Preweaning Rat Study

Male and female pups were given DBHCB once-daily at 0, 250, or 500 mg/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 3–4 h 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.

	Intact rats		Castrated rats	
Dose (mg/kg/day)	**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.

	Intact rats		Castrated rats	
Dose (mg/kg/day)	**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

[a]Values are expressed as the mean ± SD (g).
[b]Values 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.

		Dose (mg/kg/day)	
	Grade	**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 **
Decreased glycogen in hepatocytes	++	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	0.26 ± 0.04**	0.29 ± 0.04**
	(0.51 ± 0.06)	(0.50 ± 0.04)	(0.52 ± 0.04)
Lungs (g)	0.54 ± 0.04	0.46 ± 0.09**	0.47 ± 0.06**
	(0.85 ± 0.06)	(0.89 ± 0.16)	(0.86 ± 0.10)
Liver (g)	2.56 ± 0.33	3.75 ± 0.71**	4.28 ± 0.62**
	(3.99 ± 0.33)	(7.21 ± 0.61**)	(7.78 ± 0.41**)
Spleen (g)	0.38 ± 0.05	0.19 ± 0.04**	0.21 ± 0.07**
	(0.60 ± 0.06)	(0.36 ± 0.05**)	(0.38 ± 0.10**)
Kidneys (g)	0.69 ± 0.06	0.66 ± 0.10	0.67 ± 0.09
	(1.09 ± 0.07)	(1.26 ± 0.11**)	(1.21 ± 0.06**)
Adrenals (mg)	17.4 ± 2.1	12.1 ± 3.4**	13.3 ± 3.3**
	(28.8 ± 3.4)	(22.7 ± 7.0**)	(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	0.26 ± 0.03**	0.27 ± 0.02**
	(0.54 ± 0.05)	(0.51 ± 0.05)	(0.52 ± 0.04)
Lungs (g)	0.51 ± 0.03	0.41 ± 0.08**	0.43 ± 0.04**
	(0.83 ± 0.09)	(0.82 ± 0.10)	(0.82 ± 0.08)
Liver (g)	2.55 ± 0.33	3.71 ± 0.75**	4.02 ± 0.58**
	(4.13 ± 0.33)	(7.36 ± 0.65**)	(7.67 ± 0.54**)
Spleen (g)	0.36 ± 0.06	0.18 ± 0.05**	0.18 ± 0.05**
	(0.58 ± 0.09)	(0.36 ± 0.06**)	(0.34 ± 0.07**)
Kidneys (g)	0.68 ± 0.06	0.64 ± 0.09	0.65 ± 0.06
	(1.09 ± 0.10)	(1.29 ± 0.10**)	(1.26 ± 0.09**)
Adrenals (mg)	16.5 ± 2.5	11.4 ± 2.7**	11.8 ± 2.0**
	(25.6 ± 5.1)	(23.1 ± 6.0)	(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.

		Dose (mg/kg/day)		
	Grade	**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 6β-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.

ACKNOWLEDGMENT

This study was supported by the Ministry of Health, Labour and Welfare, Japan.

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Drug and Chemical Toxicology, 31:275–287, 2008

ISSN: 0148-0545 print / 1525-6014 online
DOI: 10.1080/01480540701873368

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Lack of Gender-Related Difference in the Toxicity of 2-(2′-Hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole in Preweaning Rats

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In our previous toxicity studies using young rats, we showed that an ultraviolet absorber, 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), principally affected the liver, and male rats had nearly 25 times higher susceptibility to the toxic effects than females. In the present study, the toxicity of HDBB was investigated in preweaning rats. HDBB was administered by gavage to male and female CD(SD) rats from postnatal days 4 to 21 at a dose of 0, 0.1, 0.5, 2.5, or 12.5 mg/kg/day. No substance-related deaths, clinical signs of toxicity, or body-weight changes were observed. Increased levels of albumin, AST and ALP in both sexes, BUN in males, and LDH in females were found at 12.5 mg/kg. Liver weights increased at 2.5 mg/kg and above in both sexes. Histopathologically, hepatocellular findings, such as nucleolar enlargement, anisokaryosis, increased mitosis, and/or hypertrophy, were observed at 2.5 mg/kg and above in both sexes. These results indicate no gender-related differences in the susceptibility to the toxic effects of HDBB in preweaning rats.

