using Fisher's exact test. The 5% level of probability was used as the criterion for significance.

RESULTS

At 500 mg/kg/day, 2 males died after 3 doses, and 1 male each died after 5 and 6 doses. In these dead males, discoloration and enlargement with tubular necrosis of the kidney was observed. A significant decrease in body-weight gain was found on Days 1–8 and 8–15 of the administration period at 500 mg/kg/day, as shown in Table 1. At this dose, significantly lower food consumption was also observed between Days 1 and 11 of the administration period. At 100 and 500 mg/kg/day, all surviving males showed brown urine. Discoloration of the kidney was observed in 4 of 8 surviving males and black-brown-colored spleen was observed in all surviving males at 500 mg/kg/day. Histopathological examination of these grossly abnormal organs revealed that basophilic tubules, protein cast, and granular cast in the kidney and deposits of hemosiderin in the red pulp and extramedullary hematopoiesis were observed in the spleen at 500 mg/kg/day.

At 500 mg/kg/day, 1 female each died after 4 and 25 administrations. At 100 and 500 mg/kg/day, all surviving females showed brown urine. A significant

Table 1: Body-weight gain in male and female rats given PAP.

Dose (mg/kg/day)	0 (control)	20	100	500
No. of male rats	12	12	12	12
No. of deaths	0	0	0	4
Initial body weight (g)[a]	373 ± 18	372 ± 20	371 ± 19	368 ± 18
Body weight gain (g)[a]				
Days 1–8	29 ± 7	33 ± 7	22 ± 15	−7 ± 10**
Days 8–15	29 ± 8	28 ± 8	26 ± 10	19 ± 5*
Days 15–22	32 ± 5	30 ± 11	34 ± 8	26 ± 8
Days 22–29	23 ± 5	24 ± 5	24 ± 7	21 ± 7
Days 29–36	28 ± 8	22 ± 5	23 ± 5	21± 10
Days 36–43	21 ± 8	22 ± 7	25 ± 6	26 ± 10
Days 43–50	20 ± 7	22 ± 6	21 ±5	17 ± 8
No. of female rats	12	12	12	12
No. of deaths	0	0	0	2
Initial body weight (g)[a]	225 ± 13	224 ± 12	223 ± 8	224 ± 9
Body weight gain (g)[a]				
Days 1–8	16 ± 8	14 ± 7	9 ± 5	−13 ± 11**
Days 8–15	9 ± 6	7 ± 5	9 ± 6	11 ± 16
Days 0–7 of pregnancy	36 ± 6	36 ± 7	32 ± 8	26 ± 9*
Days 7–14 of pregnancy	30 ± 4	31 ± 7	33 ± 6	30 ± 7
Days 14–20 of pregnancy	74 ± 12	74 ± 14	69 ± 15	50 ± 10
Days 0–4 of lactation	23 ± 15	19 ± 14	26 ± 7	6

[a]Values are given as the mean ± standard deviation.
***Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.

decrease in body-weight gain was found on Days 1–8 of the administration period and Days 0–7 of the pregnancy period at 500 mg/kg/day (Table 1). Significantly lower food consumption was also observed at 100 mg/kg/day between 1 and 8 days and at 500 mg/kg/day between 1 and 11 days of the administration period. In the dead females, histopathological examination revealed basophilic tubules, protein cast, tubular necrosis, and/or hyaline deposit on epithelial cells of the proximal tubule in the kidney. In surviving females, no gross abnormality was detected at the scheduled sacrifice.

Table 2 presents the reproductive organ weight in male and female rats given PAP. In males, the absolute and relative weights of the testes and epididymides were significantly decreased at 500 mg/kg/day. Histopathological examination revealed decreased spermatocyte and spermatid levels, vacuolation of Sertoli cells, degeneration/necrosis of spermatocytes in the testis, and decreased sperm counts and debris of germ cells in the epididymis lumen at 500 mg/kg/day. In females, no significant changes were found in organ weight or histopathology of the ovaries.

