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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

air-conditioned room at 25 ± 2°C, with a relative humidity of 55 ± 5%, a 12-h light-dark cycle, and ventilation with 10–20 air changes per hour.

Chemicals and Dosing

PAP was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). PAP, a white or pale-yellow crystalline powder, is slightly soluble in water, alcohol, and ether, but is insoluble in chloroform and benzene. The melting point of PAP is 189.6–190.2°C, and it has a molecular weight of 109.13 (HSDB, 1996). The purity and stability of the chemical were verified by analysis before the study. The purity of PAP (Lot No. 044K0101) used in this study was 99.0%, and it was kept in the dark at room temperature. Rats were dosed once-daily by gastric intubation with PAP at a dose of 0 (control), 20, 100, or 500 mg/kg. The dosage levels were determined based on the results of a previous 28-day repeated-dose toxicity study in rats given PAP by gavage at 0 (vehicle), 4, 20, 100, or 500 mg/kg/day (JECDB, 1995). At 500 mg/kg/day, the death in 1 male with renal necrosis, decreases in body-weight gain, food consumption, erythrocyte count, hematocrit value (Ht) and hemoglobin content (Hb), increased relative weight of the liver and spleen, basophilic tubules in the kidney, and brown urine were observed. At 100 mg/kg/day, brown urine and basophilic tubules in the kidney were also observed. No toxicological effects were detected at 4 and 20 mg/kg/day.

PAP was suspended in 0.5% (w/v) carboxymethylcellulose-Na solution. Males were dosed for a total of 49 days from 14 days before mating. Females were dosed for a total of 40–60 days, beginning 14 days before mating to Day 3 of lactation throughout the mating and gestation period. The volume of each dose was adjusted to 10 mL/kg body weight based on the latest body-weight measurement during the premating and mating period in males and females or the body weight on Day 0 of pregnancy in females after copulation. Control rats were given 0.5% (w/v) carboxymethylcellulose-Na solution. The stability of formulations in the dark at room temperature has been confirmed for up to 6 h. The formulations were prepared just before use and were used within 6 h.

Observations

All rats were observed daily for clinical signs of toxicity. Body weight was recorded twice a week in males, and twice a week during the premating and mating periods, on Days 0, 7, 14, and 20 of pregnancy and on Days 0 and 4 of lactation in females. Food consumption was determined twice a week during the premating period in males, and twice a week during the premating period, on Days 1, 7, 14, and 20 of pregnancy, and on Days 1 and 4 of lactation in females.

Rats were euthanized by exsanguination under anesthesia on the day after the last administration in males and on Day 4 of lactation in females.

The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. In males, the testes and epididymides were weighed. In females, the numbers of corpora lutea and implantation sites and weight of the ovaries were recorded. The testis and epididymis were fixed with Bouin's solution and preserved in 10% neutral buffered formalin, and the ovary was stored in 10% neutral buffered formalin. Histopathological evaluations were performed on hematoxylin-eosin-stained tissue sections of the testis, epididymis, and ovary in the control and highest dose groups, and the liver, spleen, and kidney were examined for gross alterations. The testis and epididymis of the 100-mg/kg/day group were also examined, since test-substance-related changes were found in the highest group.

Daily vaginal lavage samples of each female were evaluated for estrus cycle throughout the premating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the mating period (2 weeks) had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of the sperm in the vaginal smear and/or a vaginal plug was considered evidence of successful mating, and this day was designated as Day 0 of pregnancy.

Once insemination was confirmed, the females were checked for signs of parturition before noon from Day 20 of pregnancy. The day on which parturition was completed by 12:00 was designated as postnatal day (PND) 0. The females were allowed to deliver spontaneously and nurse their pups until PND 4. Litter size and numbers of live and dead pups were recorded. Pups were sexed, examined grossly, and weighed individually on PNDs 0 and 4. On PND 4, the pups were euthanized by exsanguination under anesthesia and gross 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, food consumption, count of estrus, precoital interval, gestation length, weight of the organs, relative organ weight, numbers of corpora lutea, implantations and live and dead pups, total number of pups, and weight of live pups were analyzed by using Bartlett's test for homogeneity of variance at the 5% level of significance. If homogeneous, the data were analyzed by using Dunnett's multiple comparison test to compare the mean of the control group with that of each dosage group. If not, the PAP-treated groups were compared with that of the control group by using Steel's multiple comparison test. The implantation, delivery index, viability index, and rate of stillborn pups were analyzed by using Wilcoxon's rank sum test. The copulation, fertility and gestation indexes, sex ratio of pups, and females showing abnormal estrus cycles were analyzed by

using Fisher's exact test. The 5% level of probability was used as the criterion for significance.

