Table 4: Blood biochemical findings in female rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.5	2.5	12.5
At completion of the 13-week administration period				
No. of animals	10	10	10	10
Total protein (g/dL)	6.2 ± 0.4	6.3 ± 0.2	6.4 ± 0.4	6.7 ± 0.5*
A/G ratio	1.78 ± 0.16	1.87 ± 0.22	1.93 ± 0.19	2.24 ± 0.31**
Protein fraction ratio				
α_1-Globulin (%)	13.8 ± 1.0	12.9 ± 1.7	12.6 ± 1.6	12.9 ± 1.8
α_2-Globulin (%)	5.6 ± 0.8	5.6 ± 0.2	5.5 ± 0.6	4.7 ± 0.5*
β-Globulin (%)	12.6 ± 0.9	12.4 ± 1.2	12.1 ± 1.4	9.9 ± 0.8**
γ-Globulin (%)	3.9 ± 0.8	4.3 ± 1.0	4.2 ± 1.0	3.6 ± 1.1
Albumin (%)	64.0 ± 2.0	64.9 ± 2.8	65.7 ± 2.2	68.9 ± 2.9**
ALP (IU/L)	92 ± 30	107 ± 25	101 ± 31	136 ± 81
Glucose (mg/dL)	119 ± 13	117 ± 10	118 ± 15	130 ± 10
BUN (mg/dL)	14.5 ± 1.7	14.3 ± 1.7	13.6 ± 1.1	14.1 ± 1.8
At completion of the 52-week administration period				
No. of animals	10	10	10	9
Total protein (g/dL)	6.4 ± 0.3	6.7 ± 0.2	6.7 ± 0.3	6.5 ± 0.5
A/G ratio	1.79 ± 0.25	1.69 ± 0.17	1.73 ± 0.17	2.00 ± 0.19
Protein fraction ratio				
α_1-Globulin (%)	13.5 ± 1.6	14.2 ± 1.6	12.8 ± 1.4	12.1 ± 1.0
α_2-Globulin (%)	4.8 ± 0.6	4.8 ± 0.5	5.0 ± 0.9	4.1 ± 0.4
β-Globulin (%)	13.2 ± 1.5	13.5 ± 0.7	13.6 ± 1.6	12.2 ± 1.2
γ-Globulin (%)	4.6 ± 0.9	4.9 ± 1.2	5.4 ± 1.2	5.0 ± 1.2
Albumin (%)	63.9 ± 3.1	62.6 ± 2.5	63.3 ± 2.3	66.5 ± 2.1
ALP (IU/L)	57 ± 26	59 ± 16	57 ± 14	86 ± 20**
Glucose (mg/dL)	103 ± 9	110 ± 9	106 ± 16	119 ± 16*
BUN (mg/dL)	13.4 ± 2.7	12.6 ± 2.8	12.7 ± 3.1	12.1 ± 2.0

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.

change was noted in the absolute weight. As observed at the end of the 13-week administration period, centrilobular hypertrophy of hepatocytes with eosinophilic granular cytoplasm was observed on histopathological examination, and the incidence was significantly increased at 0.5 mg/kg and higher in males, and at 12.5 mg/kg in females (Tables 7 and 8). In addition, significant increases in the incidence of cystic degeneration and lipofuscin deposition in hepatocytes at 2.5 mg/kg, and of altered hepatocellular foci (clear cell foci) at 0.5 mg/kg and higher were found in the liver of males.

DISCUSSION

In the present study, one male receiving the highest dose of 2.5 mg/kg died early in the dosing period. Although the cause of death was not identified on histopathological examination, it is unlikely that this death was due to treatment with HDBB because no deaths in this group occurred during the remaining dosing period. Other the deaths of two males at 0.1 mg/kg and the

Table 5: Relative organ weight in male rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.1	0.5	2.5
At completion of the 13-week administration period				
No. of animals	10	10	10	9
Body weight[a]	530.1 ± 32.1	566.3 ± 42.2	546.5 ± 40.3	450.1 ± 27.8**
Brain[b]	0.42 ± 0.02	0.40 ± 0.03	0.42 ± 0.03	0.49 ± 0.03**
Pituitary[c]	2.7 ± 0.3	2.5 ± 0.2	2.6 ± 0.2	2.8 ± 0.2
Thyroids[c]	3.8 ± 1.0	4.7 ± 0.8	4.5 ± 1.1	4.1 ± 0.7
Heart[b]	0.29 ± 0.03	0.29 ± 0.02	0.30 ± 0.02	0.33 ± 0.02**
Lungs[b]	0.29 ± 0.02	0.28 ± 0.03	0.30 ± 0.02	0.31 ± 0.03
Liver[b]	2.75 ± 0.10	2.82 ± 0.23	3.71 ± 0.21**	5.12 ± 0.72**
Kidneys[b]	0.62 ± 0.04	0.62 ± 0.02	0.67 ± 0.06	0.70 ± 0.07*
Testes[b]	0.65 ± 0.07	0.62 ± 0.07	0.61 ± 0.06	0.81 ± 0.07**
Epididymides[b]	0.26 ± 0.02	0.25 ± 0.02	0.23 ± 0.02*	0.28 ± 0.03
At completion of the 52-week administration period				
No. of animals	10	8	10	10
Body weight[a]	819.9 ± 145.4	792.5 ± 140.4	842.4 ± 136.3	614.2 ± 97.3**
Brain[b]	0.30 ± 0.04	0.31 ± 0.07	0.29 ± 0.04	0.39 ± 0.05**
Pituitary[c]	2.0 ± 0.2	2.0 ± 0.5	1.9 ± 0.3	2.8 ± 0.3**
Thyroids[c]	3.8 ± 0.9	3.9 ± 1.0	4.1 ± 0.8	4.9 ± 0.9*
Heart[b]	0.23 ± 0.02	0.25 ± 0.04	0.25 ± 0.03	0.31 ± 0.03**
Lungs[b]	0.23 ± 0.02	0.24 ± 0.05	0.23 ± 0.02	0.29 ± 0.03**
Liver[b]	2.22 ± 0.25	2.26 ± 0.20	2.95 ± 0.47**	4.13 ± 0.62**
Kidneys[b]	0.47 ± 0.05	0.48 ± 0.08	0.51 ± 0.06	0.68 ± 0.09**
Testes[b]	0.45 ± 0.06	0.47 ± 0.10	0.46 ± 0.07	0.61 ± 0.15**
Epididymides[b]	0.16 ± 0.03	0.18 ± 0.04	0.17 ± 0.02	0.22 ± 0.06*

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.
[a]Body weight after overnight starvation following the last dosing (g).
[b]g/100 g body weight.
[c]mg/100 g body weight.

moribund sacrifice of one female at 12.5 mg/kg were related to pituitary, renal, or muscular disorders, which was not observed in scheduled-sacrifice animals, and were considered incidental.