Table 3 presents the reproductive findings in rats given PAP. The count of estrus was decreased, but not significantly, during the 14-day premating period, and the incidence of females showing 4-day estrus cycles were significantly decreased at 500 mg/kg/day. At this dose, 4 females terminated their estrus cycles and showed extended diestrous vaginal smears. One pair did not copulate at 500 mg/kg/day. No significant effects of PAP were observed on precoital interval or copulation index. One female did not become impregnated in each of the control, 20-, and 500-mg/kg/day groups. No significant differences were noted in fertility index or gestation index between the control and PAP-treated groups. Gestation length was significantly prolonged at 500 mg/kg/day.

Table 2: Reproductive organ weights in rats given PAP.

Dose (mg/kg/day)	0 (control)	20	100	500
No. of male rats	12	12	12	8
Weight of testes (g)[a]	3.53 ± 0.14	3.50 ± 0.33	3.34 ± 0.24	2.40 ± 0.29*
Relative weight of testes[a, b]	0.64 ± 0.05	0.63 ± 0.04	0.62 ± 0.05	0.49 ± 0.05*
Weight of epididymides (g)[a]	1.27 ± 0.07	1.27 ± 0.08	1.23 ± 0.09	0.92 ± 0.05*
Relative weight of epididymides[a, b]	0.23 ± 0.02	0.23 ± 0.01	0.23 ± 0.02	0.19 ± 0.02*
No. of female rats	11	11	12	2
Weight of ovaries (mg)[a]	90.7 ± 9.9	95.8 ± 11.0	96.7 ± 8.2	81.7
Relative weight of ovaries[a, b]	28.1 ± 1.4	29.9 ± 3.1	30.7 ± 2.9	29.2

[a]Values are given as the mean ± standard deviation.
[b]Relative weight = organ weight/100 g of body weight.
**Significantly different from the control, $p < 0.01$.

Table 3: Reproductive findings in rats given PAP.

Dose (mg/kg/day)	0 (control)	20	100	500
No. of females examined	12	12	12	11
Count of estrus[a]	3.8 ± 0.5	3.8 ± 0.6	3.9 ± 0.9	2.6 ± 1.6
Females showing abnormal estrous cycles (%)[b]	0	8.3	0	45.5*
No. of mated (male/female)	12/12	12/12	12/12	7/10[f]
Precoital interval (day)[a]	2.5 ± 1.2	2.6 ± 1.2	2.9 ± 3.3	4.6 ± 4.0
Copulation index (%, male/female)[c]	100/100	100/100	100/100	85.7/90.0
Fertility index (%, male/female)[d]	91.7/91.7	91.7/91.7	100/100	100/88.9
Gestation index (%)[e]	100	100	100	100
Gestation length (day)[a]	22.2 ± 0.4	22.2 ± 0.4	22.6 ± 0.7	23.3 ± 0.5**

[a]Values are given as the mean ± standard deviation.
[b]Abnormal estrous cycles (%) = (no. of females showing abnormal estrous cycles /no. of females) × 100.
[c]Copulation index (%) = (no. of rats copulated/no. of pairs) × 100.
[d]Fertility index (%) = (no. of pregnant/no. of copulated) × 100.
[e]Gestation index (%) = (no. of females with live pups born/no. of pregnant females) × 100.
[f]One female was not used for mating because this female showed severely toxicological sign.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.

Table 4 shows the developmental findings in rats given PAP. There was no significant difference between the control and PAP-treated groups in the numbers of corpora lutea, implantations, stillborn pups, pups delivered, live pups delivered, implantation index, or sex ratio of live pups. At 500 mg/kg/day, the delivery index was significantly reduced and the rate of stillborn pups was increased significantly. At this dose, almost all dams neglected their pups, some dams showed cannibalism, and all pups of 6 dams died. Although no significant difference was observed in viability index at PND 0 between control and PAP-treated groups, the index was significantly decreased at PND 4 at 500 mg/kg/day. At this dose, the body weight of live male and female pups were significantly lowered on PND 0 and were decreased on PND 4.