RESULTS

At 500 mg/kg/day, 2 males died after 3 doses, and 1 male each died after 5 and 6 doses. In these dead males, discoloration and enlargement with tubular necrosis of the kidney was observed. A significant decrease in body-weight gain was found on Days 1–8 and 8–15 of the administration period at 500 mg/kg/day, as shown in Table 1. At this dose, significantly lower food consumption was also observed between Days 1 and 11 of the administration period. At 100 and 500 mg/kg/day, all surviving males showed brown urine. Discoloration of the kidney was observed in 4 of 8 surviving males and black-brown-colored spleen was observed in all surviving males at 500 mg/kg/day. Histopathological examination of these grossly abnormal organs revealed that basophilic tubules, protein cast, and granular cast in the kidney and deposits of hemosiderin in the red pulp and extramedullary hematopoiesis were observed in the spleen at 500 mg/kg/day.

At 500 mg/kg/day, 1 female each died after 4 and 25 administrations. At 100 and 500 mg/kg/day, all surviving females showed brown urine. A significant

Table 1: Body-weight gain in male and female rats given PAP.

Dose (mg/kg/day)	0 (control)	20	100	500
No. of male rats	12	12	12	12
No. of deaths	0	0	0	4
Initial body weight (g)[a]	373 ± 18	372 ± 20	371 ± 19	368 ± 18
Body weight gain (g)[a]				
Days 1–8	29 ± 7	33 ± 7	22 ± 15	−7 ± 10**
Days 8–15	29 ± 8	28 ± 8	26 ± 10	19 ± 5*
Days 15–22	32 ± 5	30 ± 11	34 ± 8	26 ± 8
Days 22–29	23 ± 5	24 ± 5	24 ± 7	21 ± 7
Days 29–36	28 ± 8	22 ± 5	23 ± 5	21± 10
Days 36–43	21 ± 8	22 ± 7	25 ± 6	26 ± 10
Days 43–50	20 ± 7	22 ± 6	21 ±5	17 ± 8
No. of female rats	12	12	12	12
No. of deaths	0	0	0	2
Initial body weight (g)[a]	225 ± 13	224 ± 12	223 ± 8	224 ± 9
Body weight gain (g)[a]				
Days 1–8	16 ± 8	14 ± 7	9 ± 5	−13 ± 11**
Days 8–15	9 ± 6	7 ± 5	9 ± 6	11 ± 16
Days 0–7 of pregnancy	36 ± 6	36 ± 7	32 ± 8	26 ± 9*
Days 7–14 of pregnancy	30 ± 4	31 ± 7	33 ± 6	30 ± 7
Days 14–20 of pregnancy	74 ± 12	74 ± 14	69 ± 15	50 ± 10
Days 0–4 of lactation	23 ± 15	19 ± 14	26 ± 7	6

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

decrease in body-weight gain was found on Days 1–8 of the administration period and Days 0–7 of the pregnancy period at 500 mg/kg/day (Table 1). Significantly lower food consumption was also observed at 100 mg/kg/day between 1 and 8 days and at 500 mg/kg/day between 1 and 11 days of the administration period. In the dead females, histopathological examination revealed basophilic tubules, protein cast, tubular necrosis, and/or hyaline deposit on epithelial cells of the proximal tubule in the kidney. In surviving females, no gross abnormality was detected at the scheduled sacrifice.

Table 2 presents the reproductive organ weight in male and female rats given PAP. In males, the absolute and relative weights of the testes and epididymides were significantly decreased at 500 mg/kg/day. Histopathological examination revealed decreased spermatocyte and spermatid levels, vacuolation of Sertoli cells, degeneration/necrosis of spermatocytes in the testis, and decreased sperm counts and debris of germ cells in the epididymis lumen at 500 mg/kg/day. In females, no significant changes were found in organ weight or histopathology of the ovaries.

