Table 3
Body weight after weaning in offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of male offspring	63	61	60	55	60
Body weight of male offspring (g)[a]					
Postnatal day 28	101 ± 7	102 ± 8	98 ± 6	100 ± 6	92 ± 9**
Postnatal day 35	161 ± 12	163 ± 13	157 ± 10	160 ± 10	151 ± 13**
Postnatal day 42	225 ± 15	230 ± 16	221 ± 14	225 ± 14	217 ± 15**
Postnatal day 49	289 ± 20	297 ± 20	285 ± 18	289 ± 18	281 ± 17*
Postnatal day 56	346 ± 24	356 ± 24	342 ± 22	345 ± 24	336 ± 22*
Postnatal day 63	390 ± 27	400 ± 27	384 ± 26	388 ± 27	379 ± 25
Postnatal day 70	424 ± 32	436 ± 30	418 ± 30	422 ± 32	414 ± 33
No. of female offspring	63	62	58	57	63
Body weight of female offspring (g)[a]					
Postnatal day 28	90 ± 7	93 ± 6	90 ± 5	90 ± 5	85 ± 7**
Postnatal day 35	135 ± 9	138 ± 11	135 ± 9	135 ± 7	130 ± 10*
Postnatal day 42	171 ± 13	175 ± 13	172 ± 12	171 ± 10	168 ± 13
Postnatal day 49	196 ± 14	204 ± 15*	200 ± 15	199 ± 12	196 ± 15
Postnatal day 56	222 ± 17	230 ± 17*	226 ± 17	222 ± 14	221 ± 18
Postnatal day 63	239 ± 19	248 ± 19*	246 ± 22	243 ± 17	241 ± 19
Postnatal day 70	254 ± 20	264 ± 22*	262 ± 22	258 ± 18	257 ± 21

[a] Values are given as the mean ± S.D.
* Significantly different from the control, $p < 0.05$.
** Significantly different from the control, $p < 0.01$.

Table 4
Physical development in offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No of litters examined	22	21	20	19	21
Age at pinna unfolding (days)[a]					
Male	2.9 ± 0.6	3.0 ± 0.5	3.0 ± 0.5	2.9 ± 0.7	3.1 ± 0.5
Female	2.8 ± 0.5	2.9 ± 0.5	3.0 ± 0.5	3.1 ± 0.6	3.1 ± 0.6
Body weight at pinna unfolding (g)[a]					
Male	8.6 ± 0.7	9.3 ± 1.3	9.0 ± 1.0	8.9 ± 0.5	8.8 ± 1.0
Female	8.1 ± 0.8	8.8 ± 1.0	8.6 ± 1.0	8.5 ± 0.5	8.4 ± 0.9
Age at fur appearance (days)[a]					
Male	9.0 ± 0.4	9.0 ± 0.4	9.0 ± 0.6	9.1 ± 0.5	9.0 ± 0.5
Female	9.1 ± 0.4	9.1 ± 0.4	9.2 ± 0.6	9.2 ± 0.5	9.1 ± 0.6
Body weight at fur appearance (g)[a]					
Male	22.2 ± 1.5	23.2 ± 1.8	21.9 ± 1.8	22.5 ± 1.6	20.9 ± 2.2*
Female	21.5 ± 1.5	22.3 ± 1.7	21.2 ± 1.8	22.0 ± 1.5	20.5 ± 2.3
Age at incisor eruption (days)[a]					
Male	10.0 ± 0.8	10.1 ± 0.5	10.1 ± 0.5	10.1 ± 0.7	10.1 ± 0.9
Female	10.0 ± 0.6	10.0 ± 0.7	10.1 ± 0.4	9.9 ± 0.6	10.0 ± 1.0
Body weight at incisor eruption (g)[a]					
Male	24.6 ± 2.2	25.7 ± 1.6	24.4 ± 2.5	25.0 ± 1.8	23.1 ± 3.2
Female	23.5 ± 1.6	24.3 ± 1.7	23.6 ± 2.5	24.0 ± 1.3	22.4 ± 3.0
Age at eye opening (days)[a]					
Male	15.3 ± 0.5	15.2 ± 0.7	15.3 ± 0.5	15.4 ± 0.7	15.3 ± 0.6
Female	15.4 ± 0.5	15.2 ± 0.5	15.2 ± 0.5	15.3 ± 0.6	15.2 ± 0.6
Body weight at eye opening (g)[a]					
Male	37.9 ± 1.6	39.4 ± 2.4	37.7 ± 2.6	38.5 ± 2.0	35.1 ± 3.1*
Female	36.9 ± 1.6	37.6 ± 2.0	36.0 ± 2.1	37.3 ± 1.7	34.1 ± 3.1*

[a] Values are given as the mean ± S.D.
* Significantly different from the control, $p < 0.05$.

