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 Schmori 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 Schmori 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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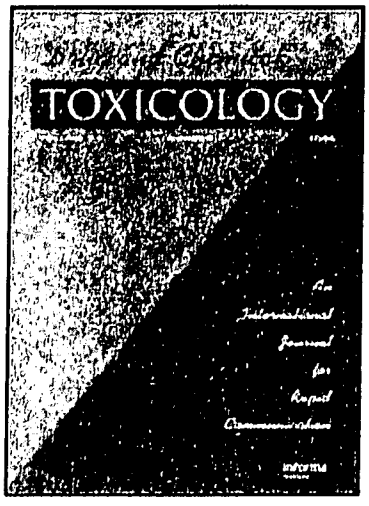

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Gonadal Influence on the Toxicity of 2-(2'-Hydroxy-3',5'-di-tert-butylphenyl) benzotriazole in Rats

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Gonadal Influence on the Toxicity of 2-(2′-Hydroxy -3′,5′-di-*tert*-butylphenyl) benzotriazole in Rats

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Previously, we showed that susceptibility of male rats to the toxicity of an ultraviolet absorber, 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), was nearly 25 times higher than that of females. In the current study, we investigated the role of sex steroids in the mediation of the gender-related difference using castrated rats. Male and female castrated CD(SD) rats were given HDBB by gavage at 0, 0.5, 2.5, or 12.5 mg/kg/day for 28 days. No deaths, clinical signs of toxicity, or changes in body weight or food consumption were found at any doses. Blood biochemical changes suggestive of hepatic damage, such as increased levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and lactate dehydrogenase, were detected at 12.5 mg/kg/day in males. Absolute and relative liver weight increased at 0.5 mg/kg/day and above in males and at 12.5 mg/kg/day in females. In the liver, histopathological changes, such as nucleolar enlargement, increased mitosis, hypertrophy in hepatocytes, and/or focal necrosis were observed at 0.5 mg/kg/day and above in males, and at 2.5 mg/kg/day and above in females. These findings indicate that castration markedly reduced the gender-related differences in toxicity of HDBB in rats.

Keywords Benzotriazole UV absorber, Castration, Gender-related difference, Rats.

INTRODUCTION

2-(2′-Hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (CAS No. 3846-71-7; HDBB) is an ultraviolet (UV) absorber used in plastic resin products, such as

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building materials and automobile components (METI, 2006). Previously, we showed a marked difference in the susceptibility of male and female rats to the toxicity of HDBB in 28-day and 52-week repeated oral dose toxicity studies (Hirata-Koizumi et al., 2007; 2008). In the 28-day study, toxic effects in the liver, heart, kidneys, thyroids, and blood were observed. 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/day. However, the NOAEL for males could not be determined because hepatic changes were noted even at the lowest dose of 0.5 mg/kg/day. In the 52-week study, the NOAEL was concluded to be 0.1 mg/kg/day in males and 2.5 mg/kg/day in females based on histopathological changes in the liver. These findings consistently showed that male rats have a nearly 25 times higher susceptibility to HDBB toxicity than female rats.

Gender-related differences in susceptibility to toxicity have been documented for other substances; for example, a subchronic toxicity study in 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, 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. Examples include the more severe adverse effects, but with greater improvement in response, to antipsychotic drugs such as chlorpromazine and fluspirilene in women (Harris et al., 1995).

Sex hormones are likely to play an important role in gender differences in toxicity responses. 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. The role of sex hormones in differences between sexes in toxicity responses seems to vary from case to case.

In the present study, we performed a repeated dose toxicity study of HDBB using male and female castrated rats to investigate the role of sex steroids in the mediation of sex difference in the susceptibility of rats to the toxicity of HDBB. Administration was conducted in the same way as the previous 28-day study using intact animals (Hirata-Koizumi et al., 2007) for comparison, and effects on the liver and heart, which were principally affected in the previous study of HDBB, were examined.

MATERIALS AND METHODS

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

Chemicals

HDBB (Lot no. AY11) was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). The HDBB used in this study was 100% pure and was kept in a light-resistant and airtight container at room temperature. Test solutions were prepared as suspensions in corn oil twice a week and kept cool in a light-resistant and airtight container until dosing. Stability under refrigerated conditions was confirmed for seven days in the previous 28-day repeated dose toxicity study using intact animals (Hirata-Koizumi et al., 2007). All other reagents used in this study were of specific purity grade.