Table 3 presents the reproductive findings in rats given PAP. The count of estrus was decreased, but not significantly, during the 14-day premating period, and the incidence of females showing 4-day estrus cycles were significantly decreased at 500 mg/kg/day. At this dose, 4 females terminated their estrus cycles and showed extended diestrous vaginal smears. One pair did not copulate at 500 mg/kg/day. No significant effects of PAP were observed on precoital interval or copulation index. One female did not become impregnated in each of the control, 20-, and 500-mg/kg/day groups. No significant differences were noted in fertility index or gestation index between the control and PAP-treated groups. Gestation length was significantly prolonged at 500 mg/kg/day.

Table 2: Reproductive organ weights in rats given PAP.

Dose (mg/kg/day)	0 (control)	20	100	500
No. of male rats	12	12	12	8
Weight of testes (g)[a]	3.53 ± 0.14	3.50 ± 0.33	3.34 ± 0.24	2.40 ± 0.29**
Relative weight of testes[a, b]	0.64 ± 0.05	0.63 ± 0.04	0.62 ± 0.05	0.49 ± 0.05**
Weight of epididymides (g)[a]	1.27 ± 0.07	1.27 ± 0.08	1.23 ± 0.09	0.92 ± 0.05**
Relative weight of epididymides[a, b]	0.23 ± 0.02	0.23 ± 0.01	0.23 ± 0.02	0.19 ± 0.02**
No. of female rats	11	11	12	2
Weight of ovaries (mg)[a]	90.7 ± 9.9	95.8 ± 11.0	96.7 ± 8.2	81.7
Relative weight of ovaries[a, b]	28.1 ± 1.4	29.9 ± 3.1	30.7 ± 2.9	29.2

[a]Values are given as the mean ± standard deviation.
[b]Relative weight = organ weight/100 g of body weight.
**Significantly different from the control, $p < 0.01$.

Table 3: Reproductive findings in rats given PAP.

Dose (mg/kg/day)	0 (control)	20	100	500
No. of females examined	12	12	12	11
Count of estrus[a]	3.8 ± 0.5	3.8 ± 0.6	3.9 ± 0.9	2.6 ± 1.6
Females showing abnormal estrous cycles (%)[b]	0	8.3	0	45.5*
No. of mated (male/female)	12/12	12/12	12/12	7/10[f]
Precoital interval (day)[a]	2.5 ± 1.2	2.6 ± 1.2	2.9 ± 3.3	4.6 ± 4.0
Copulation index (%, male/female)[c]	100/100	100/100	100/100	85.7/90.0
Fertility index (%, male/female)[d]	91.7/91.7	91.7/91.7	100/100	100/88.9
Gestation index (%)[e]	100	100	100	100
Gestation length (day)[a]	22.2 ± 0.4	22.2 ± 0.4	22.6 ± 0.7	23.3 ± 0.5**

[a]Values are given as the mean ± standard deviation.
[b]Abnormal estrous cycles (%) = (no. of females showing abnormal estrous cycles /no. of females) × 100.
[c]Copulation index (%) = (no. of rats copulated/no. of pairs) × 100.
[d]Fertility index (%) = (no. of pregnant/no. of copulated) × 100.
[e]Gestation index (%) = (no. of females with live pups born/no. of pregnant females) × 100.
[f]One female was not used for mating because this female showed severely toxicological sign.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.

Table 4 shows the developmental findings in rats given PAP. There was no significant difference between the control and PAP-treated groups in the numbers of corpora lutea, implantations, stillborn pups, pups delivered, live pups delivered, implantation index, or sex ratio of live pups. At 500 mg/kg/day, the delivery index was significantly reduced and the rate of stillborn pups was increased significantly. At this dose, almost all dams neglected their pups, some dams showed cannibalism, and all pups of 6 dams died. Although no significant difference was observed in viability index at PND 0 between control and PAP-treated groups, the index was significantly decreased at PND 4 at 500 mg/kg/day. At this dose, the body weight of live male and female pups were significantly lowered on PND 0 and were decreased on PND 4.

The results of gross examinations of pups are also shown in Table 4. At 500 mg/kg/day, pups with external malformations were found in 2 pups; 1 showed a vestigial tail and the other showed an open auricle, short tail, and kinky tail. No significant difference was observed in the incidence of pups with malformations between control and 500-mg/kg/day groups. No pups with external malformations were observed in the control or groups given PAP at 20 and 100 mg/kg/day. No pups with internal malformations were found in any groups.

DISCUSSION

In order to obtain reliable information on the reproductive and developmental toxicity of PAP, a reproductive and developmental toxicity screening study

Table 4: Developmental findings in rats given PAP.