Table 5
Sexual maturation of offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
Male preputial separation					
No. of male pups examined	22	21	20	18	21
Age (days)[a]	40.5 ± 1.5	40.5 ± 1.2	40.9 ± 1.3	40.3 ± 1.1	41.0 ± 1.3
Body weight (g)[a]	206 ± 32	205 ± 24	207 ± 22	205 ± 25	202 ± 28
Female vaginal opening					
No. of female pups examined	22	21	20	19	21
Age (days)[a]	33.3 ± 1.9	33.6 ± 1.6	33.9 ± 1.4	32.8 ± 0.9	33.4 ± 2.2
Body weight (g)[a]	124 ± 10	130 ± 14	127 ± 9	121 ± 9	121 ± 16

[a] Values are given as the mean ± S.D.

The rate of successfully conditioned responses for every 10 min test period on PNDs 60–67 is shown in Fig. 5. No significant changes were found in males and females of any PS80-treated groups when conditioned avoidance responses were determined on PNDs 60–67.

3.6. Necropsy and histopathology in offspring

There were no compound-related gross lesions in males and females at necropsy on PND 22. Table 6 shows absolute and relative organ weights on postnatal day 22 in male and female offspring. There were no significant differences in absolute and relative weights of the brain, liver, spleen, adrenal or kidney in male and female pups between control and PS80-treated groups.

No histopathological changes in the cerebrum, cerebellum, medulla oblongata, pons, spinal cord in the thoracic and lumbar regions, and sciatic nerve were noted in 22-day-old males and females of the control and 7.5% groups (data not shown).

No compound-related gross lesions were found in males and females at necropsy on PND 70 and on PNDs 103–126. There were no significant differences in the absolute and relative weights of the brain, liver, spleen, adrenal or kidney in 70-day-old male and female pups between control and PS80-treated groups. Although slight mononuclear cell infiltration in the choroid plexus was observed in the cerebrum in one male in the control group, no other histopathological changes in the cerebrum, cerebellum, medulla oblongata, pons, spinal cord in the thoracic and lumbar regions, and sciatic nerve were found in 70-day-old males and females of control and 7.5% groups (data not shown).

4. Discussion

A developmental neurotoxicity study was performed to evaluate the potential functional and morphological effects of PS80 on the developing nervous system of offspring of rats given PS80 during pregnancy and lactation. This study was designed to assess both continuous parameters, such as body weight and food and water consumption, and parameters at specific times, pre-weaning, adolescence and young adult periods, such as physical development, reflex ontogeny, sexual maturation, motor activity, motor and sensory function, learning, and pathological findings, and to further assess reproductive and developmental endpoints.

In the present study, loose stool during lactation was observed in many dams given drinking water containing PS80 at 7.5%. In a previous 2-year breeding study, diarrhea was observed in rats