In scheduled-sacrifice animals, a lowered body weight was found at 2.5 mg/kg in males. This change was not observed even at the highest dose of 62.5 mg/kg in the previous 28-day repeated dose toxicity study of HDBB (Hirata-Koizumi et al., 2007). Since increased food consumption, blood glucose, and A/G ratio were noted in both previous 28-day and present 52-week studies, prolonged disturbance of metabolic homeostasis might affect body weight gain. Increased relative weight of the brain, heart, kidneys, testes, etc., without changes in the absolute weight, which was noted at 2.5 mg/kg in males in the present study, is considered to be due to the lowering of body weight.

Anemic changes, such as decreased red blood cell count, hematocrit, and hemoglobin, were also found at 0.5 mg/kg and higher in males in the current study. In females, slight changes indicative of anemia, such as decreased hematocrit and MCV, were noted at 12.5 mg/kg at the end of the 13-week

Table 6: Relative organ weight in female rats given HDBB by gavage.

Dose (mg/kg/day)	0	0.5	2.5	12.5
At completion of the 13-week administration period				
No. of animals	10	10	10	10
Body weight[a]	304.1 ± 26.9	303.0 ± 31.0	297.0 ± 17.5	299.8 ± 23.1
Brain[b]	0.68 ± 0.06	0.69 ± 0.05	0.70 ± 0.03	0.70 ± 0.05
Pituitary[c]	5.6 ± 0.5	6.1 ± 0.7	6.4 ± 1.0*	6.2 ± 0.8
Thyroids[c]	5.5 ± 1.1	5.9 ± 0.8	6.5 ± 1.1*	6.2 ± 0.7
Heart[b]	0.32 ± 0.02	0.30 ± 0.02	0.32 ± 0.02	0.32 ± 0.03
Lungs[b]	0.37 ± 0.03	0.37 ± 0.02	0.36 ± 0.02	0.38 ± 0.03
Liver[b]	2.63 ± 0.14	2.63 ± 0.18	2.80 ± 0.18	3.88 ± 0.50**
Kidneys[b]	0.70 ± 0.25	0.64 ± 0.07	0.64 ± 0.05	0.66 ± 0.06
Ovaries[c]	26.1 ± 4.0	26.5 ± 3.2	26.9 ± 4.6	27.0 ± 4.0
Uterus[b]	0.19 ± 0.03	0.22 ± 0.04	0.19 ± 0.03	0.21 ± 0.03
At completion of the 52-week administration period				
No. of animals	10	10	10	9
Body weight[a]	423.2 ± 87.2	441.8 ± 71.4	481.0 ± 104.7	425.8 ± 71.4
Brain[b]	0.54 ± 0.12	0.51 ± 0.07	0.47 ± 0.10	0.52 ± 0.08
Pituitary[c]	6.6 ± 2.3	7.0 ± 3.8	7.1 ± 3.2	7.4 ± 2.4
Thyroids[c]	5.7 ± 1.1	5.5 ± 1.3	5.9 ± 1.1	6.4 ± 1.4
Heart[b]	0.28 ± 0.04	0.28 ± 0.04	0.26 ± 0.04	0.29 ± 0.03
Lungs[b]	0.33 ± 0.07	0.30 ± 0.05	0.29 ± 0.07	0.32 ± 0.05
Liver[b]	2.48 ± 0.39	2.42 ± 0.14	2.45 ± 0.32	3.54 ± 0.41**
Kidneys[b]	0.55 ± 0.08	0.54 ± 0.06	0.52 ± 0.13	0.63 ± 0.09
Ovaries[c]	16.0 ± 3.3	14.3 ± 4.4	13.5 ± 5.5	14.3 ± 2.5
Uterus[b]	0.24 ± 0.08	0.22 ± 0.06	0.22 ± 0.09	0.25 ± 0.08

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.
[a]Body weight after overnight starvation following the last dosing (g).
[b]g/100 g body weight.
[c]mg/100 g body weight.

administration period but not at the completion of the 52-week administration period. The previous 28-day study also showed anemic effects of HDBB at 2.5 mg/kg and higher in males (Hirata-Koizumi et al., 2007). Since no change in the serum bilirubin level or hemosiderin accumulation in the liver, spleen, or kidneys were found in both the present 52-week and previous 28-day studies, anemic changes seem at least not to come from the hemolytic action of HDBB. In order to clarify the mechanisms, further study is required.

In the previous 28-day study, histopathological changes in the liver and heart were observed at 0.5 mg/kg and higher in males, and at 12.5 mg/kg and higher in females (Hirata-Koizumi et al., 2007). At higher doses, histopathological changes were also found in the kidneys and thyroids. In the current study, histopathological changes were observed in the liver. At the end of the 13-week administration, the incidence of centrilobular hypertrophy of hepatocytes was increased at 2.5 mg/kg in males and at 12.5 mg/kg in females, and this change was accompanied with eosinophilic granular cytoplasm. In addition to these changes, increased incidences of altered

Table 7: Histopathological findings in the liver of male rats given HDBB by gavage.

		Dose (mg/kg/day)				
	Grade	0	0.1	0.5	2.5	
At completion of the 13-week administration period						
No. of animals		10	10	10	9	
Centrilobular hypertrophy of hepatocytes[a]	+	0	0	3	6	] **
	++	0	0	0	3	
Focal necrosis	+	1	0	1	2	
At completion of the 52-week administration period						
No. of animals		10	8	10	10	
Centrilobular hypertrophy of hepatocytes[a]	+	0	0	5*	7	] **
	++	0	0	0	1	
Focal necrosis	+	1	0	3	4	
Lipofuscin deposition in hepatocytes[b]	+	0	0	0	6**	
Altered hepatocellular foci	+	0	1	7**	6	] **
	++	0	0	0	1	
Cystic degeneration of hepatocytes	+	0	2	2	4*	

Values represent the number of animals with findings.
+ = mild; ++ = moderate.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$
[a]Accompanied with eosinophilic granular cytoplasm.
[b]Identified by the Schmorl method, Berlin blue staining, and the Hall method.

Table 8: Histopathological findings in the liver of female rats given HDBB by gavage.