The results of gross examinations of pups are also shown in Table 4. At 500 mg/kg/day, pups with external malformations were found in 2 pups; 1 showed a vestigial tail and the other showed an open auricle, short tail, and kinky tail. No significant difference was observed in the incidence of pups with malformations between control and 500-mg/kg/day groups. No pups with external malformations were observed in the control or groups given PAP at 20 and 100 mg/kg/day. No pups with internal malformations were found in any groups.

DISCUSSION

In order to obtain reliable information on the reproductive and developmental toxicity of PAP, a reproductive and developmental toxicity screening study

Table 4: Developmental findings in rats given PAP.

Dose (mg/kg/day)	0 (control)	20	100	500
No. of pregnant females	11	11	12	8
No. of corpora lutea[a]	15.4 ± 1.6	14.1 ± 2.0	15.3 ± 1.8	15.6 ± 1.5
No. of implantations[a]	14.4 ± 1.0	13.6 ± 2.0	14.2 ± 2.8	14.8 ± 0.9
Implantation index (%)[b]	93.5	96.8	92.9	94.4
No. of pups delivered[a]	12.8 ± 3.1	13.0 ± 2.0	13.3 ± 2.8	11.1 ± 3.5
No. of live pups delivered[a]	12.7 ± 3.2	12.9 ± 2.0	13.1 ± 2.6	10.1 ± 4.4
No. of stillborn pups [a]	0.1 ± 0.3	0.1 ± 0.3	0.3 ± 0.5	1.0 ± 1.2
Delivery index (%)[c]	88.6	94.7	92.4	68.6**
Rate of stillborn pups (%)[d]	0.7	0.7	1.9	9.0*
Sex ratio of live pups (males/females)	74/66	66/76	69/88	50/31
No. of dams delivered	11	11	12	8
No. of dams with total litter loss	0	0	0	6
Viability index (%)[e, f]				
Day 0 of lactation	99.3	99.3	98.1	91.0
Day 4 of lactation	99.3	99.3	98.7	24.7**
Body weight of pups (g)[a]				
Male				
PND 0	6.9 ± 0.6	6.9 ± 0.3	6.7 ± 0.9	4.9 ± 0.6**
PND 4	10.9 ± 1.6	11.0 ± 0.94	10.7 ± 2.2	6.1
Female				
PND 0	6.5 ± 0.7	6.5 ± 0.4	6.4 ± 0.8	4.5 ± 0.6**
PND 4	10.4 ± 1.6	10.5 ± 0.9	10.2 ± 2.0	6.9
No. of pups (litters) examined externally on PND 0	141 (11)	143 (11)	160 (12)	89 (8)
No. of pups (litters) with malformations	0	0	0	2 (2)
Open auricle	0	0	0	1 (1)
Vestigial tail	0	0	0	1 (1)
Short tail	0	0	0	1 (1)
Kinky tail	0	0	0	1 (1)
No. of pups (litters) examined internally on PND 4	139 (11)	141 (11)	155 (12)	20 (2)
No. of pups (litters) with malformations	0	0	0	0

[a]Values are given as the mean ± standard deviation.
[b]Implantation index (%) = (no. of implantations/no. of corpora lutea) × 100.
[c]Delivery index (%) = (no. of live pups delivered/no. of implantations) × 100.
[d]Rate of stillborn pups (%) = (no. of stillborns pups/total no. of pups delivered) × 100.
[e]Viability index on day 0 of lactation (%) = (no. of live pups delivered/total no. of pups delivered) × 100.
[f]Viability index on Day 4 of lactation (%) = (no. of live pups on day 4 of lactation/no. of live pups delivered) × 100.
*Significantly different from the control, $p < 0.05$; **significantly different from the control, $p < 0.01$.

was performed by using rats. The present findings show that PAP is a general and reproductive/developmental toxic, but it is unlikely to be teratogenic, in rats.