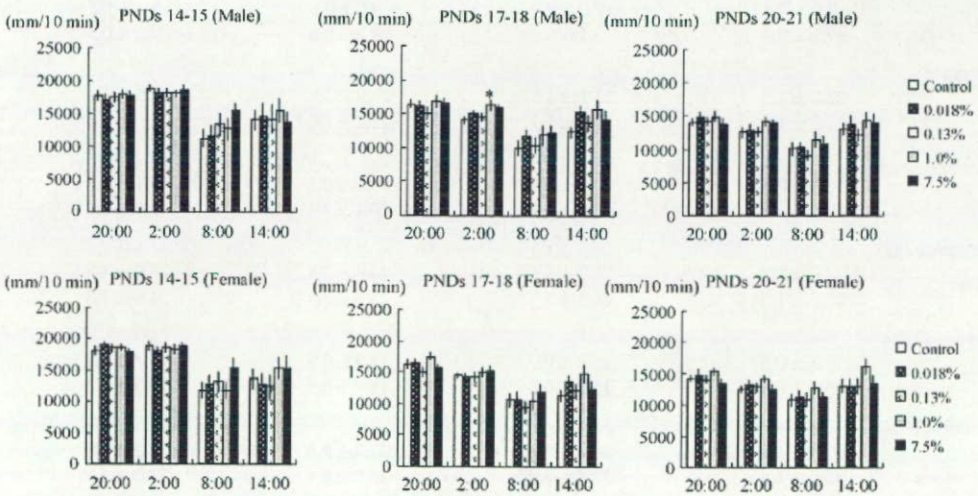

Fig. 3. Locomotor activity in pre-weaning offspring of rats given polysorbate 80 during pregnancy and lactation. Values are given as the mean ± S.E.M. *Significantly different from the control, $p < 0.05$.

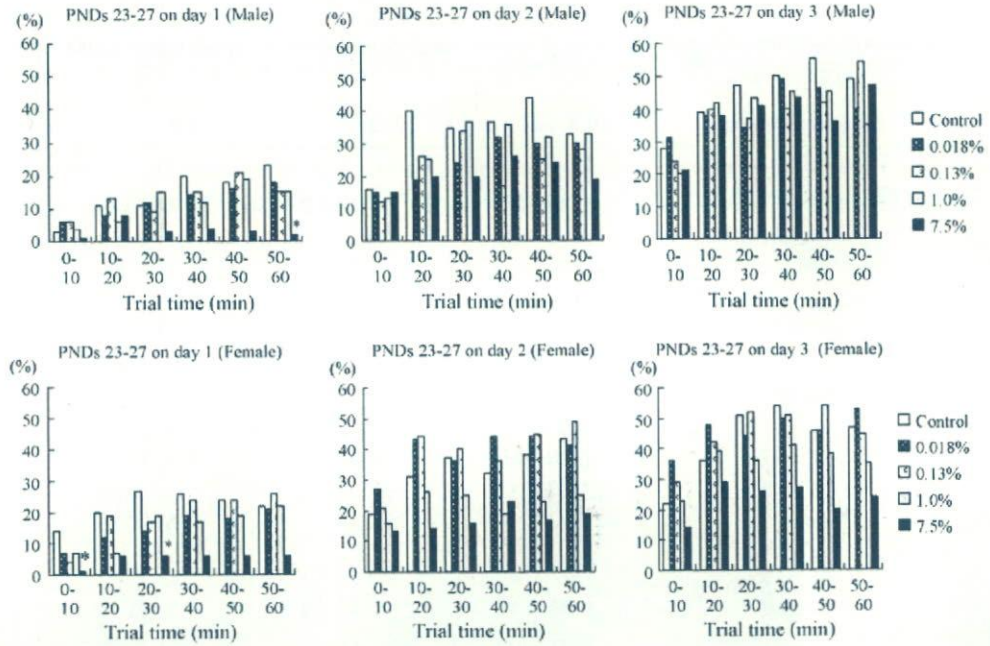

Fig. 4. The rate of successful responses in conditioned avoidance test on postnatal days 23–27 in offspring of rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

fed a diet containing PS80 at 10% and higher [12]. The diarrhea observed in feeding studies with polysorbates seems to result from having high concentrations of the unabsorbed poloxyethylene sorbitan moiety within the intestinal lumen [13,14]. The decrease in body weight and body weight gain accompanied by decreased food and water consumption was also noted at 7.5%; however, no significant findings in clinical observations, body weight, body weight gain, or food and water consumption were detected at 1.0% and below. Reduced water consumption may be due to slight characteristic scent and unpleasant and slightly bitter taste of PS80 [15]. However, lower water consumption was noted only on 2 days at the highest dose. These findings do not indicate poor palatability of PS80 in water and dose–dependent taste aversion. PS80 seems to be dosed successfully by this route. Dilatation of the cecum was also observed at 7.5%. Although increased relative weight of the kidney was

