

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, Prewaning 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 LD₅₀ for DBHCB was greater than 5,000mg/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 22–800mg/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 25mg/kg/day and above in males, but these changes were not observed in females, even at the highest dose of 250mg/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 250 mg/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 25 mg/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 250 mg/kg/day for the castration study and 250 or 500 mg/kg/day for the preweaning rat study. The daily application volume (10 mL/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 250 mg/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 1–2 h 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.

Preweaning Rat Study

Male and female pups were given DBHCB once-daily at 0, 250, or 500mg/kg by gavage from PNDs 4 to 21. A polyvinylchloride nutrient catheter (Type 3Fr; Atom Medical Corporation, Tokyo, Japan) attached to a disposable syringe was used for dosing.

All dams were observed daily for clinical signs of toxicity, and the body weight and food consumption were recorded on Days 0, 10, and 20 of pregnancy and on Days 0, 3, 10, 16, and 20 after delivery. On Day 23 after delivery, they were euthanized by exsanguination under deep ether anesthesia, and the body surface, organs and tissues were macroscopically observed.

All pups were observed daily before, just after, and 3–4h after dosing for clinical signs of toxicity. Body weight was recorded on PNDs 4, 6, 8, 10, 12, 14, 16, 18, and 21. On PND 22, blood was collected from the caudal vena cava in the abdomen of 2 male and 2 female pups per litter under deep ether anesthesia. Plasma was separated from the blood by centrifugation and examined for total protein, albumin, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), ALP, lactate dehydrogenase (LDH), creatine phosphokinase (CPK), calcium (Ca), inorganic phosphorus (IP), sodium (Na), potassium (K), and chlorine (Cl). Following the collection of blood, all pups (4 males and 4 females per litter) were euthanized by exsanguination under deep ether anesthesia, and the body surface, organs and tissues were macroscopically observed. The heart, lungs, liver, spleen, kidneys, and adrenals were then removed, weighed, and fixed in 10% neutral-buffered formalin. Histopathological examination was conducted on the liver of 1 male and 1 female per litter in all groups. Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin.

Data Analysis

In the castration study, parametric data, such as body weight gain, food consumption, and organ weights, were analyzed by the *F* test for homogeneity of distribution ($p < 0.05$). When homogeneity was recognized, the Student's *t*-test was conducted to compare the mean in the DBHCB-treated group with that in the control group ($p < 0.01$ or 0.05). If not homogenous, data were analyzed using Aspin-Welch's test ($p < 0.01$ or 0.05). For parametric data obtained in the preweaning rat study (body weight gain, food consumption, blood biochemical parameters, and organ weights), the homogeneity of variance was tested by Bartlett's test ($p < 0.01$). If the variances were homogeneous, Dunnett's test ($p < 0.01$ or 0.05) was applied, and when heterogeneous, the Dunnett-type mean rank test ($p < 0.01$ or 0.05) was used to compare control and individual treatment groups. In both studies, histopathological findings were analyzed by using the Wilcoxon rank sum test ($p < 0.01$ or 0.05).

RESULTS

Castration Study

Intact Rats

No substance-related deaths or clinical signs of toxicity were observed in male and female rats in the DBHCB-treated group. In males, body weight gain on Days 0–7 and 18–25 of the administration period and during the whole administration period was significantly higher in the DBHCB-treated group than the control group (Table 1). Food consumption was also significantly increased on Days 4–5 of the administration period and from Day 11 to the end of the administration in males given DBHCB (data not shown). In females, body weight gain was significantly increased on Days 0–4 and 11–14 of the administration period but decreased on Days 4–7 of the administration period in the DBHCB-treated group (Table 1). There was no significant difference in food consumption by females between DBHCB-treated and control groups (data not shown).

At necropsy, enlargement of the liver was observed in all males in the DBHCB-treated group. Significant increases in absolute and relative liver

Table 1: Body weight gain in male and female intact and castrated rats given DBHCB by gavage for 28 days.