Dose (mg/kg/day)	0 (control)	20	100	500
No. of pregnant females	11	11	12	8
No. of corpora lutea[a]	15.4 ± 1.6	14.1 ± 2.0	15.3 ± 1.8	15.6 ± 1.5
No. of implantations[a]	14.4 ± 1.0	13.6 ± 2.0	14.2 ± 2.8	14.8 ± 0.9
Implantation index (%)[b]	93.5	96.8	92.9	94.4
No. of pups delivered[a]	12.8 ± 3.1	13.0 ± 2.0	13.3 ± 2.8	11.1 ± 3.5
No. of live pups delivered[a]	12.7 ± 3.2	12.9 ± 2.0	13.1 ± 2.6	10.1 ± 4.4
No. of stillborn pups[a]	0.1 ± 0.3	0.1 ± 0.3	0.3 ± 0.5	1.0 ± 1.2
Delivery index (%)[c]	88.6	94.7	92.4	68.6**
Rate of stillborn pups (%)[d]	0.7	0.7	1.9	9.0*
Sex ratio of live pups (males/females)	74/66	66/76	69/88	50/31
No. of dams delivered	11	11	12	8
No. of dams with total litter loss	0	0	0	6
Viability index (%)[e, f]				
Day 0 of lactation	99.3	99.3	98.1	91.0
Day 4 of lactation	99.3	99.3	98.7	24.7**
Body weight of pups (g)[a]				
Male				
PND 0	6.9 ± 0.6	6.9 ± 0.3	6.7 ± 0.9	4.9 ± 0.6**
PND 4	10.9 ± 1.6	11.0 ± 0.94	10.7 ± 2.2	6.1
Female				
PND 0	6.5 ± 0.7	6.5 ± 0.4	6.4 ± 0.8	4.5 ± 0.6**
PND 4	10.4 ± 1.6	10.5 ± 0.9	10.2 ± 2.0	6.9
No. of pups (litters) examined externally on PND 0	141 (11)	143 (11)	160 (12)	89 (8)
No. of pups (litters) with malformations	0	0	0	2 (2)
Open auricle	0	0	0	1 (1)
Vestigial tail	0	0	0	1 (1)
Short tail	0	0	0	1 (1)
Kinky tail	0	0	0	1 (1)
No. of pups (litters) examined internally on PND 4	139 (11)	141 (11)	155 (12)	20 (2)
No. of pups (litters) with malformations	0	0	0	0

[a]Values are given as the mean ± standard deviation.
[b]Implantation index (%) = (no. of implantations/no. of corpora lutea) × 100.
[c]Delivery index (%) = (no. of live pups delivered/no. of implantations) × 100.
[d]Rate of stillborn pups (%) = (no. of stillborns pups/total no. of pups delivered) × 100.
[e]Viability index on day 0 of lactation (%) = (no. of live pups delivered/total no. of pups delivered) × 100.
[f]Viability index on Day 4 of lactation (%) = (no. of live pups on day 4 of lactation/no. of live pups delivered) × 100.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.

was performed by using rats. The present findings show that PAP is a general and reproductive/developmental toxic, but it is unlikely to be teratogenic, in rats.

Acute renal failure due to PAP may have participated in male and female deaths at 500 mg/kg/day, because PAP is known to be nephrotoxic and histopathological changes in the kidney were observed. Histopathological changes

in the kidney were also observed in surviving animals of the 500-mg/kg/day group. Brown urine observed in all surviving male and females at 100 and 500 mg/kg/day was thought to result from the nephrotoxic effects of PAP. The renal findings of the present study are supported by a 28-day repeated dose toxicity study of PAP (JECDB, 1995), in which brown urine, epithelial cells in urine, increased absolute and relative weights of the kidney, and basophilic tubules with mitotic cells were found at 500 mg/kg/day. Decreased body-weight gain was associated with reduced food consumption in males and females at 500 mg/kg/day, and decreased food consumption unassociated with decreased body-weight gain was found in females at 100 mg/kg/day. In male rats, decreased weights of the testes and epididymides and histopathological changes in these organs at 500 mg/kg/day indicated that PAP exerts testicular toxicity at this dose. These findings indicated that the dosages of PAP used in this study were sufficiently high to induce general toxicity in parental rats, and the NOAEL of PAP for general toxicity is considered to be 20 mg/kg/day.