Keywords Benzotriazole UV absorber, Preweaning rat, Gender-related difference, Hepatotoxicity.

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INTRODUCTION

A number of reports have been published on gender-related differences in the toxic effects of chemicals in rats (Agarwal et al., 1982; Coleman et al., 1990; McGovren et al., 1981; Muraoka and Itoh, 1980; Nishino et al., 1998; Ogirima et al., 2006; Raheja et al., 1983). For example, fluoranthene, a polycyclic aromatic hydrocarbon, showed greater effects on male rats than females, especially on the kidneys, in a subchronic toxicity study (Knuckles et al., 2004). In contrast, female rats exhibited greater susceptibility to hypothalamic cholinesterase inhibitory and hypothermic effects of a carbamate cholinesterase inhibitor, rivastigmine (Wang et al., 2001). Such gender-related variations are also reported in humans, mostly for medicines (Harris et al., 1995). Examples include more severe adverse effects, but with greater improvement in response, to antipsychotic drugs such as chlorpromazine and fluspirilene in women.

Previously, we reported that male and female rats showed markedly different susceptibilities to the toxicity of 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), which is an ultraviolet absorber used in plastic resin products, such as building materials and automobile components (METI, 2006). In a 28-day repeated-dose toxicity study, male and female rats were administered HDBB by gavage, and adverse effects on the liver, heart, blood, kidneys, and thyroids were found (Hirata-Koizumi et al., 2007). The no observed adverse effect level (NOAEL) for females was 2.5 mg/kg/day based on histopathological changes in the liver and heart detected at 12.5 mg/kg, but the NOAEL for males could not be determined because hepatic changes were noted even at the lowest dose of 0.5 mg/kg. In the 52-week repeated-dose toxicity study, chronic oral administration of HDBB principally affected the liver, and the NOAEL was concluded to be 0.1 mg/kg/day in males and 2.5 mg/kg/day in females (Hirata-Koizumi et al., 2008a), showing that male rats have approximately 25 times higher susceptibility to HDBB toxicity than females.

For such gender differences in toxic responses, sexual hormones are likely to play important roles. In fact, Wang et al. (2001) reported that orchidectomy completely abolished the above-mentioned sex differences in hypothalamic cholinesterase inhibition induced by rivastigmine, and testosterone treatment to gonadectomized males and females decreased the cholinesterase inhibitory effects of rivastigmine; therefore, it is apparent that testosterone interferes with the effects of rivastigmine. 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.

In order to investigate the role of sex steroids in the mediation of sex differences in the susceptibility to the toxic effects of HDBB, we recently performed a 28-day repeated-dose toxicity study using male and female

castrated rats (Hirata-Koizumi et al., 2008b). As expected, castration markedly reduced the sexual variation in HDBB toxicity, but some difference, less than five times, remained between male and female castrated rats. It is speculated that the determinants of susceptibility to HDBB toxicity are already differentiated between sexes by four weeks of age, when the castration was performed; therefore, in the present study, we determined the sexual difference in the susceptibility to HDBB toxicity in preweaning rats.

MATERIALS AND METHODS

This study was performed at Shin Nippon Biomedical Laboratories, Ltd., Drug Safety Research Laboratories (SNBL DSR; Kagoshima, Japan) in 2006–2007. 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.

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 seven-day acclimation, females were cohabited overnight with one male each. Females with vaginal plugs were regarded as pregnant, and this day was designated as Day 0 of gestation. On gestation day 20, the 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. On PND 4, the sex of the pups was determined, and the litters were adjusted randomly to four males and four females. Five litters were selected and randomly assigned to each of five dose groups, including control groups; the initial number of pups for treatment was 20/sex/group.