Animals

Crl:CD(SD) rats (SPF, three weeks old) were purchased from Hino Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). All animals were maintained in an air-conditioned room at 21.8–22.8°C, with a relative humidity of 45%–55%, a 12-h light/dark cycle, and ventilation with 15 air changes/h. Animals were housed individually in stainless cages suspended over a cage board. A basal diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water, which meets the drinking water standard under the Water Works Law of Japan, were provided *ad libitum*.

Male and female rats were castrated under ether anesthesia between five and eight days after purchase. After a two-week acclimation, they were subjected to treatment at six weeks of age. Rats found to be in good health were selected and assigned to four groups of 10 males and 10 females by stratified random sampling based on body weight. One female in the highest dose group was excluded from the present study because remnants of the left ovary were confirmed at necropsy.

Experimental Design

Male and female castrated rats were given HDBB once-daily at 0 (vehicle control), 0.5, 2.5, and 12.5 mg/kg/day by gavage for 28 days. The dosage levels were determined based on the results of our previous 28-day study using intact rats given HDBB by gavage at 0.5, 2.5, 12.5, or 62.5 mg/kg/day, at which adverse effects, mainly on the liver and heart, were found at all doses in males and at

12.5 mg/kg/day and above in females (Hirata-Koizumi et al., 2007). The volume of each dose was adjusted to 10 mL/kg based on the latest body weight.

All animals were observed daily before and one to two hours after dosing for clinical signs of toxicity. Body weight was measured on days 0, 3, 7, 10, 14, 17, 21, 24, and 28 of the dosing period, and food consumption was recorded on days 0, 3, 7, 10, 14, 17, 21, 24, and 27 of the dosing period.

On the day after the last dosing, blood was drawn from the caudal vena cava in the abdomen with a heparin-added syringe under ether anesthesia and centrifuged to obtain plasma. The plasma was examined for biochemical parameters, such as total protein, albumin, glucose, total cholesterol, triglycerides, total bilirubin, urea nitrogen (BUN), creatinine, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), lactate dehydrogenase (LDH), creatinine phosphokinase, calcium, inorganic phosphorus, sodium, potassium, and chlorine. Following the collection of blood, all animals were euthanized by exsanguination, and the surface of the body, and organs and tissues of the entire body, were examined macroscopically. The liver and heart were then removed and weighed. Both organs were fixed in 10% neutral-buffered formalin, processed routinely for embedding in paraffin, and sections were prepared for staining with hematoxylin and eosin. Histopathological observation was performed for all groups.

Data Analysis

Parametric data, such as body weight, food consumption, blood biochemical parameters, and organ weights, were analyzed by Bartlett's test (Bartlett, 1937) for homogeneity of distribution ($p < 0.05$). When homogeneity was recognized, Dunnett's test (Dunnett, 1964) was conducted to compare control and individual treatment groups ($p < 0.01$ or 0.05). If not homogenous, the data were analyzed using a Dunnett-type mean rank test ($p < 0.01$ or 0.05) (Hollander and Wolfe, 1973).

RESULTS

No deaths or clinical signs of toxicity were found in any groups. There was no significant difference in body weight between the control and HDBB-treated groups (Fig. 1). Food consumption was also not significantly changed, except for a transient increase on day 21 of the administration period at 12.5 mg/kg/day and on day 27 of the administration period at 2.5 mg/kg/day in males (data not shown).

Blood biochemical examination revealed significant increases in the level of albumin at 0.5 mg/kg/day and above in males and at 2.5 mg/kg/day and above in females, total protein at 0.5 mg/kg/day and above in females, glucose at 12.5 mg/kg/day in males, and BUN at 12.5 mg/kg/day in both sexes (Table 1).