Acute renal failure due to PAP may have participated in male and female deaths at 500 mg/kg/day, because PAP is known to be nephrotoxic and histopathological changes in the kidney were observed. Histopathological changes

in the kidney were also observed in surviving animals of the 500-mg/kg/day group. Brown urine observed in all surviving male and females at 100 and 500 mg/kg/day was thought to result from the nephrotoxic effects of PAP. The renal findings of the present study are supported by a 28-day repeated dose toxicity study of PAP (JECDB, 1995), in which brown urine, epithelial cells in urine, increased absolute and relative weights of the kidney, and basophilic tubules with mitotic cells were found at 500 mg/kg/day. Decreased body-weight gain was associated with reduced food consumption in males and females at 500 mg/kg/day, and decreased food consumption unassociated with decreased body-weight gain was found in females at 100 mg/kg/day. In male rats, decreased weights of the testes and epididymides and histopathological changes in these organs at 500 mg/kg/day indicated that PAP exerts testicular toxicity at this dose. These findings indicated that the dosages of PAP used in this study were sufficiently high to induce general toxicity in parental rats, and the NOAEL of PAP for general toxicity is considered to be 20 mg/kg/day.

Although changes in weights and histopathological findings in the testes and epididymides were detected at the highest dose, there were no adverse effects on male reproductive performance, as evidenced by no changes in the copulation index, fertility index, or precoital interval. These findings are consistent with the previous findings. It was noted previously that rodent males produce sperm in numbers that greatly exceed the minimum requirements for fertility (Amann, 1981; Parker, 2006), and sperm production can be drastically reduced (by up to 50%) without affecting fertility in SD rats (Robaire et al., 1984).

There is a general consensus that a single cycle with a diestrus period of 4 days or longer or an estrus period of 3 days or longer is aberrant, and cycles that have 4 or more days of diestrus are classified as showing persistent or prolonged diestrus (Parker, 2006). In females treated with 500 mg/kg/day, decreased incidence of females showing 4-day estrus cycles and 4 females showing extended diestrus were observed, and these phenomena might result in a prolonged precoital interval. Slightly, but significantly, increased gestation length were also found at the highest dose. These findings in females may indicate a disruptive effect of PAP on hormonal homeostasis at 500 mg/kg/day, which was high enough to cause death.

As for developmental parameters, decreases in the delivery index, viability index at PND 4, and body weights of pups at PNDs 0 and 4, and an increased rate of stillborn pups were detected at 500 mg/kg/day in the present study. These findings are essentially consistent with the previous findings reported by Burnett et al. (1989), in which decreased number and body weight of live fetuses were detected at 0.7% (520 mg/kg/day). Malformations detected in pups in the present study are of types observed spontaneously among control rat fetuses in the literature (Morita et al., 1987; Nakatsuka et al., 1997; Barnett et al., 2000), and the incidence in the PAP-treated group was very low and not

significantly different from that of the control group. However, in the present study, no skeletal examinations were performed and the adverse effects of PAP on the morphological development of offspring could not be adequately evaluated at the highest dose, because an insufficient number of pups were obtained. Previously, no teratogenic effects of PAP were reported in rats fed PAP at up to 520 mg/kg/day (Burnett et al., 1989) and in rats given PAP by oral application at up to 250 mg/kg/day (Spengler et al., 1986). The previous and present findings together suggest that PAP has little teratogenic potential in rats.

There is concern over the possibility that the PAP-induced methemoglobinemia (Smith, 1967) and nephrotoxicity (Green et al., 1969; Calder et al., 1971; Kiese et al., 1975; Newton et al., 1982; Gartland et al., 1989) in dams are associated with the reproductive and developmental toxicity of PAP. John and Schmitz (1961) indicated a possible relationship between high maternal methemoglobin levels and abortion in humans. Sinha and Sleight (1971) observed that increased incidences of abortion and fetal deaths were produced after the administration of sodium nitrite to pregnant guinea pigs, and suggest that fetal deaths resulted from hypoxia induced mainly by maternal methemoglobinemia. Methemoglobin former *p*-nitroaniline, at levels that produced significant methemoglobinemia and low-level anemia, caused no reproducible effects on reproductive performance in a combined chronic study with a two-generation reproductive toxicity study using rats (Nair et al., 1990). In a recent review, Manassaram et al. (2006) concluded that the current literature does not provide sufficient evidence of a causal relationship between exposure to nitrates in drinking water and adverse reproductive effects in experimental animals and humans. In the case of PAP, hemosiderin deposition and extramedullary hematopoiesis observed in males of the 500-mg/kg/day group in the present study suggests a possible occurrence of hemolytic anemia. However, in the previous PAP study (Burnett et al., 1989), increased postimplantation loss and reduced fetal weight were not accompanied by methemoglobinemia in rats.