		Dose (mg/kg/day)			
	Grade	0	0.5	2.5	12.5
At completion of the 13-week administration period					
No. of animals		10	10	10	10
Centrilobular hypertrophy of hepatocytes[a]	+	0	-	0	6**
Focal necrosis	+	0	-	1	0
At completion of the 52-week administration period					
No. of animals		10	10	10	9
Centrilobular hypertrophy of hepatocytes[a]	+	0	-	0	4*
Focal necrosis	+	2	-	0	0
Lipofuscin deposition in hepatocytes[b]	+	0	-	0	2

Values represent the number of animals with findings.
+ = mild; - = not examined.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.
[a]Accompanied with eosinophilic granular cytoplasm.
[b]Identified by the Schmorl method, Berlin blue staining, and the Hall method.

hepatocellular foci (clear cell foci) at 0.5 mg/kg and higher and of cystic degeneration and lipofuscin deposition in hepatocytes at 2.5 mg/kg were found in males at the completion of the 52-week administration.

Centrilobular hypertrophy of hepatocytes with eosinophilic granular cytoplasm is 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). Prolonged exposure to these substances has been shown in many studies to induce liver tumors in rats and mice (IARC, 1995), and preferential growth of altered hepatocytes, as detected in the present 52-week study, could be observed in the developmental process. In addition, increased mitosis of hepatocytes, indicating hepatocellular proliferation, was observed in the higher dose group in the previous 28-day study of HDBB (Hirata-Koizumi et al., 2007). Further longer-term studies are needed to precisely evaluate whether HDBB induces liver tumors in rats. In the current study, lipofuscin deposition in hepatocytes was also apparent at the completion of the 52-week administration. While lipofuscin accumulates in hepatocytes with aging, increased amounts of lipofuscin have also been reported in the liver of rats treated for long periods with peroxisome proliferators (IARC, 1995; Cattley and Popp, 2002). Based on these findings, HDBB might exert an effect on the liver via the mechanism of peroxisome proliferation, although the ultrastructure or peroxisome-associated enzyme was not analyzed in the current study. The hepatic changes caused by this mechanism are considered not to be significant for human risk assessment (Hasegawa et al., 2004) because primates are much less sensitive to peroxisome proliferators than rodents (Elcombe and Mitchell, 1986; Blaauboer et al., 1990). For HDBB, however, the incidence of cystic degeneration of hepatocytes was increased at the end of the current 52-week study, and increased incidence of focal necrosis, vacuolar degeneration of hepatocytes, and bile duct proliferation in the liver was found in the previous 28-day study (Hirata-Koizumi et al., 2007). These changes may not be necessarily associated with the mechanism of peroxisome proliferation. Considering the possible induction of neoplastic change in the liver by mechanisms relevant to humans, further study is required.

In the current study, histopathological changes in the heart were not detected even at the highest dose of 2.5 mg/kg in males and 12.5 mg/kg in females, at which degeneration and hypertrophy of the myocardium or cell infiltration were found in the previous 28-day study (Hirata-Koizumi et al., 2007). Although the cause of this difference between studies is not clear, the borderline dose of HDBB for affecting the heart is considered to be around 2.5 mg/kg in males and 12.5 mg/kg in females. As functional parameters are considered to be more sensitive than histopathological changes in the heart (Glaister, 1992), further studies are required to clarify the adverse effects of HDBB on cardiac function. Histopathological changes in the kidneys and

thyroids, detected in the previous 28-day study (Hirata-Koizumi et al., 2007), were also not observed in the present study, which would be due to the low dosage administered; however, changes in osmotic pressure, specific gravity, or volume of urine, and/or increase in the levels of BUN, noted at 0.5 mg/kg and higher in males and at 12.5 mg/kg in females, suggest renal effects of HDBB.

Based on these findings in the current study, the NOAEL for chronic toxicity of HDBB was concluded to be 0.1 mg/kg/day in male rats and 2.5 mg/kg/day in female rats based on the induction of altered hepatocellular foci and/or hypertrophy of hepatocytes. This result showed that male rats are nearly 25 times more susceptible to HDBB toxicity than female rats, which is consistent with the results of the previous 28-day study (Hirata-Koizumi et al., 2007). Since male rats showed higher susceptibility to various effects of HDBB (on the liver, blood, etc.) consistently, sex-related variations in toxicokinetic determinants, such as metabolism and elimination, might increase the blood concentration of causative substances (i.e., HDBB or its metabolites) in males. In order to clarify the cause of the sexual differences in the HDBB toxicity, we are planning a toxicokinetic study of HDBB, which would include the identification of metabolites and the related metabolic enzyme as well as measurement of the blood concentration of HDBB both after single and repeated administration of HDBB to rats.

Gender-related differences in toxic susceptibility have been documented for other substances. For example, a recent subchronic toxicity study using F344 rats showed that fluoranthene, a polycyclic aromatic hydrocarbon, had greater effects on males than females, especially in the kidneys (Knuckles et al., 2004). In contrast, it was reported that female rats exhibited a greater susceptibility to hypothermic effects and inhibition of hypothalamic cholinesterase by a carbamate cholinesterase inhibitor, rivastigmine (Wang et al., 2001). For such gender differences, sexual hormones must play an important role. In fact, Wang et al. (2001) reported that orchidectomy completely abolished the above-mentioned sex differences in hypothalamic cholinesterase inhibition induced by rivastigmine. Since testosterone decreased cholinesterase inhibition in gonadectomized males and females, it is apparent that testosterone interferes with the effects of rivastigmine. It is interesting to investigate the role of sex steroids in the mediation of sex differences in toxic susceptibility to HDBB; therefore, we are currently performing a repeated-dose toxicity study of HDBB using male and female castrated rats.

CONCLUSIONS

The current results showed that the oral administration of HDBB for 52 weeks principally affected the liver. The NOAEL for chronic toxicity was concluded to be 0.1 mg/kg/day in male rats and 2.5 mg/kg/day in female rats.

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A 28-Day Repeated Dose Toxicity Study of Ultraviolet Absorber 2-(2′-Hydroxy -3′,5′-di-*tert*-butylphenyl) benzotriazole in Rats

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To examine the possible repeated-dose toxicity of an ultraviolet absorber, 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), CD(SD)IGS rats were administered HDBB by gavage at a dose of 0 (vehicle: corn oil), 0.5, 2.5, 12.5, or 62.5 mg kg^{-1} day^{-1} for 28 days. At the completion of the administration period, a decrease in red blood cells, hemoglobin, and hematocrit was noted only in males at 2.5 mg/kg and more. Blood biochemical changes were noted at 0.5 mg/kg and more in males and at 62.5 mg/kg in females. Histopathologic changes were observed principally in the liver (vacuolar degeneration and hypertrophy of hepatocytes, bile duct proliferation, etc.) and in the heart (degeneration and hypertrophy of myocardium and cell infiltration). These changes were noted at 0.5 mg/kg and more in males and at 12.5 mg/kg and more in females. At higher doses, hypertrophy of tubular epithelium in the kidneys and diffuse follicular cell hyperplasia in the thyroids in both sexes and increased severity of basophilic tubules in the kidneys and extramedullary hematopoiesis in the spleen in males were also detected. After the 14-day recovery period, these changes mostly recovered in females but not in males. Based on these findings, no observed adverse effect level (NOAEL) was concluded to be less than 0.5 mg kg^{-1} day^{-1} in male rats and 2.5 mg kg^{-1} day^{-1} in female rats.