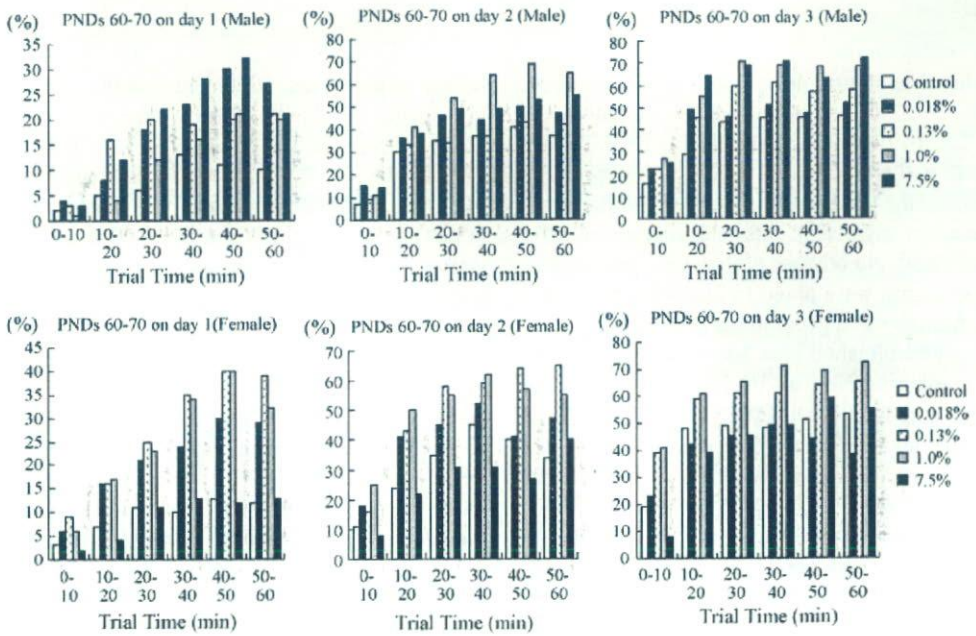

Fig. 5. The rate of successful responses in conditioned avoidance test on postnatal days 60–70 in offspring of rats given polysorbate 80 during pregnancy and lactation.

Table 6
Absolute and relative organ weights on postnatal day 22 in male and female offspring of rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of male pups	11	11	11	9	10
Body weight (g)[a]	58.9 ± 6.8	61.1 ± 5.1	60.3 ± 4.2	62.2 ± 3.6	55.0 ± 6.3
Brain (g)[a]	1.49 ± 0.05[b] 2.56 ± 0.29[c]	1.55 ± 0.06 2.55 ± 0.18	1.53 ± 0.06 2.54 ± 0.18	1.53 ± 0.05 2.47 ± 0.17	1.49 ± 0.07 2.75 ± 0.33
Liver (g)[a]	2.45 ± 0.43 4.18 ± 0.77[c]	2.38 ± 0.38 3.88 ± 0.37	2.25 ± 0.23 3.74 ± 0.29	2.44 ± 0.21 3.92 ± 0.23	2.21 ± 0.38 3.99 ± 0.33
Spleen (mg)[a]	266 ± 57 449 ± 59[d]	308 ± 65 502 ± 91	302 ± 52 500 ± 66	290 ± 32 466 ± 35	274 ± 45 495 ± 37
Adrenal (mg)[a]	21 ± 4 35.5 ± 5.7[d]	23 ± 3 38.0 ± 4.8	22 ± 5 36.8 ± 8.1	22 ± 4 35.2 ± 6.6	21 ± 4 38.9 ± 9.5
Kidney (mg)[a]	720 ± 97 1222 ± 84[d]	746 ± 80 1219 ± 70	725 ± 61 1202 ± 45	738 ± 49 1187 ± 76	666 ± 53 1218 ± 72
No. of female pups	11	11	11	9	10
Body weight (g)[a]	55.2 ± 6.3	56.5 ± 6.0	55.8 ± 3.9	57.5 ± 4.3	52.4 ± 5.6
Brain (g)[a]	1.45 ± 0.05[b] 2.65 ± 0.28[c]	1.47 ± 0.06 2.63 ± 0.29	1.45 ± 0.06 2.61 ± 0.21	1.46 ± 0.04 2.54 ± 0.16	1.43 ± 0.08 2.75 ± 0.25
Liver (g)[a]	2.09 ± 0.38 3.77 ± 0.35[c]	2.15 ± 0.34 3.78 ± 0.26	2.21 ± 0.43 3.99 ± 0.86	2.23 ± 0.21 3.87 ± 0.14	2.07 ± 0.27 3.95 ± 0.25
Spleen (mg)[a]	263 ± 44 479 ± 80[d]	295 ± 56 519 ± 75	266 ± 30 477 ± 40	265 ± 51 458 ± 64	262 ± 36 501 ± 50
Adrenal (mg)[a]	23 ± 4 41.5 ± 6.6[d]	21 ± 4 37.7 ± 7.2	22 ± 5 39.0 ± 8.8	22 ± 6 38.6 ± 10.1	19 ± 2 36.4 ± 5.5
Kidney (mg)[a]	717 ± 90 1299 ± 92[d]	724 ± 72 1282 ± 57	711 ± 65 1277 ± 99	726 ± 66 1262 ± 82	676 ± 52 1294 ± 74