It is well known that chloroform is a nephrotoxic compound that injures the proximal tubule as well as PAP (Schnellmann, 2008). Schwetz et al. (1974) reported that inhalation of chloroform on Days 6–15 of pregnancy caused decreased conception rate, increased embryonic/fetal deaths, decreased fetal weight, and increased incidences of fetuses with tail anomalies, subcutaneous edema, skeletal variation, and retarded ossification in rats. These findings suggest that the possibility that maternal methemoglobinemia and/or nephrotoxicity participate in the developmental toxicity of PAP. The relationship between alteration of maternal physiology and offspring development is still controversial. Adverse effects of PAP on offspring observed in the present study are suggested to be due to a combination of effects of PAP and/or its metabolites and altered maternal physiology.

CONCLUSIONS

In conclusion, PAP caused death and decreased body weight gain in both sexes at 500 mg/kg/day, decreased food consumption in males at 500 mg/kg/day and females at 100 and 500 mg/kg/day, and brown urine in both sexes at 100 and 500 mg/kg/day. Terminated estrus cycles, longer gestation period, decreased delivery index, lowered pup weight, increased stillborns, and decreased viability index of pups were observed. The no observed adverse effect levels of PAP for general and reproductive/developmental toxicity were 20 and 100 mg/kg/day, respectively, in rats.

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REFERENCES

Amman, R. P. (1981). A critical review of methods for evaluation of spermatogenesis from seminal characteristics. *J. Androl.* 2:37–58.

Barnett, J. F., Jr., Lewis, D., Tappen, A., Hoberman, A. M., Christian, M. S. (2000). Reproductive indices, fetal gross, visceral and skeletal alterations, sexual maturation, passive avoidance and water maze data, a comparison of results in CD(SD)IGS rats and CD(SD) rats. In: Matsuzawa, T., Inoue, H. (eds). *Biological Reference Data on CD(SD)IGS Rats-2000* (pp. 159–173). Yokohama: CD(SD)IGS Study Group, c/o Charles River Japan, Inc.

Burnett, C. M., Re, T. A., Rodriguez, S., Leoehr, R. F., Dressler, W. E. (1989). The toxicity of *p*-aminophenol in the Sprague-Dawley rat: Effects on growth, reproduction, and fetal development. *Food Chem. Toxicol.* 27:691–698.

Calder, I. C., Funder, C. C., Green, C. R., Ham, K. N., Tange, J. D. (1971). Comparative nephrotoxicity of aspirin and phenacetin derivatives. *Br. Med. J.* 4:518–521.

Clayson, D. B., and Krewski, D. R. (1990). Objectives of toxicity testing. In: Arnold, D. L., Grice, H. C., Krewski, D. R., eds. *Handbook of In Vivo Toxicity Testing* (pp. 3–18). San Diego, California, USA: Academic Press.

EA, MHW, and MITI (Environment Agency, Ministry of Health and Welfare, and Ministry of International Trade and Industry of Japan). (1988). Research of Designated Chemical Substances, Planning and Coordination Bureau, Environment Agency, No. 39, Environmental Health Bureau, Ministry of Health and Welfare, No. 229, Basic Industries Bureau, Ministry of International Trade and Industry, No. 85, March 31, 1984, and amendments, November 18, 1988.

Gartland, K. P. R., Bonner, F. W., Timbrell, J. A., Nicholson, J. K. (1989). Biochemical characterisation of *para*-aminophenol-induced nephrotoxic lesions in the F 344 rat. *Arch. Toxicol.* 63:97–106.

Gemborys, M. W., Mudge, G. H. (1981). Formation and disposition of the minor metabolites of acetaminophen in the hamster. *Drug. Metab. Dispos.* 9:340–351.

Green, C. R., Ham, K. N., Tange, J. D. (1969). Kidney lesions induced in rats by *p*-aminophenol. *Br. Med. J.* 1:162–164.