Keywords **Benzotriazole, Gender-related difference, Rat, Repeated dose toxicity, UV absorber**

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INTRODUCTION

Plastic generally ages rapidly under the effects of light, oxygen, and heat, leading to loss of strength, reduced flexibility and electric properties, discoloration, scratching, and loss of gloss (Commerce Online, 2006; Tenkazai.com, 2006). In particular, ultraviolet (UV), possessing considerable energy (e.g., approximately 70 kcal/mol at 400 nm and 110 kcal/mol at 250 nm), directly breaks polymer bonds and promotes oxidative degradation in the presence of oxygen; therefore, UV absorbers are added to plastics to improve their long-term weather resistance and stability.

Benzotriazole UV absorbers, which have a phenolic group attached to the benzotriazole structure, are known to have the most excellent absorption capacity with a full spectrum of UV absorption (Tenkazai.com, 2006), and are therefore used in a variety of polymers. In 1999, the Phenolic Benzotriazole Association voluntarily agreed to participate in the U.S. High Production Volume Chemical Challenge Program (U.S. EPA, 2001). The existing data on four benzotriazole UV absorbers (2-(2′-hydroxy-5′-methylphenyl)benzotriazole, 2-(2′-hydroxy-5′-octylphenyl)benzotriazole, 2-(2′-hydroxy-3′,5′-di-*tert*-amylphenyl)benzotriazole, and 2-(2H-benzotriazole-2-yl)-4,6-bis(1-methyl-1-phenylethyl)phenol), reviewed in this program, showed low acute mammalian toxicity, moderate toxicity with repeated exposure (effect typically in the liver and kidney), and a lack of genotoxicity in this category of chemicals.

2-(2′-Hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (CAS no. 3846–71–7; HDBB) is a benzotriazole UV absorber added at ~0.02–2% mainly to unsaturated polyester resin, polycarbonate, vinyl chloride resin, polyacrylic acid ester, polyacetal, polyolefin, polymethacrylic acid ester, and polyamide (METI, 2006). From these resins, plastic resin products such as building materials and automobile components are manufactured. In addition, HDBB is also used in printing or sensitive materials and coating compounds, all intended for UV absorption. Although 257.5 tons were produced in Japan from April 2002 to March 2003, only limited toxicity information as a short abstract written in Japanese, which was distributed to the Committee on Safety of Chemical Substances in Chemical Substances Council of Japan, was available (METI, 2006). HDBB was selected as an object substance in an existing chemical testing program by the Japanese Government (MHLW, 2003). In this program, a 28-day repeated-dose toxicity study of HDBB was performed using rats to obtain information on its toxicity. We report the details here.

MATERIALS AND METHODS

This study was performed in compliance with the Test Guideline of the Japanese Chemical Control Act (law concerning examination and regulation of manufacture, etc., of chemical substances), "Twenty-eight-day Repeated Dose

Toxicity Test in Mammalian Species" (EA, MHW and MITI, 1986), and in accordance with the principles for Good Laboratory Practice (OECD, 1998; EA, MHW and MITI, 2000) at the Biosafety Research Center, Foods, Drugs and Pesticides (An-pyo Center, Iwata, Japan).

Chemicals

HDBB was obtained from Shipro Kasei Kaisha, Ltd. (Osaka, Japan). The HDBB (lot no. S4-034-1) used in this study was 100 wt% pure, and it was kept at room temperature. Test solutions were prepared as suspension in corn oil once a week and kept cool until dosing because stability for 7 days was confirmed under these conditions. The concentration was adjusted in such a way that the volume of each dose is constantly 5 mL/kg based on the latest body weight. The test solutions were confirmed to be 94.2% to 104.3% of the target concentration by analysis using high-performance liquid chromatography. All other reagents used in this study were specific purity grade.

Animals

Crj;CD (SD) IGS rats (SPF, 4 weeks old) were purchased from Charles River Laboratories Japan, Inc. (Yokohama, Japan). All animals were maintained in an air-conditioned room at 21.4–25.9°C, with a relative humidity of 51–75%, a 12-h light/dark cycle, and ventilation with 20 air changes per hour. They were housed individually in stainless steel wire mesh cages with anterior surfaces of aluminum. A basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water were provided *ad libitum*. Male and female rats were assigned to each dose group by stratified random sampling based on body weight. The initial numbers of rats were 10/sex in control and the highest dose group, and 5/sex in other dose groups. After 8-day acclimation, they were subjected to treatment at 5 weeks of age. This experiment was approved by the Institutional Animal Care and Use Committee of An-pyo Center and performed in accordance with the ethics criteria contained in the bylaws of the committee of An-pyo Center.

Experimental Design

The dosage levels were determined based on the findings in a 14-day dose-finding study, in which an increase in absolute and relative liver weight was observed at all doses of 100, 300, and 1000 mg kg^{-1} day^{-1}. Rats were given HDBB once daily at 0 (vehicle control), 0.5, 2.5, 12.5, or 62.5 mg kg^{-1} day^{-1} by gavage for 28 days. The day after the last dosing, five males and five females from each group were euthanized for the assessment of hematology, blood biochemistry, organ weights, and macroscopic and microscopic findings. The remaining five rats/sex at 0 and 62.5 mg/kg were kept without treatment for 14 days as a recovery period and then fully examined.

All animals were observed before and 1 h and 5 h after dosing for clinical signs of toxicity. During the recovery period, observation was made twice a day (morning and afternoon). Body weight was recorded on days 0, 7, 14, 21, and 27 of the dosing period and days 0, 7, and 13 of the recovery period. Food consumption was measured on days 7, 14, 21, and 27 of the dosing period and days 7 and 13 of the recovery period. On day 25 of the dosing period and day 11 of the recovery period, urine was collected for 3 h and analyzed for dipstick parameters, such as occult blood, pH, protein, glucose, ketone bodies, bilirubin, and urobilinogen. In addition, a 24-h urine sample was also collected for color, sediment, osmotic pressure, and volume of the urine.