Although changes in weights and histopathological findings in the testes and epididymides were detected at the highest dose, there were no adverse effects on male reproductive performance, as evidenced by no changes in the copulation index, fertility index, or precoital interval. These findings are consistent with the previous findings. It was noted previously that rodent males produce sperm in numbers that greatly exceed the minimum requirements for fertility (Amann, 1981; Parker, 2006), and sperm production can be drastically reduced (by up to 50%) without affecting fertility in SD rats (Robaire et al., 1984).

There is a general consensus that a single cycle with a diestrus period of 4 days or longer or an estrus period of 3 days or longer is aberrant, and cycles that have 4 or more days of diestrus are classified as showing persistent or prolonged diestrus (Parker, 2006). In females treated with 500 mg/kg/day, decreased incidence of females showing 4-day estrus cycles and 4 females showing extended diestrus were observed, and these phenomena might result in a prolonged precoital interval. Slightly, but significantly, increased gestation length were also found at the highest dose. These findings in females may indicate a disruptive effect of PAP on hormonal homeostasis at 500 mg/kg/day, which was high enough to cause death.

As for developmental parameters, decreases in the delivery index, viability index at PND 4, and body weights of pups at PNDs 0 and 4, and an increased rate of stillborn pups were detected at 500 mg/kg/day in the present study. These findings are essentially consistent with the previous findings reported by Burnett et al. (1989), in which decreased number and body weight of live fetuses were detected at 0.7% (520 mg/kg/day). Malformations detected in pups in the present study are of types observed spontaneously among control rat fetuses in the literature (Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000), and the incidence in the PAP-treated group was very low and not

significantly different from that of the control group. However, in the present study, no skeletal examinations were performed and the adverse effects of PAP on the morphological development of offspring could not be adequately evaluated at the highest dose, because an insufficient number of pups were obtained. Previously, no teratogenic effects of PAP were reported in rats fed PAP at up to 520 mg/kg/day (Burnett et al., 1989) and in rats given PAP by oral application at up to 250 mg/kg/day (Spengler et al., 1986). The previous and present findings together suggest that PAP has little teratogenic potential in rats.

There is concern over the possibility that the PAP-induced methemoglobinemia (Smith, 1967) and nephrotoxicity (Green et al., 1969; Calder et al., 1971; Kiese et al., 1975; Newton et al., 1982; Gartland et al., 1989) in dams are associated with the reproductive and developmental toxicity of PAP. John and Schmitz (1961) indicated a possible relationship between high maternal methemoglobin levels and abortion in humans. Sinha and Sleight (1971) observed that increased incidences of abortion and fetal deaths were produced after the administration of sodium nitrite to pregnant guinea pigs, and suggest that fetal deaths resulted from hypoxia induced mainly by maternal methemoglobinemia. Methemoglobin former *p*-nitroaniline, at levels that produced significant methemoglobinemia and low-level anemia, caused no reproducible effects on reproductive performance in a combined chronic study with a two-generation reproductive toxicity study using rats (Nair et al., 1990). In a recent review, Manassaram et al. (2006) concluded that the current literature does not provide sufficient evidence of a causal relationship between exposure to nitrates in drinking water and adverse reproductive effects in experimental animals and humans. In the case of PAP, hemosiderin deposition and extramedullary hematopoiesis observed in males of the 500-mg/kg/day group in the present study suggests a possible occurrence of hemolytic anemia. However, in the previous PAP study (Burnett et al., 1989), increased postimplantation loss and reduced fetal weight were not accompanied by methemoglobinemia in rats.

It is well known that chloroform is a nephrotoxic compound that injures the proximal tubule as well as PAP (Schnellmann, 2008). Schwetz et al. (1974) reported that inhalation of chloroform on Days 6–15 of pregnancy caused decreased conception rate, increased embryonic/fetal deaths, decreased fetal weight, and increased incidences of fetuses with tail anomalies, subcutaneous edema, skeletal variation, and retarded ossification in rats. These findings suggest that the possibility that maternal methemoglobinemia and/or nephrotoxicity participate in the developmental toxicity of PAP. The relationship between alteration of maternal physiology and offspring development is still controversial. Adverse effects of PAP on offspring observed in the present study are suggested to be due to a combination of effects of PAP and/or its metabolites and altered maternal physiology.