Dose (mg/kg/day)	Intact rats		Castrated rats	
	0 (control)	250	0 (control)	250
No. of males	6	6	6	6
Days 0–4	33.3 ± 2.1	42.8 ± 5.0**	27.5 ± 6.2	26.8 ± 1.8
Days 4–7	21.5 ± 5.8	31.8 ± 4.0**	20.7 ± 4.1	21.2 ± 1.5
Days 7–11	36.8 ± 5.7	38.3 ± 6.9	26.0 ± 3.5	26.2 ± 3.1
Days 11–14	21.7 ± 3.9	24.7 ± 6.8	19.2 ± 5.6	21.3 ± 4.3
Days 14–18	25.0 ± 7.5	30.8 ± 3.5	23.0 ± 6.2	22.5 ± 4.2
Days 18–21	18.3 ± 2.7	22.5 ± 3.2*	19.0 ± 5.9	20.0 ± 3.0
Days 21–25	21.5 ± 2.7	27.0 ± 5.8*	17.8 ± 4.3	21.7 ± 2.7*
Days 25–28	14.0 ± 5.8	18.3 ± 6.9	13.5 ± 4.5	12.3 ± 3.2
Days 0–28	192.2 ± 29.5	236.3 ± 26.8*	166.7 ± 31.5	172.0 ± 10.1
No. of females	6	6	6	6
Days 0–4	12.5 ± 2.7	17.5 ± 4.0*	24.8 ± 3.6	23.7 ± 3.9
Days 4–7	13.0 ± 2.5	10.2 ± 2.3*	20.3 ± 1.8	17.5 ± 3.1*
Days 7–11	16.0 ± 3.1	15.2 ± 4.1	20.0 ± 4.6	22.8 ± 2.6
Days 11–14	8.7 ± 4.1	13.0 ± 2.3*	18.7 ± 3.1	19.0 ± 3.2
Days 14–18	14.5 ± 3.3	14.2 ± 3.1	20.5 ± 3.8	17.5 ± 3.2
Days 18–21	8.3 ± 4.5	5.5 ± 3.8	14.3 ± 2.7	13.8 ± 4.0
Days 21–25	8.0 ± 3.3	11.3 ± 3.9	20.7 ± 2.4	17.0 ± 8.0
Days 25–28	8.5 ± 4.5	7.5 ± 7.3	9.2 ± 2.6	8.5 ± 7.3
Days 0–28	89.5 ± 9.6	94.3 ± 16.6	148.5 ± 10.9	139.8 ± 37.9

Values are expressed as the mean ± SD (g).

*Significantly different from the respective control, $p < 0.05$.

**Significantly different from the respective control, $p < 0.01$.

weights were found in males given DBHCB (Table 2). In females, no gross abnormality was detected. The relative liver weight was significantly increased in females in the DBHCB-treated group, although no significant change in absolute weight was found (Table 2). Histopathological examination revealed hypertrophy, eosinophilic granular change, and decreased glycogen in hepatocytes in the liver of males receiving DBHCB, as shown in Table 3. No substance-related microscopical findings of the liver were detected in females.

Castrated Rats

No deaths or clinical signs were observed in male and female castrated rats in the DBHCB-treated or control group. In the DBHCB-treated group,

Table 2: Absolute and relative liver weights of male and female intact and castrated rats given DBHCB by gavage for 28 days.

Dose (mg/kg/day)	Intact rats		Castrated rats	
	0 (control)	250	0 (control)	250
No. of males	6	6	6	6
Body weight ^a	418 ± 43	489 ± 44	382 ± 44	372 ± 26
Absolute liver weight ^a	17.1 ± 1.9	41.9 ± 4.8**	15.6 ± 2.5	21.6 ± 2.7**
Relative liver weight ^b	4.10 ± 0.10	8.62 ± 1.18**	4.08 ± 0.22	5.81 ± 0.75**
No. of females	6	6	6	6
Body weight ^a	269 ± 19	280 ± 30	351 ± 14	328 ± 36
Absolute liver weight ^a	10.2 ± 1.0	11.4 ± 1.3	14.0 ± 1.3	13.4 ± 2.4
Relative liver weight ^b	3.79 ± 0.20	4.07 ± 0.19*	3.98 ± 0.28	4.06 ± 0.37

^aValues are expressed as the mean ± SD (g).

