It is generally assumed that the biological effects produced by chemical compounds should be studied in laboratory animals to investigate their possible influences on human health, and the results of toxicity studies of chemicals in animals are relevant to humans (Clayson and Krewski, 1990). However, little information on the toxicity of PAP, except for its nephrotoxicity, has been published. The Material Safety Data Sheet (MSDS) of this compound (Mallinckrodt Inc., 2003) noted that PAP is harmful if swallowed, inhaled, or absorbed through the skin and may cause methemoglobinemia. It is reported that the oral LD_{50} value of PAP was determined as 671 mg/kg in rats, and PAP caused mild conjunctival reaction and mild skin irritation in rabbits (Lloyd et al., 1977). PAP is also noted to be a fairly potent methemoglobin-producing agent in mice (Smith et el., 1967). In rats, no increase in the level of methemoglobin has been reported, other than slight reductions in total erythrocytes and hemoglobin in females fed a diet containing PAP at 0.7% for 13 weeks (Burnett et al., 1989).

There are many studies available concerning PAP-induced nephrotoxicity. The therapeutic dosage of acetaminophen was not toxic, but large overdoses produced an acute nephrotoxicity in rats (Newton et al., 1983). The toxic potential of PAP as a nephrotoxicant was stronger than acetaminophen in F344 rats (Newton et al., 1982, 1983, 1985). The nephropathy was also noted in male and female Sprague-Dawley (SD) rats given dietary PAP at 0.7% for 13 or 27 weeks (Burnett et al., 1989). The mechanism of PAP's nephrotoxicity has been investigated thoroughly, and its toxicity is known to be site-specific for the S3 segment of the proximal tubule (Green et al., 1969; Calder et al., 1971; Kiese et al., 1975; Newton et al., 1982; Gartland et al., 1989).

Although the areas of reproductive and developmental toxicology are becoming increasingly important parts of the overall toxicology profile for chemicals, only a few reports are available on the developmental toxicity of this compound. PAP was teratogenic in hamsters administered on Day 8 of pregnancy by intraperitoneal and intravenous injection, but not by gavage at

200 mg/kg/day (Rutkowski and Ferm, 1982). No teratogenicity was found in rats given PAP at up to 250 mg/kg/day by oral application on Days 6 to 15 of pregnancy (Spengler et al., 1986). Increased postimplantation loss, reduced fetal weight, and reduced ossification and increased skeletal variations in fetuses were observed at a dose that also induced lower maternal weight in rats given dietary PAP at 0.7% (equivalent to about 520 mg/kg/day) during the 13-week premating and pregnancy period (Burnett et al., 1989). The above toxicology reports on PAP can be regarded as not totally adequate for the toxicological assessment for PAP, because these studies were non–Good Laboratory Practice (GLP) studies or did not fully comply with a specific testing guideline (Klimisch et al., 1997; OECD, 2005). Therefore, PAP was selected as a target substance for the Safety Examination of Existing Chemicals in Japan to obtain reliable information on the possible effects on the reproduction and development in compliance with the OECD Test Guideline and in accordance with the principles for GLP. The present paper reports the results of reproductive/developmental toxicity screening tests of PAP in rats.

MATERIALS AND METHODS

This study was performed in compliance with OECD guideline 421 of the Reproduction/Developmental Toxicity Screening Test (OECD, 1995) and in accordance with the principles for GLP (OECD, 1998; EA, MHW, and MITI, 1988) and Guidance for Animal Care and Use of Mitsubishi Chemical Safety Institute Ltd. (Uto, Japan).

Animals

International Genetic Standard (Crj:CD(SD)IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in studies on toxicity, including reproductive and developmental toxicity, and historical control data are available. Males and females at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimated to the laboratory for 13 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Vaginal smears of each female were recorded, and only females showing a 4-day estrous cycle were used in the experiment. Male and female rats were distributed on a random basis into four groups of 12 males and 12 females each. Rats were housed individually, except during the acclimation, mating, and nursing periods. From Day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared by using wooden chips (White Flake; Charles River Japan, Inc.) as bedding.

Animals were reared on a sterilized basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water *ad libitum* and maintained in an