Prior to necropsy at the end of dosing and recovery periods, blood was collected from the abdominal aorta under deep ether anesthesia after overnight starvation. One portion of the blood was treated with EDTA-2K and examined for hematologic parameters such as red blood cell count, hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count, platelet count, reticulocyte count, and differential leukocyte count. Another blood sample was treated with 3.13% sodium citrate, and blood clotting parameters such as prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen were examined. Serum from the remaining portions of blood was analyzed for blood biochemistry [total protein, albumin, albumin-globulin (A/G) ratio, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ-glutamyl transpeptidase, calcium, inorganic phosphorus, sodium, potassium, chlorine]. After the collection of blood, all animals were sacrificed by exsanguination, and the surface and cavity of the body and the organs and tissues of the entire body were macroscopically observed. The brain, pituitary, thymus, thyroids (including parathyroids), heart, liver, spleen, kidneys, adrenals, testes, epididymides, and ovaries were then removed and weighed (after formalin fixation of the pituitary and thyroids). The trachea, lungs (including bronchus), pancreas, lymph nodes (mesenteric and mandibular), stomach, duodenum, jejunum, ileum, cecum, colon, rectum, urinary bladder, eyeballs, spinal cord (cervical, pectoral, and lumbar part), sciatic nerve, seminal vesicles, prostates, uterus, vagina, bone marrow (femur), skeletal muscle (femur) as well as the above organs were fixed in 10% neutral-buffered formalin phosphate (after formalin acetate fixation for testes and epididymides). Histopathologic examination was conducted for all these organs of the control and the highest dose groups. In addition, the liver, heart, kidneys, spleen, and thyroids of the other groups were examined, as test-substance-related changes were found in the highest group. Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin.

Data Analysis

Parametric data such as body weight, food consumption, urinalysis findings (urine volume and osmotic pressure), hematologic and biochemical findings, and organ weights were analyzed by Bartlett's test (Bartlett, 1937) for homogeneity of distribution. When homogeneity was recognized, Dunnett's test (Dunnett, 1964) was conducted for comparison between control and individual treatment groups. If not homogenous, the data were analyzed using Steel's multiple comparison test (Steel, 1959). For histopathologic findings, Fisher's exact test (Fisher, 1973) was performed. The 5% level of probability was used as the criterion for significance.

RESULTS

No death or clinical sings of toxicity were found in any groups. There were also no significant changes in body weight, but a significant increase in food consumption was noted on dosing days 14 and 21 in males and on dosing days 21 and 27 in females at 62.5 mg/kg. No dose-related changes were found in the findings of urinalysis.

At the end of the 28-day administration period, a significant decrease in red blood cell count, hematocrit and hemoglobin at 2.5 mg/kg and more, decrease in MCHC at 12.5 mg/kg and more, and increase in platelet count at 62.5 mg/kg were noted in males, but these changes were not found in females (Table 1). For clotting factors, a significant decrease in fibrinogen was noted at 2.5 mg/kg and more in males and at 62.5 mg/kg in females (Table 1) but no significant prolongation of PT or APTT.

Blood biochemical examination revealed significant increases in the A/G ratio at 0.5 mg/kg and more, and levels of glucose at 2.5 mg/kg and more, albumin, ALT, and ALP at 12.5 mg/kg and more, and BUN and AST at 62.5 mg/kg in males (Table 2). On the other hand, for females, a significant increase in the levels of glucose, A/G ratio, total cholesterol, triglyceride, and ALT was noted only at 62.5 mg/kg.

At necropsy, absolute liver weight was significantly increased at 2.5 mg/kg and more in males and at 12.5 mg/kg and more in females with a significant increase in the relative weight at all doses in males and at 12.5 mg/kg and more in females (Table 3). In the highest dose group, there was also a significant increase in absolute and relative kidney weight in males and in absolute heart weight in females. No test-substance-related significant change was detected in other organs. Macroscopically, enlargement of the liver was observed at all doses in males and at 12.5 mg/kg and more in females. In the liver, a white patch/zone was found at 2.5 mg/kg and more in males and at 62.5 mg/kg in females.

Table 1: Principal hematologic values in male and female rats given HDBB by gavage for 28 days.

	At the completion of the administration period					At the completion of the recovery period	
	0 mg kg^{-1} day^{-1}	0.5 mg kg^{-1} day^{-1}	2.5 mg kg^{-1} day^{-1}	12.5 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}	0 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}
Male							
No. of animals	5	5	5	5	5	5	5
Red blood cells (10^6/mm^3)	7.89 ± 0.18	7.65 ± 0.32	7.23 ± 0.33*	7.18 ± 0.27**	7.16 ± 0.46**	8.26 ± 0.16	7.65 ± 0.38*
Hemoglobin (g/dL)	15.2 ± 0.4	14.8 ± 0.5	13.9 ± 0.8**	13.6 ± 0.3**	13.2 ± 0.3**	15.3 ± 0.3	13.2 ± 0.9**
Hematocrit (%)	45.6 ± 1.8	44.6 ± 1.5	42.5 ± 2.4*	41.9 ± 1.2**	40.7 ± 0.9**	44.6 ± 1.0	40.1 ± 2.7**
MCV (μm^3)	57.8 ± 1.9	58.3 ± 1.5	58.7 ± 1.2	58.3 ± 1.7	57.0 ± 2.7	54.0 ± 1.6	52.5 ± 2.6
MCH (pg)	19.3 ± 0.7	19.4 ± 0.6	19.3 ± 0.4	19.0 ± 0.9	18.4 ± 0.9	18.5 ± 0.5	17.3 ± 0.9*
MCHC (%)	33.4 ± 0.7	33.2 ± 0.5	32.8 ± 0.2	32.5 ± 0.7*	32.3 ± 0.3*	34.2 ± 0.3	32.9 ± 0.6**
Reticulocyte (%)	2.8 ± 0.3	3.3 ± 0.4	3.2 ± 0.3	3.9 ± 0.5*	3.2 ± 1.0	2.5 ± 0.4	4.4 ± 0.2**
Platelet count (10^3/mm^3)	1202 ± 75	1265 ± 107	1280 ± 116	1572 ± 430	1639 ± 227*	1196 ± 145	1502 ± 134**
Fibrinogen (mg/dL)	249 ± 13	224 ± 8	189 ± 15**	198 ± 21**	193 ± 20**	240 ± 24	214 ± 13
Female							
No. of animals	5	5	5	5	5	5	5
Red blood cells (10^6/mm^3)	7.81 ± 0.38	7.62 ± 0.61	7.79 ± 0.22	7.46 ± 0.30	7.49 ± 0.30	7.80 ± 0.27	7.64 ± 0.38
Hemoglobin (g/dL)	15.1 ± 0.9	14.9 ± 1.3	15.2 ± 0.4	14.8 ± 0.7	14.1 ± 0.6	14.9 ± 0.5	14.2 ± 0.6
Hematocrit (%)	43.7 ± 1.7	43.5 ± 3.1	44.0 ± 1.3	43.1 ± 1.8	41.6 ± 1.6	42.2 ± 1.0	40.6 ± 1.6
MCV (μm^3)	56.0 ± 1.1	57.1 ± 1.6	56.4 ± 0.8	57.7 ± 1.4	55.6 ± 1.0	54.2 ± 0.8	53.2 ± 1.8
MCH (pg)	19.3 ± 0.4	19.6 ± 0.6	19.5 ± 0.4	19.8 ± 0.5	18.9 ± 0.4	19.1 ± 0.3	18.6 ± 0.6
MCHC (%)	34.5 ± 0.8	34.3 ± 0.8	34.5 ± 0.4	34.4 ± 0.3	34.0 ± 0.4	35.2 ± 0.3	35.1 ± 0.4
Reticulocyte (%)	2.1 ± 0.4	3.5 ± 1.7	2.6 ± 0.4	2.5 ± 0.2	2.4 ± 0.3	2.7 ± 0.4	2.6 ± 0.3
Platelet count (10^3/mm^3)	1295 ± 118	1360 ± 155	1367 ± 79	1368 ± 138	1350 ± 194	1166 ± 64	1410 ± 95**
Fibrinogen (mg/dL)	193 ± 11	222 ± 46	186 ± 9	184 ± 29	155 ± 10*	210 ± 7	241 ± 7**