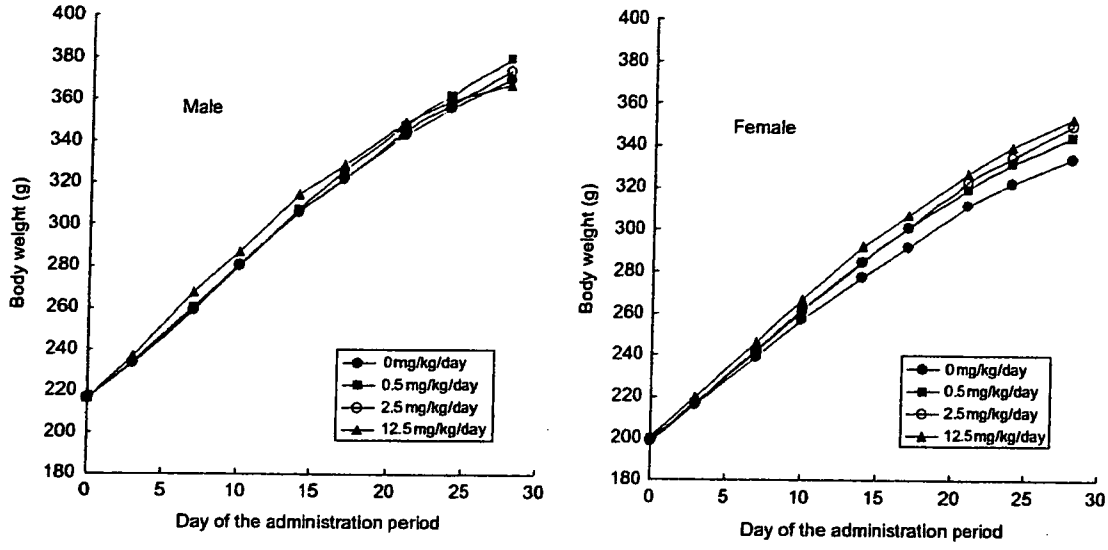

Figure 1: Body weight of male and female castrated rats given HDBB by gavage for 28 days.

Table 1: Blood biochemical findings in male and female castrated rats given HDBB by gavage for 28 days.

Dose (mg/kg/day)	**0**	**0.5**	**2.5**	**12.5**
Male				
No. of animals	10	10	10	10
Total protein (g/dL)	6.19 ± 0.32	6.44 ± 0.23	6.45 ± 0.40	6.26 ± 0.31
Albumin (g/dL)	4.43 ± 0.18	4.90 ± 0.17**	4.99 ± 0.25**	5.03 ± 0.18**
AST (IU/L)	61.0 ± 6.2	54.4 ± 3.5	63.6 ± 8.0	91.4 ± 24.0**
ALT (IU/L)	40.2 ± 8.9	37.9 ± 4.2	46.2 ± 8.6	55.5 ± 7.2**
ALP (IU/L)	868 ± 200	995 ± 267	989 ± 344	1552 ± 538**
LDH (IU/L)	112 ± 28	129 ± 18	173 ± 30*	403 ± 189**
Glucose (mg/dL)	176 ± 12	199 ± 13**	176 ± 7	196 ± 22*
BUN (mg/dL)	15.8 ± 2.0	15.3 ± 2.1	16.0 ± 1.8	19.7 ± 1.6**
Creatinine (mg/dL)	0.208 ± 0.020	0.174 ± 0.022**	0.176 ± 0.027**	0.175 ± 0.016**
Na (mEq/L)	145 ± 1	145 ± 1	145 ± 1	142 ± 1**
Cl (mEq/L)	107 ± 1	106 ± 2	106 ± 2	104 ± 2**
Female				
No. of animals	10	10	10	9[a]
Total protein (g/dL)	5.81 ± 0.21	6.17 ± 0.26**	6.15 ± 0.18*	6.41 ± 0.34**
Albumin (g/dL)	4.19 ± 0.12	4.39 ± 0.22	4.55 ± 0.19**	5.14 ± 0.32**
AST (IU/L)	54.8 ± 3.5	62.4 ± 5.1*	57.4 ± 6.2	58.4 ± 10.0
ALT (IU/L)	39.1 ± 4.6	43.2 ± 7.8	39.5 ± 5.9	45.8 ± 8.7
ALP (IU/L)	727 ± 164	742 ± 122	703 ± 199	1026 ± 217**
LDH (IU/L)	138 ± 44	254 ± 27**	209 ± 44*	235 ± 116*
Glucose (mg/dL)	202 ± 25	181 ± 13	182 ± 10	216 ± 16
BUN (mg/dL)	20.0 ± 1.6	20.2 ± 2.1	18.2 ± 2.6	23.2 ± 2.2**
Creatinine (mg/dL)	0.230 ± 0.022	0.229 ± 0.025	0.196 ± 0.022*	0.208 ± 0.030
Na (mEq/L)	142 ± 1	143 ± 1	144 ± 1**	141 ± 1
Cl (mEq/L)	104 ± 1	105 ± 2	106 ± 1**	102 ± 2*

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.
[a]One female was excluded because left ovary remnants were found at autopsy.

The levels of LDH at 2.5 mg/kg/day and above in males and at 0.5 mg/kg/day and above in females, ALP at 12.5 mg/kg/day in both sexes, and AST and ALT at 12.5 mg/kg/day in males were also significantly increased. In addition, significant decreases in the levels of creatinine at 0.5 mg/kg/day and above, of sodium at 12.5 mg/kg/day in males, and of chloride at 12.5 mg/kg/day in both sexes were detected.