Throughout the study, the animals were maintained in an air-conditioned room at 21.5–22.4°C, with a relative humidity of 43–55%, 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

HDBB (CAS No. 3846-71-7, Lot no. AY11) was 100% pure and was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan); it was kept in a dark place at room temperature under airtight conditions. Dosing

solutions were prepared as a suspension in corn oil (Wako Pure Chemical Industries, Ltd., Osaka, Japan) once or twice a week and kept cool in a dark place under airtight conditions until dosing. Stability under refrigerated conditions was confirmed for seven days in the previous 28-day repeated-dose toxicity study using young animals (Hirata-Koizumi et al., 2007).

Male and female preweaning rats were given HDBB by gavage once-daily from PNDs 4 to 21. Control rats received the vehicle only. A nutrient catheter (Type 3Fr; Atom Medical Corporation, Tokyo, Japan), attached to a disposable syringe, was used for dosing. The volume of each dose was adjusted to 10 mL/kg of body weight, based on the latest body weight.

The dosage levels of HDBB were determined to be 0.1, 0.5, 2.5, or 12.5 mg/kg/day, based on the results of our previous 28-day repeated-dose toxicity study using young rats (Hirata-Koizumi et al., 2007). In this previous study, male and female young rats were given HDBB by gavage at 0.5, 2.5, 12.5, or 62.5 mg/kg/day, and adverse effects, mainly on the liver and heart, were found at all doses in males and at 12.5 mg/kg and above in females.

Observations

All dams were observed daily for clinical signs of toxicity, and body weight was recorded on Days 0, 10, and 20 of pregnancy and on Days 0, 10, 20, and 22 after delivery. On Day 22 after delivery, they were euthanized by exsanguination under deep ether anesthesia, and the surface, organs, and tissues of the entire body were macroscopically observed.

All pups were observed daily before and three to four hours after dosing for clinical signs of toxicity. Body weight was recorded on PNDs 0, 4, 6, 8, 10, 12, 14, 16, 18, 21, and 22. On PND 22, blood was collected from the caudal vena cava in the abdomen of two male and two female pups per litter under deep ether anesthesia. Plasma separated from the blood by centrifugation was examined for total protein, albumin, glucose, total cholesterol, triglycerides, total bilirubin, blood urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatine phosphokinase, calcium, inorganic phosphorus, sodium, potassium, and chlorine. Following the collection of blood, all pups (four males and four females per litter) were euthanized by exsanguination under deep ether anesthesia, and the surface, organs, and tissues of the entire body were macroscopically observed. The heart, lungs, liver, spleen, kidneys, and adrenals were then collected and weighed. The liver and heart were histopathologically examined in one male and one female per litter. The organs were fixed in 10% neutral-buffered formalin, and paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin.

Data Analysis

Body weight, blood biochemical parameters, and organ weights of pups were analyzed by Bartlett's test (Bartlett, 1937) for homogeneity of distribution ($p < 0.01$). When homogeneity was recognized, Dunnett's test (Dunnett, 1964) was conducted to compare between control and individual treatment groups ($p < 0.01$ or 0.05). If not homogenous, data were analyzed using the mean rank test of Dunnett's type (Hollander and Wolfe, 1973) ($p < 0.01$ or 0.05). Histopathological findings were analyzed using Wilcoxon's rank sum test (Wilcoxon, 1945) ($p < 0.01$ or 0.05).

RESULTS

HDBB, orally administered to pups from PNDs 4 to 21, did not induce any clinical signs of toxicity or affect the body weight of maternal rats (data not shown). At necropsy, no gross abnormality was found in the dams.

One male pup each at 0 or 0.5 mg/kg and one female pup each at 0, 0.5, or 12.5 mg/kg died, which was confirmed to be due to gavage error. No substance-related clinical signs of toxicity were found in pups of any groups. There were also no significant changes in the body weight of male and female pups, as shown in Figure 1.

Principle blood biochemical values are summarized in Table 1. In males, the levels of albumin, AST, ALP, and BUN were significantly increased at 12.5 mg/kg. In females, significant increases in the levels of albumin, AST, ALP, and LDH were found at the same dose. There were no substance-related changes in other blood biochemical parameters.