CONCLUSIONS

In conclusion, PAP caused death and decreased body weight gain in both sexes at 500 mg/kg/day, decreased food consumption in males at 500 mg/kg/day and females at 100 and 500 mg/kg/day, and brown urine in both sexes at 100 and 500 mg/kg/day. Terminated estrus cycles, longer gestation period, decreased delivery index, lowered pup weight, increased stillborns, and decreased viability index of pups were observed. The no observed adverse effect levels of PAP for general and reproductive/developmental toxicity were 20 and 100 mg/kg/day, respectively, in rats.

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Gender-Related Difference in the Toxicity of 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 absorber. Previously, we showed that male rats had more than a 100 times higher susceptibility to the toxic effects of DBHCB than females. In order to investigate the role of sex steroids in the mediation of this gender-related difference, DBHCB (0 or 250 mg/kg/day) was given to male and female young intact and castrated rats by gavage for 28 days in the current study. In intact rats, relative liver weight increased to more than two times that of the control in males, while the rate of change was less than 10% in females. On histopathology, hypertrophy of hepatocytes was observed in males but not in females. In castrated rats, an approximately 40% increase in the relative liver weight was found only in males, and no histopathological changes in the liver were detected in either sex. The gender-related difference was also determined in preweaning rats administered DBHCB at 0, 250, or 500 mg/kg/day by gavage from postnatal days 4 to 21. Blood biochemical changes, including increases in the levels of AST, ALT, and ALP, 80–95% increase in the relative liver weight and histopathological changes in the liver, such as hypertrophy and single cell necrosis of hepatocytes, were observed at both doses in both sexes. In conclusion, the gender-related difference in the toxicity of DBHCB, which was observed in young rats, was markedly reduced by castration and abolished in preweaning rats.

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Keywords Benzotriazole ultraviolet absorber, Gender-related difference, Castration, Preweaning rats.

INTRODUCTION

2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (CAS No. 3864-99-1; DBHCB) is an ultraviolet (UV) absorber belonging to the benzotriazole class of UV absorbers (Great Lakes Chemical Corporation, 2007). Regarding toxicity, it is reported that oral LD_{50} for DBHCB was greater than 5,000 mg/kg in rats, and DBHCB caused minimal irritation to the skin and slight irritation to the eyes in rabbits (Everlight Chemical Industrial Corporation, 2002). A 90-day feeding study of DBHCB in rats resulted in dose-dependent increases in liver weights and signs of liver toxicity at 22–800 mg/kg/day, but no detailed information is available on this study. Previously, we showed that DBHCB exerted no effects on the reproduction and development of rats in a prenatal developmental toxicity study (Ema et al., 2006) and in a combined repeated dose and reproductive/developmental toxicity screening test (Ema et al., 2008). In the latter combined study, increases in serum levels of ALP, albumin and/or A/G ratio, and in absolute and relative liver weight were found at 25 mg/kg/day and above in males, but these changes were not observed in females, even at the highest dose of 250 mg/kg/day. These findings indicate that male rats have more than a 100 times higher susceptibility to DBHCB toxicity than females.

Gender-related differences in the susceptibility of rats to toxicity have been documented for many other industrial chemicals (Hirata-Koizumi et al., 2007, 2008a; Muraoka and Itoh, 1980), environmental pollutants (Knuckles et al., 2004), insecticides (Agarwal et al., 1982; Carlson and DuBois, 1970), and pharmaceuticals (Coleman et al., 1990; Stern et al., 2007; Wang et al., 2001). For example, fluoranthene, a polycyclic aromatic hydrocarbon, showed greater effects on the kidneys of male rats, as compared to those of females, in a subchronic toxicity study (Knuckles et al., 2004). In contrast, female rats exhibited greater susceptibility to the acetylcholinesterase inhibitory effects of an organophosphorus insecticide, parathion (Agarwal et al., 1982). Such sexual variations are also reported in humans, mostly for medicines (Harris et al., 1995). Examples include the more severe adverse effects, but with greater improvement in response, of antipsychotic drugs, such as chlorpromazine and fluspirilene in women.

For such gender differences in toxic responses, sexual hormones are likely to play important roles. Agarwal et al. (1982) reported that gonadectomy abolished sex differences in acetylcholinesterase inhibition induced by parathion. Since gonadectomy increased the susceptibility of males and the administration of testosterone led to recovery from the increased sensitivity to the antiacetylcholinesterase activity of parathion, it is apparent that testosterone interferes with the effects of parathion. On the other hand, estrogen has been shown to act

as a dopamine antagonist (Harris et al., 1995), which is considered to contribute, at least in part, to sex differences in response to antipsychotic drugs. The role of sex hormones in toxicity responses seems to vary from case to case.