[a] Values are expressed as the mean ± S.D.
[b] Absolute organ weight.
[c] Relative organ weight = organ weight (g)/100 g body weight.
[d] Relative organ weight = organ weight (mg)/100 g body weight.

observed at 7.5%, this change was not thought to have toxicological meaning because of no changes in the gross pathology or in absolute weight. These findings indicate that the NOAEL for general toxicity in maternal rats was 1.0% (1.864 ml/kg bw/day).

In previous studies, the reproductive and developmental effects of PS80 were investigated in rats and mice given relatively high doses of PS80. No adverse effects on reproductive and developmental outcome were noted in rats fed a diet containing PS80 at 2% through three generations [16]. Fertility and offspring survival were diminished in a 2-year breeding study using rats fed a diet containing PS80 at 20%, but not at 5 or 10% [17]. A prenatal developmental toxicity study revealed no clear adverse effects in dams and fetuses of rats given PS80 at 500 and 5000 mg/kg bw/day by gavage on days 6–15 of pregnancy [18]. Administration of PS80 at 2500 mg/kg bw/day by gavage on days 8–12 of pregnancy caused no adverse effects on dams or offspring of mice [19]. In these previous studies, no detailed information on reproductive and developmental parameters was reported. Although a few females showed reproductive difficulties in PS80-treated groups in the present study, necropsy of the reproductive organs revealed no evidence of reproductive failure in these rats. No changes in the fecundity index, gestation length or gestation index were noted in any PS80-treated groups; however, the number of pups born was significantly decreased at 7.5%. One possible explanation for this decrease may be the slight decrease in the number of implantations. It is not known whether the decreased number of implantations was attributable to a decreased number of corpora lutea or an increase of number of pre-implantation embryonic loss, because the dams were sacrificed 21 days after delivery and the number of corpora lutea was not determined in the present study. No information on the adverse effects of PS80 on formation of the corpora lutea, implantation process and pre-implantation embryonic loss is available in previous reproductive and developmental toxicity studies of PS80 [16–19]. In the present study, acaudate and anal atresia were found in one pup at 0.018%; however, the incidence of malformations was very low, not dose-related and not significantly different from that in the control group. The external malformations observed in the present study were of the types that occur spontaneously among control rat fetuses reported in the literature [20–23]; therefore, it seems unlikely that the morphological changes in pups observed in the present

study indicate a teratogenic response. Maternal administration of PS80 at 7.5% caused the low body weight of male and female offspring during the pre-weaning period, and these changes were accompanied with the decreased weight of maternal rats. Body weight of offspring during the post-weaning period was also lower at 7.5%. The effect on pup weight showed up later but may actually have been present at birth in the 7.5% group, because the smaller number of litter mates per dam might have heavier body weight of pups in this group. These findings indicate that the dose level of 7.5% used in this study was potent enough to have adverse effects on growth of the offspring.

In the present study, the body weights at the age of completed fur appearance in male pups and eye opening in male and female pups were reduced at 7.5%; however, no significant changes were found in the age of completed these developmental landmarks. No changes were detected in reflex ontogeny and sensory function in male and female offspring given PS80. In addition, PS80 did not cause any changes in indicators of the onset of sexual maturity. It seems unlikely that PS80 affects the functional development and sexual maturation of offspring.