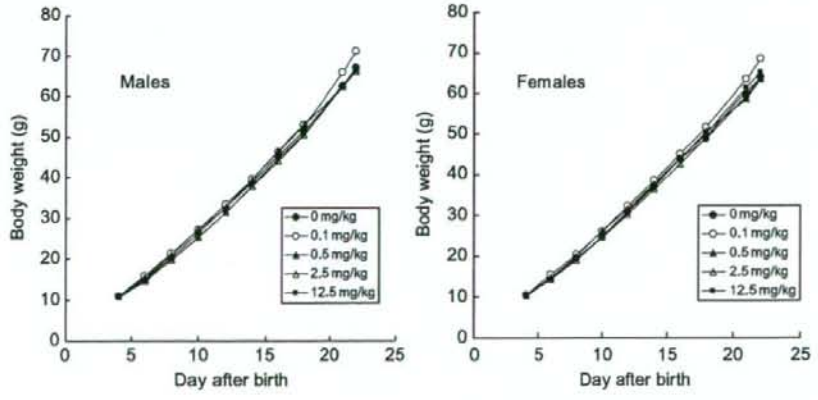

Figure 1: Body weight curves of male and female preweaning rats given HDBB by gavage.

Table 1: Principle blood biochemical values in male and female preweaning rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.1	0.5	2.5	12.5
No. of males	10	10	10	10	10
Total protein (g/dL)	4.49 ± 0.28	4.53 ± 0.22	4.48 ± 0.26	4.43 ± 0.17	4.42 ± 0.18
Albumin (g/dL)	3.62 ± 0.24	3.60 ± 0.24	3.59 ± 0.21	3.74 ± 0.27	4.04 ± 0.17**
BUN (mg/dL)	11.4 ± 1.5	14.1 ± 2.6	13.7 ± 5.3	12.9 ± 1.8	14.7 ± 2.3**
AST (IU/L)	91.4 ± 15.9	85.2 ± 4.8	88.7 ± 5.2	91.6 ± 12.2	100.2 ± 8.5*
ALT (IU/L)	34.8 ± 5.7	34.0 ± 6.3	29.4 ± 5.3	30.7 ± 5.5	35.9 ± 6.1
ALP (IU/L)	1557 ± 203	1529 ± 240	1412 ± 279	1286 ± 249	2054 ± 444**
LDH (IU/L)	198 ± 123	165 ± 16	184 ± 40	236 ± 170	326 ± 221
No. of females	10	10	10	10	10
Total protein (g/dL)	4.49 ± 0.24	4.54 ± 0.24	4.53 ± 0.28	4.55 ± 0.18	4.50 ± 0.14
Albumin (g/dL)	3.59 ± 0.28	3.66 ± 0.24	3.70 ± 0.26	3.80 ± 0.25	4.04 ± 0.16**
BUN (mg/dL)	12.5 ± 2.0	15.4 ± 1.5	13.5 ± 4.0	14.1 ± 4.1	15.5 ± 3.3
AST (IU/L)	87.3 ± 9.4	85.1 ± 8.2	86.5 ± 6.3	85.2 ± 6.6	101.3 ± 9.2**
ALT (IU/L)	30.7 ± 5.9	30.7 ± 3.6	27.1 ± 5.5	27.1 ± 4.5	35.9 ± 4.2
ALP (IU/L)	1470 ± 136	1394 ± 215	1287 ± 105	1339 ± 183	1872 ± 259**
LDH (IU/L)	175 ± 52	176 ± 36	179 ± 35	139 ± 28	370 ± 295*

Values are expressed as the mean ± SD.
BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; ALP, alkaline phosphatase; LDH, lactate dehydrogenase.
*Significantly different from the control group ($p < 0.05$).
**Significantly different from the control group ($p < 0.01$).