In the present study, in order to investigate the role of sex steroids in the mediation of gender-related differences in the susceptibility of rats to the toxicity of DBHCB, we performed a 28-day repeated-dose toxicity study of DBHCB, using male and female intact and castrated rats (castration study). Further, we determined sexual variations in DBHCB toxicity in preweaning rats, which were considered under the limited influence of sexual hormones (preweaning rat study).

MATERIALS AND METHODS

This study was performed at Shin Nippon Biomedical Laboratories, Ltd., Drug Safety Research Laboratories (SNBL DSR; Kagoshima, Japan) in 2005–2006. The experiment was approved by the Institutional Animal Care and Use Committee of SNBL DSR and was performed in accordance with the ethics criteria contained in the bylaws of the Committee of SNBL DSR.

Animals and Housing Conditions

Eleven-week-old male and 10-week-old female Crl: CD (SD) rats were purchased from Hino Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan) and individually housed in stainless steel cages suspended over a cage board. After a 7 day acclimation, females were cohabited overnight with 1 male each. Females with vaginal plugs were regarded as pregnant, and this day was designated as Day 0 of gestation. Twelve pregnant rats were assigned each for the castration and preweaning rat study. On Day 20 of gestation, pregnant females were transferred to aluminum cages with wooden chips as bedding (White Flake; Charles River Laboratories Japan, Inc.) and allowed to deliver spontaneously and rear their pups. The day of birth was defined as postnatal day (PND) 0. The sex of pups was determined on PND 0.

In the castration study, after weaning on PND 21, male and female pups found to be in good health were selected, and half of them were castrated under ether anesthesia on PNDs 25–29. Intact or castrated animals were randomly divided into DBHCB-treated and control groups of 6 males and 6 females each. They were subjected to treatment at 6 weeks of age.

In the preweaning rat study, the litters were adjusted randomly to 4 males and 4 females on PND 3. Four litters were selected and assigned to each of three dose groups, including control groups, by stratified random sampling based on body weight; the initial number of pups for treatment was 16/sex/group.

Animals were maintained in an air-conditioned room at 21.7–23.1°C, with relative humidity of 44–67%, a 12-h light/dark cycle, and ventilation with 15

air changes/hour. A basal diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water, which met the drinking water standard under the Water Works Law of Japan, were provided *ad libitum*.

Chemicals and Doses

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 was kept in a dark place at room temperature under airtight conditions. The test article was suspended in 5w/v% gum Arabic solution, 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 and dark place under airtight conditions until dosing. The stability of the formulations under these conditions had been confirmed for up to 14 days in the previous combined repeated-dose and reproductive/developmental toxicity screening test (Ema et al., 2008).

In the previous combined study, male and female rats were given DBHCB by gavage for 55–69 days at 0, 2.5, 25, or 250mg/kg/day. Increases in absolute and relative liver weight and in serum levels of alkaline phosphatase (ALP) and albumin and/or A/G ratio were observed at 25mg/kg/day and above in males, but no changes in these parameters were found in females. Taking into account these previous results, the dose levels of DBHCB in the present study were set as 250mg/kg/day for the castration study and 250 or 500mg/kg/day for the preweaning rat study. The daily application volume (10mL/kg body weight) was calculated according to the latest body weight.

Experimental Design

Castration Study

Male and female young intact and castrated rats were given DBHCB once-daily at 0 or 250mg/kg by gavage for 28 days. A Teflon gastric tube for rats (RZ-2; CLEA Japan, Inc., Tokyo, Japan), attached to a disposable syringe, was used for dosing.

All animals were observed daily before and 1–2h after dosing for clinical signs of toxicity. Body weight was measured on Days 0, 4, 7, 11, 14, 18, 21, 25, and 28 of the dosing period, and food consumption was recorded twice a week.

On the day after the last dosing, all animals were euthanized by exsanguination under deep ether anesthesia, and the body surface, organs, and tissues were examined macroscopically. The liver was then removed, weighed, and fixed in 10% neutral-buffered formalin. Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin. Histopathological examination of the liver was conducted for all animals.