Values are expressed as the mean ± SD.
*Significantly different from the control, $p \leq 0.05$.
**Significantly different from the control, $p \leq 0.01$.

Table 2: Principal blood biochemical values in male and female rats given HDBB by gavage for 28 days.

	At the completion of the administration period					At the completion of the recovery period	
	0 mg kg^{-1} day^{-1}	0.5 mg kg^{-1} day^{-1}	2.5 mg kg^{-1} day^{-1}	12.5 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}	0 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}
Male							
No. of animals	5	5	5	5	5	5	5
Total protein (g/dL)	6.84 ± 0.34	6.52 ± 0.10	6.55 ± 0.24	6.72 ± 0.22	6.86 ± 0.40	6.02 ± 0.19	5.95 ± 0.49
Albumin (g/dL)	3.78 ± 0.22	3.90 ± 0.17	4.06 ± 0.20	4.43 ± 0.18**	4.40 ± 0.41**	3.75 ± 0.1	4.22 ± 0.45*
A/G ratio	1.85 ± 0.18	2.43 ± 0.23*	2.75 ± 0.29**	3.47 ± 0.25**	3.05 ± 0.55**	1.66 ± 0.11	2.46 ± 0.34*
Glucose (mg/dL)	122 ± 13	132 ± 15	170 ± 18**	170 ± 10**	156 ± 16**	166 ± 13	182 ± 22
Total cholesterol (mg/dL)	59 ± 11	46 ± 9	45 ± 4	49 ± 13	52 ± 20	62 ± 13	55 ± 19
Triglyceride (mg/dL)	25.5 ± 8.4	24.3 ± 4.5	34.5 ± 7.1	44.8 ± 20.9	45.8 ± 12.5	68.0 ± 52.0	47.5 ± 26.6
BUN (mg/dL)	13.0 ± 2.5	12.9 ± 0.5	15.5 ± 1.7	15.8 ± 1.3	17.2 ± 2.4**	14.5 ± 2.4	19.0 ± 1.9*
AST (U/L)	72 ± 7	71 ± 11	65 ± 5	83 ± 22	115 ± 16*	61 ± 7	68 ± 22
ALT (U/L)	30 ± 5	28 ± 4	32 ± 3	42 ± 5*	48 ± 10**	25 ± 5	49 ± 29**
ALP (U/L)	757 ± 175	992 ± 220	1089 ± 168	1569 ± 427**	1462 ± 250**	622 ± 123	906 ± 169*
Female							
No. of animals	5	5	5	5	5	5	5
Total protein (g/dL)	5.68 ± 0.14	5.61 ± 0.18	5.53 ± 0.19	5.93 ± 0.33	5.85 ± 0.19	5.91 ± 0.29	6.50 ± 0.30*
Albumin (g/dL)	3. 81 ± 0.23	3.67 ± 0.43	3.72 ± 0.12	4.12 ± 0.14	4.21 ± 0.18	3.85 ± 0.32	4.27 ± 0.10*
A/G ratio	2.04 ± 0.26	1.95 ± 0.44	2.09 ± 0.27	2.30 ± 0.25	2.59 ± 0.29*	1.89 ± 0.25	1.93 ± 0.18
Glucose (mg/dL)	110 ± 15	120 ± 20	114 ± 16	127 ± 22	151 ± 8**	117 ± 8	149 ± 16**
Total cholesterol (mg/dL)	49 ± 10	59 ± 5	50 ± 7	54 ± 6	84 ± 16**	63 ± 6	91 ± 14**
Triglyceride (mg/dL)	12.3 ± 5.6	12.1 ± 2.6	8.8 ± 3.7	12.2 ± 1.1	31.9 ± 4.8**	18.8 ± 7.6	37.7 ± 18.8
BUN (mg/dL)	16.1 ± 4.3	15.5 ± 1.5	16.6 ± 3.8	15.8 ± 2.4	16.9 ± 1.3	16.6 ± 1.2	16.8 ± 0.8
AST (U/L)	68 ± 5	69 ± 11	66 ± 7	68 ± 9	76 ± 12	66 ± 13	65 ± 19
ALT (U/L)	21 ± 2	22 ± 4	23 ± 3	27 ± 4	33 ± 6**	25 ± 4	36 ± 21
ALP (U/L)	490 ± 110	409 ± 86	414 ± 85	433 ± 83	633 ± 199	381 ± 138	247 ± 63

Values are expressed as the mean ± SD.
*Significantly different from the control, $p \leq 0.05$.
**Significantly different from the control, $p \leq 0.01$.

Table 3: Principal organ weights of male and female rats given HDBB by gavage for 28 days.