At necropsy, no gross abnormality was observed. Absolute and relative organ weights of scheduled sacrifice animals are shown in Table 2. In males, absolute liver weight at 12.5 mg/kg and relative weight at 2.5 mg/kg and above were significantly increased. In addition, absolute and relative weights of the lungs and spleen were significantly decreased at 12.5 mg/kg. In females, significant increases in absolute liver weight at 12.5 mg/kg and relative liver weight at 2.5 mg/kg and above, and decreases in relative spleen weight and absolute and relative adrenal weight at 12.5 mg/kg, were found. No substance-related changes were detected in other organ weights.

Histopathological findings in the liver are presented in Table 3. In males, nucleolar enlargement, anisokaryosis, and increased mitosis of hepatocytes were observed at 2.5 mg/kg and above. In the 12.5 mg/kg group, hypertrophy of hepatocytes accompanied with eosinophilic granular changes was also observed. Further, increased incidence and/or severity of decreased glycogen in hepatocytes was found at 2.5 mg/kg and above. Similarly, in females, nucleolar enlargement, anisokaryosis, and increased mitosis of hepatocytes at 2.5 mg/kg and above, and hypertrophy and eosinophilic granular change of hepatocytes at 12.5 mg/kg were detected, and the incidence and/or severity of decreased glycogen in hepatocytes was higher at 12.5 mg/kg. No substance-related histopathological changes were detected in the heart in both sexes.

Table 2: Organ weights of male and female preweaning rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.1	0.5	2.5	12.5
No. of males	19	20	19	20	20
Body weight (g)	67.2 ± 7.3	71.3 ± 6.9	67.3 ± 5.8	66.2 ± 9.6	66.2 ± 5.0
Heart (g)	0.37 ± 0.04	0.37 ± 0.04	0.36 ± 0.05	0.36 ± 0.05	0.35 ± 0.04
	(0.55 ± 0.04)	(0.52 ± 0.04)	(0.53 ± 0.05)	(0.54 ± 0.03)	(0.53 ± 0.04)
Lung (g)	0.58 ± 0.07	0.58 ± 0.04	0.53 ± 0.03*	0.59 ± 0.08	0.53 ± 0.04*
	(0.87 ± 0.07)	(0.82 ± 0.09)	(0.80 ± 0.06*)	(0.90 ± 0.09)	(0.80 ± 0.06*)
Liver (g)	2.83 ± 0.47	2.88 ± 0.34	2.75 ± 0.44	3.24 ± 0.68	4.54 ± 0.61**
	(4.19 ± 0.36)	(4.04 ± 0.26)	(4.07 ± 0.42)	(4.87 ± 0.40**)	(6.84 ± 0.53**)
Spleen (g)	0.37 ± 0.09	0.40 ± 0.05	0.34 ± 0.08	0.38 ± 0.77	0.29 ± 0.05**
	(0.55 ± 0.10)	(0.57 ± 0.06)	(0.51 ± 0.10)	(0.57 ± 0.08)	(0.44 ± 0.06**)
Kidneys (g)	0.72 ± 0.09	0.74 ± 0.06	0.72 ± 0.08	0.68 ± 0.10	0.71 ± 0.07
	(1.07 ± 0.07)	(1.04 ± 0.07)	(1.07 ± 0.08)	(1.03 ± 0.05)	(1.07 ± 0.08)
Adrenals (mg)	17.5 ± 3.7	19.3 ± 3.7	18.1 ± 3.3	21.5 ± 5.2*	17.0 ± 2.4
	(26.2 ± 5.1)	(27.3 ± 5.8)	(27.4 ± 5.8)	(32.4 ± 6.8**)	(25.6 ± 3.3)
No. of females	19	20	19	20	19
Body weight (g)	64.0 ± 7.1	68.6 ± 7.5	63.6 ± 4.7	63.6 ± 8.9	65.3 ± 4.1
Heart (g)	0.35 ± 0.05	0.35 ± 0.05	0.33 ± 0.03	0.34 ± 0.05	0.35 ± 0.04
	(0.54 ± 0.04)	(0.51 ± 0.05)	(0.52 ± 0.06)	(0.53 ± 0.04)	(0.53 ± 0.04)
Lung (g)	0.54 ± 0.08	0.54 ± 0.06	0.55 ± 0.06	0.57 ± 0.09	0.51 ± 0.05
	(0.85 ± 0.11)	(0.80 ± 0.09)	(0.86 ± 0.10)	(0.90 ± 0.12)	(0.78 ± 0.06)
Liver (g)	2.72 ± 0.47	2.77 ± 0.41	2.62 ± 0.38	3.01 ± 0.54	4.47 ± 0.39**
	(4.23 ± 0.43)	(4.02 ± 0.24)	(4.12 ± 0.44)	(4.71 ± 0.27*)	(6.84 ± 0.41**)
Spleen (g)	0.36 ± 0.12	0.37 ± 0.06	0.32 ± 0.07	0.33 ± 0.06	0.28 ± 0.07
	(0.55 ± 0.15)	(0.53 ± 0.07)	(0.50 ± 0.10)	(0.52 ± 0.08)	(0.43 ± 0.09*)
Kidneys (g)	0.70 ± 0.07	0.71 ± 0.07	0.67 ± 0.06	0.66 ± 0.09	0.72 ± 0.07
	(1.09 ± 0.05)	(1.04 ± 0.04**)	(1.05 ± 0.05)	(1.04 ± 0.05*)	(1.10 ± 0.07)
Adrenals (mg)	19.2 ± 3.7	18.8 ± 4.5	16.9 ± 2.3	19.9 ± 3.7	15.4 ± 3.5**
	(29.9 ± 4.6)	(27.5 ± 6.8)	(26.8 ± 4.2)	(31.4 ± 5.2)	(23.5 ± 4.8**)