^bValues are expressed as the mean ± SD (g/100g body weight).

*Significantly different from the respective control, $p < 0.05$.

**Significantly different from the respective control, $p < 0.01$.

Table 3: Histopathological findings in the liver of male intact rats given DBHCB by gavage for 28 days.

	Grade	Dose (mg/kg/day)	
		0 (control)	250
No. of males		6	6
Hypertrophy of hepatocytes	+	0	1
	++	0	4
	+++	0	1
Eosinophilic granular change of hepatocytes	+	0	1
	++	0	5
	+++	0	4

Values represent the number of animals with findings.

+: Very slight.

++: Slight.

+++ : Moderate.

**Significantly different from the control, $p < 0.01$.

body weight gain was significantly increased on Days 21–25 of the administration period in males and decreased on Days 4–7 of the administration period in females (Table 1). No significant difference in food consumption was found between the control and DBHCB-treated groups in either sex (data not shown).

At necropsy, no gross abnormality was found in the DBHCB-treated or control group. As shown in Table 2, absolute and relative liver weights were significantly increased in males, but no such changes were found in females. On histopathology, no substance-related changes in the liver were detected in either sex.

Preweaning Rat Study

DBHCB, orally administered to pups from PNDs 4 to 21, did not induce any clinical signs of toxicity nor affect the body weight or food consumption of maternal rats (data not shown). At necropsy, no gross abnormality was found in the dams.

No deaths or clinical signs were found in any pups in the DBHCB-treated or control groups. Body weight gain of male pups was significantly decreased on PNDs 10–12 and 16–21 at 250 and 500 mg/kg/day, and on PNDs 12–14 at 500 mg/kg/day, as shown in Table 4. In females, a significant reduction of body

Table 4: Body weight gain in male and female preweaning rats given DBHCB by gavage for 18 days.

Dose (mg/kg/day)	0 (control)	250	500
No. of males	16	16	16
PNDs 4–6	4.09 ± 0.68	3.78 ± 0.67	3.83 ± 1.48
PNDs 6–8	4.43 ± 1.19	4.36 ± 0.91	4.82 ± 1.00
PNDs 8–10	5.48 ± 1.19	4.89 ± 1.05	5.05 ± 0.90
PNDs 10–12	5.86 ± 0.60	4.93 ± 0.50**	5.28 ± 1.21*
PNDs 12–14	5.73 ± 0.84	5.07 ± 1.21	4.35 ± 0.53**
PNDs 14–16	5.23 ± 1.26	4.40 ± 1.00	4.87 ± 1.58
PNDs 16–18	5.65 ± 0.86	4.13 ± 1.27**	4.49 ± 0.88**
PNDs 18–21	12.37 ± 1.48	6.63 ± 1.79**	7.97 ± 1.99**
PNDs 4–21	48.84 ± 4.46	38.19 ± 5.47**	40.65 ± 6.01**
No. of females	16	16	16
PNDs 4–6	4.03 ± 0.54	3.72 ± 0.51	3.51 ± 1.11
PNDs 6–8	4.68 ± 0.76	4.33 ± 0.90	4.28 ± 0.95
PNDs 8–10	4.94 ± 0.75	4.80 ± 0.98	5.04 ± 0.60
PNDs 10–12	5.74 ± 0.74	4.97 ± 0.88*	5.26 ± 0.82
PNDs 12–14	5.69 ± 1.16	4.39 ± 0.97**	4.61 ± 0.78**
PNDs 14–16	5.46 ± 1.20	4.18 ± 1.59*	4.59 ± 1.31
PNDs 16–18	5.13 ± 1.04	3.92 ± 1.70*	4.42 ± 0.74
PNDs 18–21	11.68 ± 2.36	6.77 ± 1.64**	7.38 ± 1.36**
PNDs 4–21	47.36 ± 4.98	37.09 ± 6.33**	39.08 ± 4.09**

Values are expressed as the mean ± SD (g).

*Significantly different from the control, $p < 0.05$.