At necropsy, no gross abnormality was found at any dose. Absolute and relative liver weight was significantly increased at 0.5 mg/kg/day and above in males and at 12.5 mg/kg/day in females (Table 2). No significant change was found in the absolute and relative heart weight.

Histopathological findings in the liver are summarized in Table 3. Diffuse hypertrophy of hepatocytes were observed at 0.5 mg/kg/day and above in males and at 2.5 mg/kg/day and above in females. The cytoplasm of the hepatocytes was slightly eosinophilic. At these doses, anisokaryosis, nucleolar enlargement, and decreased glycogen in hepatocytes were also found. In addition, focal coagulative necrosis at 12.5 mg/kg/day in males and at 2.5 mg/kg/day and above in females, and increased mitosis of hepatocytes at 2.5 mg/kg/day and above and mononuclear cell infiltration at 12.5 mg/kg/day in males, were detected. No substance-related histopathological findings were detected in the heart.

DISCUSSION

The current study was designed to investigate the role of sex steroids in the mediation of gender-related differences in HDBB toxicity. The dosage of HDBB used in the present study was sufficiently high to be expected to induce

Table 2: Organ weight of the heart and liver in male and female castrated rats given HDBB by gavage for 28 days.

Dose (mg/kg/day)	0	0.5	2.5	12.5
Male				
No. of animals	10	10	10	10
Heart (g)	1.30 ± 0.07	1.25 ± 0.09	1.35 ± 0.12	1.37 ± 0.12
	(0.352 ± 0.022)[a]	(0.331 ± 0.028)	(0.362 ± 0.020)	(0.373 ± 0.030)
Liver (g)	15.5 ± 1.5	18.2 ± 2.7*	21.6 ± 3.0**	26.9 ± 1.9**
	(4.18 ± 0.27)	(4.78 ± 0.47*)	(5.76 ± 0.61**)	(7.32 ± 0.40**)
Female				
No. of animals	10	10	10	9[b]
Heart (g)	1.14 ± 0.07	1.11 ± 0.09	1.15 ± 0.10	1.25 ± 0.14
	(0.342 ± 0.027)	(0.322 ± 0.027)	(0.329 ± 0.024)	(0.352 ± 0.035)
Liver (g)	14.5 ± 1.9	14.8 ± 1.4	16.2 ± 2.5	27.0 ± 3.3**
	(4.33 ± 0.34)	(4.28 ± 0.19)	(4.63 ± 0.32)	(7.63 ± 0.87**)

Values are expressed as the mean ± SD.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.
[a]Relative organ weight (g/100 g body weight).
[b]One female was excluded because left ovary remnants were found at autopsy.

Table 3: Histopathological findings in the liver of male and female castrated rats given HDBB by gavage for 28 days.

		Dose (mg/kg/day)			
	Grade	**0**	**0.5**	**2.5**	**12.5**
Male					
No. of animals		10	10	10	10
Anisokaryosis of hepatocytes	±	0	1	8	3
	+	0	0	0	7
Nucleolar enlargement in hepatocytes	±	0	1	10	5
	+	0	0	0	5
Increased mitosis of hepatocytes	±	0	0	1	4
Hypertrophy of hepatocytes	±	0	4	10	10
Decreased glycogen in hepatocytes	±	0	1	6	8
	+	0	0	0	2
Focal necrosis	±	0	0	0	3
Mononuclear cell infiltration	±	1	0	0	5
Female					
No. of animals		10	10	10	9[a]
Anisokaryosis of hepatocytes	±	0	0	5	8
Nucleolar enlargement in hepatocytes	±	0	0	5	9
Hypertrophy of hepatocytes	±	0	0	2	9
Decreased glycogen in hepatocytes	±	0	0	2	8
Focal necrosis	±	0	0	3	2
Mononuclear cell infiltration	±	1	1	1	0

Values represent the number of animals with the findings.
± = very slight; + = slight.
[a]One female was excluded because left ovary remnants were found at autopsy.

toxicological effects on the liver, based on the results of the previous 28-day and 52-week repeated dose toxicity study using intact rats (Hirata-Koizumi et al., 2007; 2008). As expected, absolute and relative liver weight increased at 0.5 mg/kg/day and above in males and at 12.5 mg/kg/day in females, and histopathological changes in the liver, including anisokaryosis, nucleolar enlargement, increased mitosis, hypertrophy and decreased glycogen in hepatocytes, focal necrosis, and/or mononuclear cell infiltration, were observed at 0.5 mg/kg/day and above in males and at 2.5 mg/kg/day and above in females. Blood biochemical changes, such as increases in the level of total protein, albumin, AST, ALT, ALP, and/or LDH, were also found at all doses in both sexes. Although these changes in blood biochemical parameters were mostly slight and lacked dose dependence in some cases, simultaneous increase in hepatic enzymes (AST, ALT, ALP, and LDH) at 12.5 mg/kg/day in males is considered to be related to hepatic damage caused by HDBB.