Haz-Map. (2007). Information on Hazardous Chemicals and Occupational Diseases by Jay A. Brown, M.D., M.P.H. Accessed on December 17, 2007. Available at: http://hazmap.nlm.nih.gov/cgi-bin/hazmap_search?tbl=TblAll&queryx=123–30–8

HSDB (Hazardous Substances Data Bank). (1996). Comprehensive, peer-reviewed toxicology data for about 5,000 chemicals Accessed on December 17, 2007. Available at: http://toxnet.nlm.nih.gov/cgi-bin/sis/search/f?./temp/~LZgIGD:1

JECDB (Japan Existing Chemical Data Base). (1995). Twenty-eight-day repeat dose oral toxicity test of 4-aminophenol in rats (Japanese). Accessed on December 17, 2007. Available at: http://dra4.nihs.go.jp/mhlw_data/home/paper/paper123-30-8b.html

John, T., Schmitz, M. D. (1961). Methemoglobinemia—a cause of abortions?: preliminary report. *Obstet. Gynecol.* 17:413–415.

Kiese, M., Szinicz, L., Thiel, N., Weger N. (1975). Ferrihemoglobin and kidney lesions in rats produced by 4-aminophenol or 4-dimethylaminophenol. *Arch. Toxicol.* 34:337–340.

Klimisch, H. J., Andreae, M., Tillmann, U. (1997). A systematic approach for evaluating the quality of experimental toxicological and ecotoxicological data. *Regul. Toxicol. Pharmacol.* 25:1–5.

Lloyd, G. K., Liggett, M. P., Kynoch, S. R., Davies, R. E. (1977). Assessment of the acute toxicity and potential irritancy of hair dye constituents. *Food. Cosmet. Toxicol.* 15:607–610.

Mallinckrodt Inc. (2003). Material Safety Data Sheet (MSDS) Accessed on December 17, 2007. Available at: http://bulkpharm.mallinckrodt.com/_attachments/msds/amnol.htm

Manassaram, D. M., Backer, L. C., Moll, D. M. (2006). A review of nitrates in drinking water: maternal exposure and adverse reproductive and developmental outcomes. *Environ. Health Perspect.* 114:320–327.

Morita, H., Ariyuki, F., Inomata, N., Nishimura, K., Hasegawa, Y., Miyamoto, M., Watanabe, T. (1987). Spontaneous malformations in laboratory animals: frequency of external, internal, and skeletal malformations in rats, rabbits, and mice. *Cong. Anom.* 27:147–206.

Nair, R. S., Auletta, C. S., Schroeder, R. E., Johannsen, F. R. (1990). Chronic toxicity, oncogenic potential, and reproductive toxicity of *p*-nitroaniline in rats. *Fundam. Appl. Toxicol.* 15:607–621.

Nakatsuka, T., Horimoto, M., Ito, M., Matsubara, Y., Akaike, M., Ariyuki, F. (1997). Japan Pharmaceutical Manufacturers Association (JPMA) survey on background control data of developmental and reproductive toxicity studies in rats, rabbits, and mice. *Cong. Anom.* 37:47–138.

Newton, J. F., Kuo, C. H., Gemborys, M. W., Mudge, G. H., Hook, J. B. (1982). Nephrotoxicity of *p*-aminophenol, a metabolite of acetaminophen in the Fischer 344 rat. *Toxicol. Appl. Pharmacol.* 65:336–344.

Newton, J. F., Bailie, M. B., Hook, J. B. (1983). Acetaminophen nephrotoxicity in the rat. Renal metabolic activation *in vitro*. *Toxicol. Appl. Pharmacol.* 70:433–444.

Newton, J. F., Kuo, C. H., Deshore, G. M., Hoefle, D., Bernstein, J., Hook, J. B. (1985). The role of *p*-aminophenol in acetaminophen-induced nephrotoxicity: effect of bis(*p*-nitrophenyl) phosphate on acetaminophen and *p*-aminophenol nephrotoxicity and metabolism in Fischer 344 rats. *Toxicol. Appl. Pharmacol.* 81:416–430.