**Significantly different from the control, $p < 0.01$.

weight gain was found on PNDs 10–21 at 250 mg/kg/day and on PNDs 12–14 and 18–21 at 500 mg/kg/day. In both sexes, body weight gain during the whole administration period was significantly decreased at 250 and 500 mg/kg/day.

Principle blood biochemical values are shown in Table 5. In males, the levels of AST, ALT, ALP, total bilirubin, and BUN were significantly increased at 250 mg/kg/day and above. Significant decreases in the levels of CPK and glucose were found at 500 mg/kg/day. Significant increases in the levels of AST, ALT, ALP, total bilirubin, and BUN and a significantly decreased level of glucose were also found in females at 250 and 500 mg/kg/day. In addition, the levels of total protein and IP were significantly decreased at both doses in females. There were no substance-related changes in other blood biochemical parameters.

Table 5: Principle blood biochemical findings in male and female preweaning rats given DBHCB by gavage for 18 days.

Dose (mg/kg/day)	0 (control)	250	500
No. of males	8	8	8
Total protein (g/dL)	4.66 ± 0.23	4.03 ± 0.19**	4.45 ± 0.32
Albumin (g/dL)	3.84 ± 0.19	3.53 ± 0.15*	4.00 ± 0.34
AST (IU/L)	77.6 ± 7.5	591.5 ± 779.2**	141.0 ± 29.7*
ALT (IU/L)	28.9 ± 5.7	137.6 ± 148.6**	49.1 ± 7.2*
ALP (IU/L)	1115 ± 193	2788 ± 614**	2722 ± 500**
LDH (IU/L)	140 ± 35	1211 ± 1621**	246 ± 91
CPK (IU/L)	277 ± 48	644 ± 1139	221 ± 52*
Total bilirubin (mg/dL)	0.076 ± 0.035	0.186 ± 0.108*	0.210 ± 0.119**
Glucose (mg/dL)	173 ± 28	149 ± 19	143 ± 13*
BUN (mg/dL)	16.3 ± 2.4	21.0 ± 3.9*	22.1 ± 2.6**
IP (mg/dL)	9.52 ± 1.17	9.03 ± 1.25	9.42 ± 1.86
Ca (mg/dL)	10.3 ± 0.7	9.8 ± 0.8	10.1 ± 0.4
Na (mEq/L)	144 ± 3	145 ± 1	145 ± 2
K (mEq/L)	5.88 ± 1.36	5.50 ± 1.15	5.21 ± 0.78
Cl (mEq/L)	111 ± 3	112 ± 3	111 ± 2
No. of females	8	8	8
Total protein (g/dL)	4.78 ± 0.12	4.09 ± 0.16**	4.39 ± 0.19**
Albumin (g/dL)	3.95 ± 0.05	3.66 ± 0.17*	3.94 ± 0.28
AST (IU/L)	81.1 ± 9.8	360.1 ± 199.3**	146.3 ± 18.2*
ALT (IU/L)	27.1 ± 6.4	84.1 ± 29.9**	50.8 ± 5.9*
ALP (IU/L)	1073 ± 95	2330 ± 278**	2148 ± 447**
LDH (IU/L)	147 ± 20	482 ± 309**	257 ± 172
CPK (IU/L)	283 ± 61	293 ± 231	226 ± 97
Total bilirubin (mg/dL)	0.085 ± 0.032	0.129 ± 0.029*	0.156 ± 0.042**
Glucose (mg/dL)	175 ± 18	153 ± 13*	149 ± 11**
BUN (mg/dL)	15.8 ± 3.5	21.7 ± 3.1**	23.3 ± 1.8**
IP (mg/dL)	9.68 ± 0.89	8.36 ± 0.66**	8.67 ± 0.74*
Ca (mg/dL)	10.4 ± 0.3	9.7 ± 0.4*	9.9 ± 0.5
Na (mEq/L)	145 ± 2	144 ± 2	144 ± 2
K (mEq/L)	4.91 ± 1.26	4.78 ± 0.58	4.64 ± 0.37
Cl (mEq/L)	111 ± 2	111 ± 2	112 ± 2

Values are expressed as the mean ± SD.