A previous 28-day study using intact rats showed the cardiac toxicity of HDBB; degeneration and hypertrophy of the myocardium or cell infiltration were found at 2.5 mg/kg/day and above in males and at 12.5 mg/kg/day and above in females (Hirata-Koizumi et al., 2007). In the present study, using castrated rats, however, histopathological changes in the heart were not

detected even at the highest dose of 12.5 mg/kg/day. Considering that histopathological effects on the heart were also not found at the highest dose of 2.5 mg/kg/day in males and 12.5 mg/kg/day in females in the previous 52-week study using intact rats (Hirata-Koizumi et al., 2008), the present results would not necessarily mean that castration caused a change in the cardiac effect of HDBB. Although the cause of the difference in the cardiac toxicity of HDBB in our studies is not clear, further study is required to investigate the toxicological effects of HDBB on the heart in more detail, including the effect on cardiac function (e.g., electrocardiographic parameters, blood pressure, etc.).

In the previous 28-day study, male and female intact rats were given HDBB by gavage at 0.5, 2.5, 12.5, or 62.5 mg/kg/day (Hirata-Koizumi et al., in press). Histopathological findings similar to those observed in the present study were detected in the liver at all doses in males and at 12.5 mg/kg/day and above in females. The changes were accompanied with an increase in the absolute and/or relative liver weight. Serum levels of hepatic enzymes increased at 12.5 mg/kg/day and above in males and slightly at 62.5 mg/kg/day in females. When comparing the sensitive endpoint for hepatotoxicity of HDBB, histopathological changes in the liver, between sexes, the changes detected at 0.5 mg/kg/day in male rats were comparable in severity and incidence to those at 12.5 mg/kg/day in females. Thus, it was considered that male rats showed a nearly 25 times higher susceptibility to the hepatotoxicity of HDBB than females. In the present study, using castrated rats, histopathological findings in the liver were detected in males but not in females at the lowest dose of 0.5 mg/kg/day. The hepatic changes at 0.5 mg/kg/day in males were slightly milder than those at 2.5 mg/kg/day in females, showing that the difference in the susceptibility of male and female castrated rats was less than five times. Thus, castration markedly reduced gender-related differences in the hepatotoxicity of HDBB. As shown in Figure 2, a comparison of the rate of changes in the relative liver weight provided a more clear description of a nearly 25 times difference in the susceptibility of male and female intact rats to HDBB hepatotoxicity and the marked reduction by castration.

When comparing the histopathological findings of the liver from the previous 28-day study using intact rats and the present study using castrated rats, those in males were approximately equivalent at the same dose. On the other hand, for females, hepatic changes were observed at 12.5 mg/kg/day and above in intact rats, but clear changes in the histopathology of the liver were detected in castrated rats at a lower dose of 2.5 mg/kg/day. Therefore, castration of female rats enhanced the adverse effects of HDBB on the liver, suggesting suppressive effects of estrogen on HDBB hepatotoxicity in rats. Comparison of the relative liver weight change (Fig. 2) showed decreased male susceptibility as well as increased female susceptibility by castration. Androgen might have an enhancing effect on the hepatotoxicity of HDBB.