	At the completion of the administration period					At the completion of the recovery period	
	0 mg kg^{-1} day^{-1}	0.5 mg kg^{-1} day^{-1}	2.5 mg kg^{-1} day^{-1}	12.5 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}	0 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}
Male							
No. of animals	5	5	5	5	5	5	5
Brain (g)	2.02 ± 0.08	2.03 ± 0.07	2.12 ± 0.06	2.08 ± 0.05	2.06 ± 0.09	2.10 ± 0.10	2.07 ± 0.10
	(0.624 ± 0.009)[a]	(0.622 ± 0.038)	(0.633 ± 0.062)	(0.628 ± 0.044)	(0.630 ± 0.046)	(0.527 ± 0.046)	(0.580 ± 0.034)
Heart (g)	1.09 ± 0.09	1.10 ± 0.11	1.17 ± 0.14	1.18 ± 0.07	1.23 ± 0.19	1.20 ± 0.10	1.28 ± 0.16
	(0.337 ± 0.026)	(0.336 ± 0.028)	(0.346 ± 0.011)	(0.355 ± 0.017)	(0.374 ± 0.028)	(0.298 ± 0.008)	(0.356 ± 0.016**)
Liver (g)	9.40 ± 0.58	11.65 ± 1.90	17.11 ± 3.46*	21.64 ± 2.73*	24.47 ± 5.06*	11.8 ± 1.64	20.61 ± 3.36**
	(2.908 ± 0.139)	(3.533 ± 0.296*)	(5.045 ± 0.506*)	(6.507 ± 0.536*)	(7.413 ± 1.283*)	(2.930 ± 0.133)	(5.746 ± 0.527**)
Kidneys (g)	2.43 ± 0.22	2.54 ± 0.17	2.74 ± 0.29	2.88 ± 0.40	3.04 ± 0.45*	2.83 ± 0.23	2.91 ± 0.40
	(0.753 ± 0.075)	(0.775 ± 0.046)	(0.814 ± 0.053)	(0.865 ± 0.080)	(0.927 ± 0.119**)	(0.706 ± 0.046)	(0.814 ± 0.066*)
Testes (g)	2.90 ± 0.16	2.84 ± 0.12	2.88 ± 0.15	2.91 ± 0.15	2.92 ± 0.14	3.13 ± 0.11	3.07 ± 0.18
	(0.901 ± 0.080)	(0.871 ± 0.084)	(0.865 ± 0.121)	(0.879 ± 0.046)	(0.891 ± 0.068)	(0.787 ± 0.099)	(0.861 ± 0.043)
Female							
No. of animals	5	5	5	5	5	5	5
Brain (g)	1.94 ± 0.10	1.92 ± 0.08	1.95 ± 0.07	1.90 ± 0.12	1.90 ± 0.03	1.99 ± 0.02	1.94 ± 0.05
	(0.931 ± 0.053)	(0.884 ± 0.012)	(0.901 ± 0.052)	(0.857 ± 0.046)	(0.841 ± 0.058*)	(0.838 ± 0.086)	(0.802 ± 0.084)
Heart (g)	0.75 ± 0.07	0.77 ± 0.03	0.75 ± 0.02	0.78 ± 0.05	0.84 ± 0.06*	0.79 ± 0.04	0.87 ± 0.06
	(0.357 ± 0.019)	(0.356 ± 0.008)	(0.348 ± 0.007)	(0.351 ± 0.009)	(0.371 ± 0.024)	(0.333 ± 0.022)	(0.357 ± 0.028)
Liver (g)	6.39 ± 0.87	6.84 ± 0.63	6.73 ± 0.26	8.67 ± 1.16**	12.43 ± 0.89**	6.80 ± 0.86	8.85 ± 0.99**
	(3.053 ± 0.178)	(3.146 ± 0.197)	(3.112 ± 0.107)	(3.885 ± 0.324**)	(5.497 ± 0.172**)	(2.836 ± 0.076)	(3.626 ± 0.117**)
Kidneys (g)	1.70 ± 0.14	1.61 ± 0.08	1.71 ± 0.09	1.72 ± 0.11	1.87 ± 0.19	1.77 ± 0.18	1.86 ± 0.13
	(0.816 ± 0.057)	(0.742 ± 0.033*)	(0.789 ± 0.029)	(0.776 ± 0.040)	(0.827 ± 0.042)	(0.744 ± 0.075)	(0.766 ± 0.070)
Ovaries (mg)	87 ± 22	96 ± 18	82 ± 11	97 ± 9	89 ± 18	88 ± 12	101 ± 11
	(0.041 ± 0.007)	(0.044 ± 0.008)	(0.038 ± 0.005)	(0.044 ± 0.005)	(0.039 ± 0.008)	(0.037 ± 0.004)	(0.041 ± 0.003)

Values are expressed as the mean ± SD.
[a] Relative organ weight (organ weight per body weight) (%).
*Significantly different from the control, $p \leq 0.05$.
**Significantly different from the control, $p \leq 0.01$.

On histopathology, test-substance-related changes were observed in the liver, heart, kidneys, thyroids, and spleen as shown in Table 4. In the liver, hypertrophy of hepatocytes in males at 0.5 mg/kg and more and in females at 12.5 and 62.5 mg/kg; bile duct proliferation and decreased incidence of hepatocellular fatty change in males at 0.5 mg/kg and more and in females at 62.5 mg/kg; vacuolar degeneration of hepatocytes in males at 2.5 mg/kg and more and in females at 62.5 mg/kg; focal necrosis in males at 2.5 mg/kg and more; increased mitosis of hepatocytes in males at 62.5 mg/kg and in females at 12.5 and 62.5 mg/kg; and hepatocellular pigmentation and/or cytoplasmic inclusion bodies in males at 62.5 mg/kg were observed. In the heart, cell infiltration at 2.5 mg/kg and more in males, and degeneration and/or hypertrophy of the myocardium at 12.5 and 62.5 mg/kg in both sexes were noted. Furthermore, hypertrophy of the tubular epithelium was observed in the kidneys of males at 12.5 and 62.5 mg/kg and of females at 62.5 mg/kg, and increased severity of basophilic tubules was found in males at 62.5 mg/kg. In the thyroids and spleen, diffuse follicular cell hyperplasia at 62.5 mg/kg in both sexes and extramedullary hematopoiesis at 2.5 mg/kg and more in males, respectively, were detected.