*Significantly different from the control, $p < 0.05$.

**Significantly different from the control, $p < 0.01$.

At necropsy, enlargement of the liver was observed in 3/16 males and 6/16 females at 250mg/kg/day, and in 11/16 males and 8/16 females at 500mg/kg/day. Absolute and relative organ weights are shown in Table 6. In males, absolute and relative liver weights and relative kidney weight were significantly increased at 250 and 500mg/kg/day. Absolute weights of the heart and lungs, and absolute and relative weights of the spleen and adrenals, were significantly decreased at both doses in males. Similar changes, except for no significant change in the relative adrenal weight, were found in organ weights of females at both doses.

Histopathological findings in the liver are presented in Table 7. In males, hypertrophy, eosinophilic granular change, single cell necrosis, and decreased glycogen in hepatocytes were observed at 250 and 500mg/kg/day. These findings were also detected with nearly identical incidences in females at either dose.

Table 6: Organ weights of male and female preweaning rats given DBHCB by gavage for 18 days.

Dose (mg/kg/day)	0 (control)	250	500
No. of males	16	16	16
Body weight (g)	64.0 ± 4.1	51.9 ± 7.0	55.0 ± 7.1
Heart (g)	0.32 ± 0.03 (0.51 ± 0.06)	0.26 ± 0.04** (0.50 ± 0.04)	0.29 ± 0.04** (0.52 ± 0.04)
Lungs (g)	0.54 ± 0.04 (0.85 ± 0.06)	0.46 ± 0.09** (0.89 ± 0.16)	0.47 ± 0.06** (0.86 ± 0.10)
Liver (g)	2.56 ± 0.33 (3.99 ± 0.33)	3.75 ± 0.71** (7.21 ± 0.61**)	4.28 ± 0.62** (7.78 ± 0.41**)
Spleen (g)	0.38 ± 0.05 (0.60 ± 0.06)	0.19 ± 0.04** (0.36 ± 0.05**)	0.21 ± 0.07** (0.38 ± 0.10**)
Kidneys (g)	0.69 ± 0.06 (1.09 ± 0.07)	0.66 ± 0.10 (1.26 ± 0.11**)	0.67 ± 0.09 (1.21 ± 0.06**)
Adrenals (mg)	17.4 ± 2.1 (28.8 ± 3.4)	12.1 ± 3.4** (22.7 ± 7.0**)	13.3 ± 3.3** (23.8 ± 5.0*)
No. of females	16	16	16
Body weight (g)	61.8 ± 4.2	50.2 ± 7.7	52.2 ± 5.0
Heart (g)	0.33 ± 0.03 (0.54 ± 0.05)	0.26 ± 0.03** (0.51 ± 0.05)	0.27 ± 0.02** (0.52 ± 0.04)
Lungs (g)	0.51 ± 0.03 (0.83 ± 0.09)	0.41 ± 0.08** (0.82 ± 0.10)	0.43 ± 0.04** (0.82 ± 0.08)
Liver (g)	2.55 ± 0.33 (4.13 ± 0.33)	3.71 ± 0.75** (7.36 ± 0.65**)	4.02 ± 0.58** (7.67 ± 0.54**)
Spleen (g)	0.36 ± 0.06 (0.58 ± 0.09)	0.18 ± 0.05** (0.36 ± 0.06**)	0.18 ± 0.05** (0.34 ± 0.07**)
Kidneys (g)	0.68 ± 0.06 (1.09 ± 0.10)	0.64 ± 0.09 (1.29 ± 0.10**)	0.65 ± 0.06 (1.26 ± 0.09**)
Adrenals (mg)	16.5 ± 2.5 (25.6 ± 5.1)	11.4 ± 2.7** (23.1 ± 6.0)	11.8 ± 2.0** (21.9 ± 4.0)

Values are expressed as the mean ± SD.

Values in parentheses are relative organ weights (g or mg/100g body weight).

*Significantly different from the control group ($p < 0.05$).