In the previous developmental neurotoxicity study of PS80 [6], daily locomotor activity and diurnal locomotor activity were increased in male offspring of rats given PS80 at 0.125% in their drinking water. The locomotor activity of male pups was determined during the pre-weaning period using a cage consisting of two sections, a home cage section with exploration holes that allowed movement of the pups back and forth to a second section, the exploration cage while restricting the movement of the dam to the home cage [24]. The OECD Draft Test Guideline 426 Developmental Neurotoxicity Study [7] noted that motor activity should be monitored during the pre-, peri- and post-weaning periods, including the young adult period, by an automated activity recording apparatus and that the animals should be tested individually. Monitoring nocturnal activity in rodents is important for toxicological studies [25,26], because rodents are more active during the nocturnal period [27–29] and neurotoxicants may be more effective during this period. In the present study, the motor activity of pups was individually determined during diurnal and nocturnal periods in the pre-, and peri-weaning, and young adult periods using automated activity recording apparatus. Although higher activity was detected in male pups at 2:00 on PND 18 in the 1.0% group, this change was discontinuous, inconsistent across sexes and not dose-related; therefore, this change was not thought to be due to the administration of PS80 and had no toxicological significance. No changes in locomotor activity were observed in PS80-treated pups of both sexes during other test periods. These findings indicate that PS80 is ineffective on locomotor activity in male and female pups of rats fed this compound during pregnancy and lactation.

Although a decreased rate of successfully conditioned avoidance responses was found at 7.5% in males and females on PNDs 23–27, no changes were found in both sexes of any PS80-treated groups on PNDs 60–67. It is likely that PS80 at 7.5% caused a transient suppression of conditioned avoidance responses in pups of both sexes. However, necropsy and histopathological examinations, including the nervous system, revealed no evidence of developmental disorders in pups on PNDs 22 and 70. The magnitude of decrease in body weight of pups was more pronounced during the younger stage than the older stage. It is noted that light body weight mice performed worse than heavy body weight mice in a learning task [30]. The possibility remains that the lowered conditioned avoidance response determined on PNDs 23–27 may be due to the reduced body weight of pups.

As for growth retardation of offspring, it is known that there are strong positive correlations between developmental landmark parameters and the weight of pups [31] and the best indicator of physical development is body weight [32] in experimental animal studies. Neurobehavioral teratology studies of some organic solvents have shown that decreased birth weight and functional impairment can be caused by the same chemicals at the same dosage levels [33]. In humans, the association of intrauterine growth retardation has been amply demonstrated with respect to neurological dysfunction [34]. Furthermore, human infants who show evidence of growth retardation have a 33–50% likelihood of having a learning disability [33,35]. These reports indicate that developmental neurotoxicity parameters are often associated with growth retardation, which is also an important parameter in developmental neurotoxicity studies. In the present study, transient decrease of successful responses in the conditioned avoidance test and reduced body weight were found at 7.5%, but no neuropathological changes were detected. The exposure of pups during the lactation period may be partly indirect via maternal milk and partly direct. Rat pups may gradually start to drink treated water from around PND 14, and on a mg-test-substance per kg-body-weight basis may actually be consuming a higher dose than adults during their second week of the lactation period [36]. It is needed to clarify the exposure levels of pups to PS80 produced adverse effects and clarify whether the adverse effects are attributable to the direct effects of PS80 on the developing nervous system or secondary effects via growth retardation.

In the present study, the toxicological effects were noted at 7.5% (16.783 ml/kg bw/day) and the NOAEL in this study was considered to be 1.0% (1.864 ml/kg bw/day). The value of the NOAEL is equivalent to 2013 mg/kg bw/day. It is estimated that daily intake of polysorbates from food is 12–111 mg/human in European and American countries [37]. The estimated human intake of polysorbates is equivalent to 0.20–1.85 mg/kg bw/day and is well below the NOAEL in this study.

Acknowledgment

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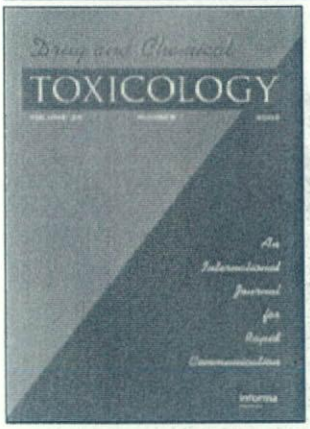

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Gonadal Influence on the Toxicity of 2-(2'-Hydroxy-3',5'-di-**tert**-butylphenyl) benzotriazole in Rats

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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).