At the end of the recovery period, a significant decrease in red blood cell count, hematocrit, hemoglobin and MCHC, and increase in platelet count were still observed in males, and a significant decrease in MCH and increase in reticulocyte in males and increase in platelet count in females were additionally found (Table 1). A significant increase in serum levels of albumin, A/G ratio, BUN, ALT, and ALP in males, and in total protein, albumin, glucose and total cholesterol in females was also noted (Table 2). At necropsy, grossly enlarged liver was still observed, and the absolute and relative weight was significantly increased in both sexes (Table 3). In males, the liver was brown, and some were accompanied with a red or white patch/zone. A significant increase in the relative weight of the heart and kidneys was also noted in males (Table 3). Histopathologically, except for increased mitosis of hepatocytes, hepatic changes were observed with similar incidence as observed at the end of the administration period in males (Table 4). Degeneration of the myocardium and cell infiltration in the heart, diffuse follicular cell hyperplasia in the thyroid, and extramedullary hematopoiesis in the spleen were also detected in males. In females, hypertrophy of hepatocytes was found, but other histopathologic changes observed at the end of the administration period were not detected. In the liver, focal necrosis and hepatocellular pigmentation were also found in females.

DISCUSSION

The current study was conducted to obtain initial information on the possible repeated-dose toxicity of HDBB in rats. The dosage of HDBB used in this

Table 4: Histopathologic findings in the principal organs of male and female rats given HDBB by gavage for 28 days.

		At the completion of the administration period					At the completion of the recovery period	
	Grade	0 mg kg^{-1} day^{-1}	0.5 mg kg^{-1} day^{-1}	2.5 mg kg^{-1} day^{-1}	12.5 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}	0 mg kg^{-1} day^{-1}	62.5 mg kg^{-1} day^{-1}
Male								
No. of animals		5	5	5	5	5	5	5
Liver								
Hypertrophy of hepatocytes	+	0	3	5**	5**	5**	0	5**
Fatty change of hepatocytes	+	5	0**	0**	0**	0**	5	0**
Bile duct proliferation	+	0	1	1	4*	4*	0	4*
Vacuolar degeneration of hepatocytes	+	0	0	5**	5**	5**	0	4*
Focal necrosis	+	0	0	1	2	4*	0	3
Increased mitosis of hepatocytes	+	0	0	0	0	4*	0	0
Pigment deposit of hepatocytes	+	0	0	0	0	1	0	2
Cytoplasmic inclusion bodies	+	0	0	0	0	1	0	1
Heart								
Cell infiltration	+	0	1	5**	4*	4*	1	3
Degeneration of myocardium	+	0	0	0	5**	5**	0	4*
Hypertrophy of myocardium	+	0	0	0	3	4*	0	0
Kidney								
Hypertrophy of tubular epithelium	+	0	0	0	2	5**	0	0
Basophilic tubules	+	2	3	4	3	3	4	4
	++	0	0	0	0	2	0	0
Thyroid								
Diffuse follicular cell hyperplasia	+	0	0	0	0	2	0	3

Spleen								
Extramedullary hematopoiesis	+	0	0	3	2	2	0	3
Female								
No. of animals		5	5	5	5	5	5	5
Liver								
Hypertrophy of hepatocytes	+	0	0	0	5**	5**	0	3
Fatty change of hepatocytes	+	5	5	5	3	0**	5	4
Bile duct proliferation	+	0	0	0	0	1	0	0
Vacuolar degeneration of hepatocytes	+	0	0	0	0	2	0	0
Focal necrosis	+	0	0	0	0	0	0	2
Increased mitosis of hepatocytes	+	0	0	0	1	2	0	0
Pigment deposit of hepatocytes	+	0	0	0	0	0	0	1
Heart								
Cell infiltration	+	0	1	0	0	1	0	0
Degeneration of myocardium	+	0	0	0	3	5**	0	0
Hypertrophy of myocardium	+	0	0	0	1	3	0	0
Kidney								
Hypertrophy of tubular epithelium	+	0	0	0	0	2	0	0
Basophilic tubules	+	1	2	2	0	3	4	4
Thyroid								
Diffuse follicular cell hyperplasia	+	0	0	0	0	2	0	0
Spleen								
Extramedullary hematopoiesis	+	0	1	0	0	0	0	0

Values represent the number of animals with findings.
+: slight; ++: moderate.
*Significantly different from the control, $p \leq 0.05$.
**Significantly different from the control, $p \leq 0.01$.

study was sufficiently high to be expected to induce toxicity in the liver. As expected, histopathologic changes including vacuolar degeneration and hypertrophy of hepatocytes were observed in the liver. These findings showed that one of the toxicologically main targets of HDBB was the liver. Increased food consumption without body weight changes, increased blood glucose, total cholesterol and triglyceride, and decreased incidence of fatty changes of hepatocytes were noted after HDBB administration for 28 days. These changes indicate metabolic derangement and suggest possible adverse effects of HDBB in metabolic homeostasis. The current study showed that the heart was another toxicologic target organ for HDBB. Although degeneration and hypertrophy of the myocardium and cell infiltration were observed after HDBB administration, cardiac function was not evaluated in the current study. Further studies are required to clarify the adverse effects of HDBB on cardiac function, because functional parameters are considered to be more susceptible than histopathologic changes in the heart (Glaister, 1992). In our study, HDBB also caused anemic changes (decreased red blood cell count, hematocrit, hemoglobin and MCHC, and extramedullary hematopoiesis), and adverse effects on the kidneys (hypertrophy of tubular epithelium and increased severity of basophilic tubules with increased BUN) and the thyroids (diffuse follicular cell hyperplasia) at higher doses. Adverse effects on the liver and kidneys, and anemia, but not adverse effects on the heart and thyroid, were reported in the 90-day repeated feeding study on the structural analogue, 2-(2′-hydroxy-3′,5′-di-*tert*-amylphenyl)benzotriazole, in rats (U.S. EPA, 2001). Further studies are needed to clarify the differences in the toxicological profiles between the current study and study on the analogue.

The results of the current study clearly showed sex differences in the toxic susceptibility of rats to HDBB. In males, the development of anemia and histopathologic changes in the liver, heart, kidneys, thyroid, and spleen accompanied with related blood biochemical and organ weight changes were seen. Hypertrophy of hepatocytes, decreased incidence of fatty change of hepatocytes, bile duct proliferation, increase in relative liver weight and serum A/G ratio were noted even at 0.5 mg/kg. Most of the changes were not improved after a 14-day recovery period in the highest dose group. In females, however, no anemic effects of HDBB were observed, and other effects observed in males were noted only at 12.5 mg/kg and more in females. These changes in females mostly recovered after the recovery period. These findings suggest that male rats have a nearly 25 times higher susceptibility to HDBB toxicity than female rats.

Gender-related differences in toxic susceptibility have been documented for some other substances. For example, a recent subchronic toxicity study using F344 rats showed that fluoranthene, a polycyclic aromatic hydrocarbon, had greater effects on males than females (especially on the kidneys) (Knuckles et al., 2004). In contrast, it was reported that female rats exhibited a greater