**Significantly different from the control group ($p < 0.01$).

Table 7: Histopathological findings in the liver of male and female preweaning rats given DBHCB by gavage for 18 days.

	Grade	Dose (mg/kg/day)		
		0 (control)	250	500
No. of males		4	4	4
Hypertrophy of hepatocytes	+	0	2	1
	++	0	2	1
	+++	0	0	2
Eosinophilic granular change of hepatocytes	+	0	2	1
	++	0	2	1
	+++	0	0	2
Decreased glycogen in hepatocytes	++	0	4	2
	+++	0	0	2
Single cell necrosis of hepatocytes	+	0	4*	4*
No. of females		4	4	4
Hypertrophy of hepatocytes	+	0	3	1
	++	0	1	2
	+++	0	0	1
Eosinophilic granular change of hepatocytes	+	0	0	1
	++	0	3	2
	+++	0	1	1
Decreased glycogen in hepatocytes	++	0	4	3
	+++	0	0	1
Single cell necrosis of hepatocytes	+	0	4*	4*

Values represent the number of animals with findings.

+: Very slight.

++: Slight.

+++ : Moderate.

*Significantly different from the control, $p < 0.05$.

DISCUSSION

The current study was designed to investigate the role of sex steroids in the mediation of gender-related differences in DBHCB toxicity. As expected from the results of our previous study of DBHCB (Ema et al., 2008), male rats showed much higher susceptibility to the toxic effects of DBHCB than females. This gender-related difference in the toxicity of DBHCB was markedly reduced by castration and abolished in preweaning rats.

Following DBHCB administration to young intact rats, the relative liver weight was increased by more than 100% in males but only by less than 10% in females. Histopathological changes in hepatocytes, including hypertrophy, decreased glycogen, and eosinophilic granular cytoplasm, were observed in males but not in females. Decreased glycogen could be considered another manifestation of hypertrophy, because glycogen is occasionally obscured by the proliferation of subcellular organelles (for example, smooth endoplasmic reticulum). Eosinophilic granular cytoplasm suggests peroxisome proliferation in hepatocytes because this change is widely known to be a characteristic

change observed in rodents administered with peroxisome proliferators, such as fibrate hypolipidemic drugs and phthalate plasticizers (Cattley and Popp, 2002). In order to ensure the precise mechanism of DBHCB hepatotoxicity, further study is needed to clarify the ultrastructural change or alteration in peroxisome-associated enzymes. In our previous combined study using adult rats, no histopathological change of hepatocytes was detected at the same dose of 250 mg/kg/day, the highest dose tested (Ema et al., 2008). Therefore, it is possible that young rats are more susceptible to DBHCB-induced hepatotoxicity than adult rats.

Castration of male rats reduced the degree of increased relative liver weight from 110% (intact males) to 42% (castrated males). Histopathology of the liver was not affected by DBHCB administration in castrated rats. These findings suggest an enhancing effect of testosterone on the hepatotoxicity of DBHCB in rats. In preweaning rats, DBHCB caused histopathological changes in the liver in both sexes with similar incidence and degree. Comparable increase in the relative liver weight of males (81–95%) and females (78–86%) clearly indicated a lack of gender-related difference in DBHCB hepatotoxicity in preweaning rats. Based on these findings, it seems likely that unknown factors developing at around 3–6 weeks of age under the influence of testosterone may participate in the induction of DBHCB toxicity.

Histopathological changes observed in the liver of preweaning rats included single cell necrosis of hepatocytes, which was not detected in young intact animals given DBHCB in the present study. Increased plasma levels of AST, ALT, and ALP are considered to result from such hepatic damage. In our previous comparative study of the toxicity of 1,3-dibromopropane in young and preweaning rats, single cell necrosis of hepatocytes was also observed only in preweaning rats (Hirata-Koizumi et al., 2005). Alexander et al. (1997) noted that the liver structure dramatically changed toward a highly regulated and structured regime on PNDs 8–28 in rats. These findings indicate that an immature and rapidly developing liver might be vulnerable to necrotic effects by chemicals.