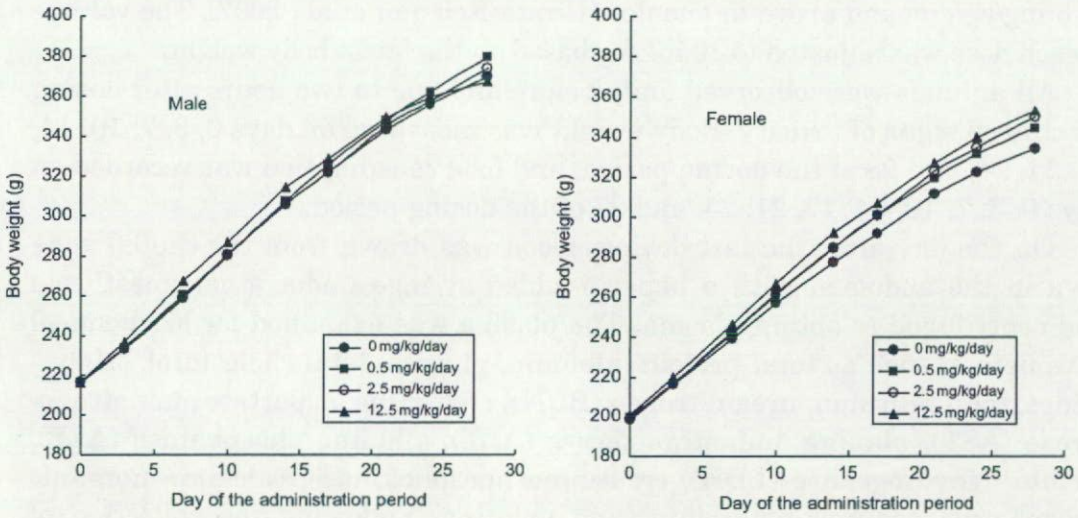

Figure 1: Body weight of male and female castrated rats given HDBB by gavage for 28 days.

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.

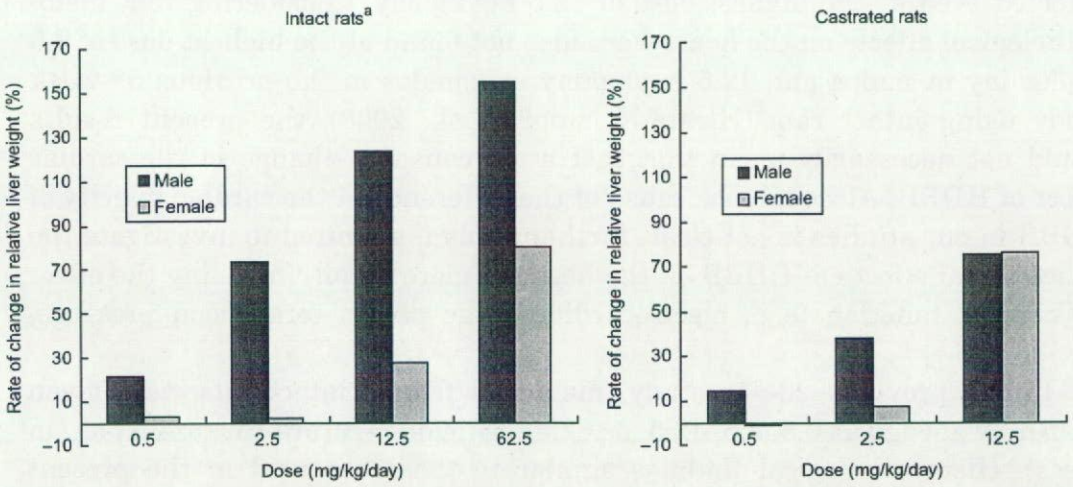

Figure 2: Comparison of change in relative liver weight of male and female intact and castrated rats given HDBB by gavage.
[a]The result of the previous 28-day repeated dose toxicity study, in which male and female intact rats were given HDBB once-daily at 0 (vehicle control), 0.5, 2.5, 12.5, and 62.5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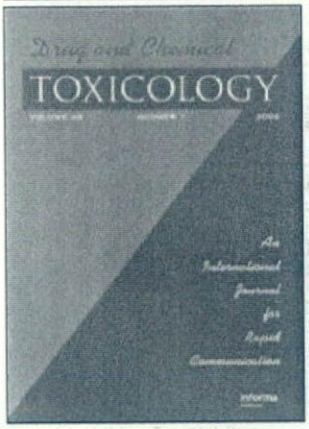

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Lack of Gender-Related Difference in the Toxicity of 2-(2'-Hydroxy-3',5'-di-**tert**-butylphenyl)benzotriazole in Preweaning Rats

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