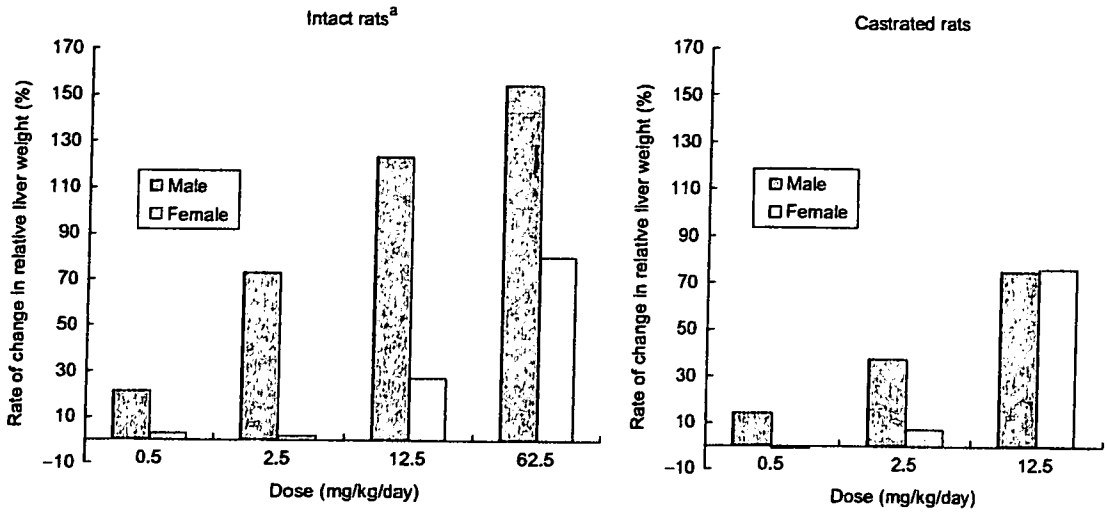

Figure 2: Comparison of change in relative liver weight of male and female intact and castrated rats given HDBB by gavage.
[a]The result of the previous 28-day repeated dose toxicity study, in which male and female intact rats were given HDBB once-daily at 0 (vehicle control), 0.5, 2.5, 12.5, and 62.5 mg/kg/day by gavage (Hirata-Koizumi et al., 2007)

The current study showed that the gender-related difference in susceptibility to HDBB hepatotoxicity was reduced, but not abolished, by castration. Sexual differences found in the present study were considered to be due to exposure to sexual hormones before four weeks of age, when castration was conducted. In female rats, serum estradiol concentration during the first three weeks after birth is as high as or higher than the level during the proestrus stage in young adults (Döhler and Wuttke, 1975); however, because serum estradiol concentration is similarly high during this preweaning period in male rats, it is unlikely that exposure to estradiol during this period contributes to the sexual difference in susceptibility of rats to the toxicity of HDBB. On the other hand, serum androgen levels before four weeks of age are much higher in male than female rats (Döhler and Wuttke, 1975). Ketelslegers et al. (1978) reported that plasma testosterone level in male rats was as high as 50 ng/100 mL two days after birth and it remained at the same level until day 8. The progressive decline occurred from days 8–24, and the testosterone level remained low, at the limit of detection of the assay (18 ng/100 mL), until day 30. There is a possibility that neonatal exposure to testosterone plays some role in the different susceptibility of male and female rats to the toxicity of HDBB. In fact, neonatal exposure to androgen irreversibly programs brain centers involved in the hypothalamo-pituitary control of hepatic sex-dependent metabolism (Gustafsson et al., 1981). We are currently in the process of performing a repeated dose toxicity study of HDBB using preweaning rats to clarify when gender-related differences in susceptibility to the toxicity of HDBB develop.

As in the case of HDBB, the male-predominant induction of toxicity in rats has been reported for many other substances, such as adenine (Ogirima et al.,

2006), acetaminophen (Raheja et al., 1983), dapsone (Coleman et al., 1990), fluoranthene (Knuckles et al., 2004), 3-nitropropionic acid (Nishino et al., 1998), and mercuric chloride (Muraoka and Itoh, 1980). Various causes of such gender-related differences are indicated mainly for toxicokinetic determinants. It is well known that hepatic metabolism differs between the sexes, with male rats generally having higher activity than females (Gad, 2006). Furthermore, gender differences in membrane transport in various organs, including the kidneys, liver, intestine, and brain, have emerged relatively recently (Morris et al., 2003). In the case of HDBB, male rats consistently showed greater susceptibility to various effects of HDBB (e.g., on the liver, blood, etc.) in the previous 28-day and 52-week studies (Hirata-Koizumi et al., 2007; 2008); therefore, such differences in metabolism or transport between the sexes might increase the blood concentration of causative substances (i.e., HDBB or its metabolites) in males.