In the current preweaning rat study, DBHCB also caused an inhibition of body weight gain, mainly during the late administration period. Such changes in body weight gain were not found in the previous combined study using adult rats (Ema et al., 2008) or in the present 28-day study using young intact rats. DBHCB would inhibit body weight gain more effectively during the preweaning period because rapid weight gain occurs during this period (Koizumi et al., 2001, 2002, 2003; Hirata-Koizumi et al., 2005). In the present study, the blood glucose level decreased; therefore, nutritional intake might be reduced by DBHCB administration. Changes in absolute heart and lung weights and relative kidney weight are considered to be secondary effects due to the inhibition of body weight gain, because the corresponding absolute or relative weight was not changed.

Increased plasma levels of total bilirubin and BUN found in the present preweaning rat study suggests hemolytic action and renal effects of DBHCB, respectively. Decreased spleen and adrenal weights might indicate specific effects on these organs. These changes were not observed in the previous combined study (Ema et al., 2008). In order to investigate the adverse effects of DBHCB on the blood, kidneys, spleen, and adrenals during the preweaning period, further studies, including hematological examination and histopathological observation of these organs, are required.

Alteration of gender-related differences by castration and age was also found in the toxicity of a structural analog, 2-(2'-hydroxy-3',5'-di-*tert*-butylphenyl)benzotriazole (HDBB), which is also used as UV absorber (Hirata-Koizumi et al., 2007, 2008a, 2008b, 2008c). In a 28-day repeated-dose toxicity study using young rats, HDBB principally affected the liver at much lower doses than DBHCB and also exerted adverse effects on the blood, heart, kidneys, and thyroids. Male rats consistently showed higher susceptibility to the toxic effects of HDBB; therefore, sexual variation in the toxicity of these benzotriazole UV absorbers might be explained by the difference in the blood concentration of causative substances (DBHCB, HDBB, or these metabolites) between sexes.

A number of reports have been published on gender-related variation in toxicokinetic determinants in rats, such as hepatic metabolism (Gad, 2006) and membrane transporter in various organs, including the kidneys and intestine (Morris et al., 2003). In particular, many mechanistic studies have been reported on the metabolic enzyme, cytochrome P450 (Waxman and Chang, 2005). In rats, a subset of P450s is expressed in a sex-dependent fashion, and gonadal hormones play an essential role in determining the expression of the major sex-specific rat liver P450 forms. Castration of male rats at birth abolishes the normal adult expression of male-specific P450s, and that of female rats reduces the expression of female-specific/predominant liver P450 enzymes (Bandiera and Dworschak, 1992; Dannan et al., 1986; McClellan-Green et al., 1989; Waxman et al., 1988). Because the current results showed that the higher susceptibility of male rats to DBHCB toxicity was markedly reduced by castration, there is a possibility that male-specific metabolic enzymes may be closely involved in the toxic activation of DBHCB. Interestingly, in the rat liver, the difference in P450 expression between sexes is not apparent until puberty (Waxman and Chang, 2005). For example, one of the male-specific liver P450s, the steroid 6 β -hydroxylase CYP3A2, is expressed in the prepubertal rat liver at similar levels in both sexes, but is selectively suppressed at puberty in females. One possible explanation for DBHCB exerting equivalent effects on the liver of male and female preweaning rats in the present study might be the contribution of such a male-specific P450 enzyme to the toxic activation of DBHCB. Gustafsson et al. (1981) reported that brain centers involved in the hypothalamo-pituitary control of hepatic sex-dependent

metabolism in adults are irreversibly programmed by neonatal androgen exposure, which might explain why sexual variation in DBHCB toxicity was not completely abolished by castration at PNDs 25–29 in the present study.

In order to clarify the cause of the gender-related difference in the toxicity of DBHCB, a toxicokinetic study is required. The study should include the identification of metabolites and related metabolic enzymes, as well as the measurement of the blood concentration of DBHCB after administration to both young and preweaning rats.

CONCLUSION

The current results showed that the oral administration of DBHCB for 28 days to young rats caused hepatotoxicity, and male rats had a much higher susceptibility to the toxic effects than females. This gender-related difference was markedly reduced by castration and abolished in preweaning rats.

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