Values are expressed as the mean ± SD.
Values in parentheses are relative organ weights (g or mg/100 g body weight).
*Significantly different from the control group ($p < 0.05$).
**Significantly different from the control group ($p < 0.01$).

Table 3: Histopathological findings in the liver of male and female preweaning rats given HDBB by gavage.

		Dose (mg/kg/day)				
	Grade	0	0.1	0.5	2.5	12.5
No. of males		5	5	5	5	5
Nucleolar enlargement in hepatocytes	±	0	0	0	1	4 **
	+	0	0	0	0	1
Anisokaryosis of hepatocytes	±	0	0	0	1	2 **
	+	0	0	0	0	3
Increased mitosis of hepatocytes	±	0	1	0	2	1 **
	+	0	0	0	1	3
	++	0	0	0	0	1
Hypertrophy of hepatocytes	±	0	0	0	0	4 **
	+	0	0	0	0	1
Eosinophilic granular change of hepatocytes	+	0	0	0	0	5**
Decreased glycogen in hepatocytes	±	1	1	2	4	2 *
	+	0	0	0	0	3
No. of females		5	5	5	5	5
Nucleolar enlargement in hepatocytes	±	0	0	0	2	4 **
	+	0	0	0	0	1
Anisokaryosis of hepatocytes	±	0	0	0	1	3 **
	+	0	0	0	0	2
Increased mitosis of hepatocytes	±	0	1	0	1	1 **
	+	0	0	0	2	3
	++	0	0	0	0	1
Hypertrophy of hepatocytes	±	0	0	0	0	3 **
	+	0	0	0	0	2
Eosinophilic granular change of hepatocytes	±	0	0	0	0	1 **
	+	0	0	0	0	4
Decreased glycogen in hepatocytes	±	1	0	2	2	3 *
	+	0	0	0	0	2

Values represent the number of animals with the finding.
±, very slight; +, slight; ++, moderate.
*Significantly different from the control ($p < 0.05$).
**Significantly different from the control ($p < 0.01$).

DISCUSSION

In the current study, the toxicity of HDBB was investigated in preweaning rats. Based on our previous results of a 28-day repeated-dose toxicity study using young rats (Hirata-Koizumi et al., 2008a), the dosage of HDBB used in this study was sufficiently high to be expected to induce adverse effects on the liver and heart. As expected, increased absolute and/or relative liver weight and histopathological changes of hepatocytes were observed at 2.5 mg/kg and above in both sexes.