For gender-related variations in toxicokinetic determinants, many mechanistic studies on the metabolic enzyme cytochrome P450 have been reported (Waxman and Chang, 2005). In rats, a subset of P450s is expressed in a sex-dependent fashion and is subject to endocrine control. Whereas testosterone has a major positive regulatory influence on male-specific P450 forms, estrogen plays a somewhat lesser role in the expression of the female-specific/predominant liver P450 enzymes. If the male-specific/predominant metabolic enzymes have an intimate involvement in the toxic activation of HDBB, our results, showing the higher susceptibility of male rats to HDBB toxicity than females and decreased susceptibility by castration of male rats, could be explained. Interestingly, it was reported that estradiol suppressed the expression of male-specific/predominant P450 enzymes (Waxman and Chang, 2005). This is consistent with our results that female susceptibility to the hepatotoxicity of HDBB was increased by castration, given that the male-specific/predominant P450 enzymes activate HDBB. Since the expression of female-specific/predominant P450 enzymes is reduced by testosterone treatment as well as by castration of females (Waxman and Chang, 2005), there is also the possibility that these enzymes might be involved in the detoxication of HDBB. In order to clarify the cause of the sexual differences in susceptibility of rats to the toxicity of HDBB, 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 a single and repeated administration of HDBB to rats.

CONCLUSIONS

The current results showed that an oral administration of HDBB to castrated rats for 28 days caused hepatotoxicity at 0.5 mg/kg/day and above in males and at 2.5 mg/kg/day and above in females. Castration markedly reduced gender-related differences in the toxicity of HDBB in male and female rats.

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Potential adverse effects of phthalic acid esters on human health: A review of recent studies on reproduction

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Abstract

Various phthalic acid esters (PAEs) have been used for a wide range of products. PAEs and their metabolites produce reproductive and developmental toxicities in laboratory animals. These findings have raised concern about the possibility of PAEs as contributors to reproductive and developmental adverse effects in humans. This paper focuses on PAE exposure and health effects in human populations and summarizes recent studies. The exposure data in human populations indicate that the current methodology of estimation of PAE exposure is inconsistent. It is therefore important to obtain improved data on human PAE exposure and better understanding of the toxicokinetics of PAEs in each subpopulation. Studies on health effects of PAEs in humans have remained controversial due to limitations of the study designs. Some of findings in human populations are consistent with animal data suggesting that PAEs and their metabolites produce toxic effects in the reproductive system. However, it is not yet possible to conclude whether phthalate exposure is harmful for human reproduction. Studies in human populations reviewed in this paper are useful for showing the strength of the association. It is sometimes claimed that the use of animal data for estimating human risk does not provide strong scientific support. However, because it is difficult to find alternative methods to examine the direct toxic effects of chemicals, animal studies remain necessary for risk assessment of chemicals including PAEs.

Keywords: Phthalic acid ester; Human health; Reproduction; Development

1. Introduction

Various phthalic acid esters (PAEs) have been used for a wide range of products, and the largest use of these esters is in plasticizers for polyvinyl chloride (PVC) products (Autian, 1973). When used as plasticizers, PAEs are not irreversibly bound to the polymer matrix; therefore, they can migrate from the plastic to the external environment under certain conditions. PAEs are ubiquitous environmental pollutants because of their widespread manufacture, use, and disposal as well as their high concentration in and ability to migrate from plastics (Marx, 1972; Mayer et al., 1972). Humans are exposed to PAEs from food contaminated during growth, processing, and packaging or from storage and indoor air. Di-(2-ethylhexyl) phthalate (DEHP), di-*n*-butyl phthalate (DBP), and butyl benzyl phthalate (BBP) were particularly found in fatty foods including dairy products (Kavlock et al., 2002a,b,c). Women have been exposed to DEHP, DBP, and diethyl phthalate (DEP) in cosmetics on a daily basis (Koo and Lee, 2004).

Some PAEs and their metabolites produce reproductive and developmental toxicities in laboratory animals. The major toxicities are known to be testicular effects (Zhang et al., 2004), embryolethality (Ema et al., 1994, 1997a; Tyl et al., 1988), malformations such as cleft palate and fusion of the sternebrae, and adverse effects on sexual differentiation (Ema et al., 1997b, 1998; Gray et al., 2000). There are considerable homologies among different

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mammalian species for androgen activities during sex differentiation (Gray et al., 1994). Chemicals that adversely affect human sex differentiation (Schardein, 2000) also produce predictable alterations of this process in rodents (Gray et al., 1994). The anti-androgenic effects of some PAEs were observed in a Hershberger assay in castrated male rats (Stroheker et al., 2005; Lee and Koo, 2007) or in an AR reporter gene assay (Satoh et al., 2004). These findings have raised concern about the possibility of PAEs as contributors to reproductive and developmental adverse effects in humans. Available data on primates are currently limited but show significant differences from rodents regarding the reproductive effects of PAEs (Kurata et al., 1998; Pugh et al., 2000; Tomonari et al., 2006), indicating the possibility of species-related differences.

The lower sensitivity of primates is thought to arise from differences between rodents and primates in the absorption, distribution, metabolism, and excretion (ADME) of PAEs. Monoester metabolites of PAEs such as mono-2-ethylhexyl phthalate (MEHP) and mono-butyl phthalate (MBP) have been reported to be the active metabolites responsible for adverse effects (Elcombe and Mitchell, 1986; Ema and Miyawaki, 2001; Tomita et al., 1986). DEHP is hydrolyzed to MEHP by the catalytic action of lipase (Ito et al., 2005). Lipase activities in the liver, small intestine, and kidneys are higher in rodents than in primates (Ito et al., 2005). The maximum concentrations of MEHP in the blood of marmosets were up to 7.5 times lower than in rats (Kessler et al., 2004). In rats, MEHP is oxidized to other secondary metabolites, and both MEHP and secondary metabolites are found in the blood and amniotic fluid primarily in their free form (Kurata et al., 2005; Calafat et al., 2006). Urinary MEHP was mostly found as a glucuronide conjugate in rats (Calafat et al., 2006). On the other hand, in humans and primates, MEHP is present in blood and urine primarily as glucuronide conjugates, which enhance urinary excretion and reduce the biological activity of the active metabolites (Ito et al., 2005; Kurata et al., 2005; Silva et al., 2003), but DEHP metabolites with a carboxylated ester side-chain were found as both conjugates and free forms in human urine (Silva et al., 2006a). Plasma radioactivity measurements of DEHP in rats and marmosets revealed that radioactivity in rat testis was about 20-fold higher than that in marmosets. About 60% of the dose was excreted in urine in rats primarily as unconjugated MEHP-metabolites. For marmosets, the majority of the dose was excreted in the feces (Kurata et al., 1998).

The potential of PAEs to produce adverse effects in humans has been the subject of considerable discussion. Many toxicity studies have been conducted in laboratory animals, especially in rats, and review papers are available based on these animal data (Corton and Lapinskas, 2005; Ema, 2002; Foster, 2006); however, studies in human populations have not been adequate to assess the toxic potential on human health. Lately, several review papers were published regarding PAE exposure in human populations (Koch et al., 2006; Latini, 2005; Schettler, 2006). These review studies are worthwhile for knowing exposure levels and routes of PAE exposure in human populations; however, review works regarding the relationships between PAE exposure and human health are not adequate. In the late 20th century, only a few papers have reported a relationship between environmental PAE exposures and human health (Aldyreva et al., 1975; Fredricsson et al., 1993; Murature et al., 1987). Studies in human populations have been receiving much attention for the last 2 or 3 years, and the number of studies in human populations has increased. Some recent studies have suggested possible associations between environmental exposure to PAEs and adverse effects on human reproductive health. It will be useful to review them to determine whether there is concordance between animal models and human populations in order to develop hypotheses for future studies. This paper focuses on the PAE exposure and health effects in human populations and summarizes recent human studies published up to 2006.

2. Exposure to PAEs

Many studies have suggested that PAEs and their metabolites produce reproductive and developmental toxicities in laboratory animals. Although the most of these animals were exposed to PAEs at relatively high level to exam toxicological effects, some studies showed that relatively low doses of PAEs caused toxic effects (Arcadi et al., 1998; Lee et al., 2004; Poon et al., 1997). Thus, there is a question of whether humans are exposed to PAEs at a severe enough level to generate human health effects. Several studies have been conducted to estimate the exposure level of PAEs in humans.

2.1. Estimate of PAE exposure in human populations

Levels of human exposure to PAEs were estimated from the urinary metabolite of PAEs. Table 1 shows the urinary PAE metabolite in US populations. A pilot study was conducted for measurement of levels of seven urinary phthalate metabolites, MEHP, MBP, mono-benzyl phthalate

Table 1
Total urinary phthalate monoester concentrations (in μg/g of creatinine)

Metabolites	Diester	Measurement in 289 individuals (Blount et al., 2000)		Measurement in 2541 individuals (Silva et al., 2004a)	
		Geometric mean	95th percentile	Geometric mean	95th percentile
MEP	DEP	345	2610	163	1950
MBP	DBP/BBP	36.9	162	22.4	97.5
MBzP	BBP	20.2	91.9	14.0	77.4
McHP	DcHP	0.3	1.0	<LOD	3.00
MEHP	DEHP	3.0	15.2	3.12	18.5
MOP	DOP	0.5	2.1	<LOD	3.51
MINP	DINP	1.3	6.8	<LOD	4.29

LOD, limit of detection.