NITE. (2004). National Institute of Technology and Evaluation: Total search system for chemical substances. Chemical profile for 4-aminophenol (Searched by CAS No.; 123-30-8). Accessed on December 17, 2007. Available at: http://www.safe.nite.go.jp/data/sougou/pkc_e_search_frm.html

OECD (Organization for Economic Co-operation and Development). (1995). OECD Test Guideline for Testing of Chemicals, No. 421, Reproduction/Developmental Toxicity Screening Test. Adopted by the Council on 27 July 1995. Paris: OECD.

OECD (Organization for Economic Co-operation and Development). (1998). *OECD Principles on Good Laboratory Practice* (as revised in 1997). OECD Series on Principles of Good Laboratory Practice and Compliance Monitoring, No. 1. Paris: OECD.

OECD (Organization for Economic Co-operation and Development). (2005). Manual for Investigation of HPV Chemicals Accessed on December 17, 2007. Available at: http://www.oecd.org/dataoecd/13/15/36045203.pdf

Parker, R. M. (2006). Testing for reproductive toxicity, In: Hood, R.D. (eds). Developmental and reproductive toxicology, 2nd ed (pp. 425–487). Boca Raton, Florida, USA: CRC Press (Taylor & Francis Group).

Robaire, B., Smith, S., Hales, B. F. (1984). Suppression of spermatogenesis by testosterone in adult male rats: effect on fertility, pregnancy outcome, and progeny. *Biol. Reprod.* 31:221–230.

Rutkowski, J. V., Ferm, V. H. (1982). Comparison of the teratogenic effects of the isomeric forms of aminophenol in the Syrian golden hamster. *Toxicol. Appl. Pharmacol.* 63:264–269.

Schnellmann, R. G. (2008). Toxic responses of kidney. In: Klaassen, C. D. (eds). *Casarett & Doull's Toxicology,* 7th ed, New York: McGraw-Hill Press, pp. 583–608.

Schwetz, B. A., Leong, B. K. J., Gehring, P. J. (1974). Embryo- and fetotoxicity of inhaled chloroform in rats. *Toxicol. Appl. Pharmacol.* 28:442–451.

Scorecard. (2005).. The Environmental Defense Fund's Chemical Scorecard summarizes information about health effects, hazard rankings, industrial and consumer product uses, environmental releases and transfers, risk assessment values, and regulatory coverage. Chemical profile for 4-aminophenol (CAS No.: 123-30-8) Accessed on December 17, 2007. Available at: http://www.scorecard.org/chemical-profiles/summary.tcl?edf_substance_id=+123–30–8

Sinha, D. P., Sleight, S. D. (1971). Pathogenesis of abortion in acute nitrite toxicosis in guinea pigs. *Toxicol. Appl. Pharmacol.* 18:340–347.

Smith, R. P., Alkaitis, A. A., Shafer, P. R. (1967). Chemically induced methemoglobinemias in the mouse. *Biochem. Pharmacol.* 16:317–328.

Spengler, J., Osterburg, I., Korte, R. (1986). Teratogenic evaluation of *p*-toluenediamine sulphate, resorcinol, and *p*-aminophenol in rats and rabbits [meeting abstract]. *Teratology* 33:31A.

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Gender-Related Difference in the Toxicity of Ultraviolet Absorber 2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole in Rats

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2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (DBHCB) is widely used as an ultraviolet absorber. Previously, we showed that male rats had more than a 100 times higher susceptibility to the toxic effects of DBHCB than females. In order to investigate the role of sex steroids in the mediation of this gender-related difference, DBHCB (0 or 250 mg/kg/day) was given to male and female young intact and castrated rats by gavage for 28 days in the current study. In intact rats, relative liver weight increased to more than two times that of the control in males, while the rate of change was less than 10% in females. On histopathology, hypertrophy of hepatocytes was observed in males but not in females. In castrated rats, an approximately 40% increase in the relative liver weight was found only in males, and no histopathological changes in the liver were detected in either sex. The gender-related difference was also determined in preweaning rats administered DBHCB at 0, 250, or 500 mg/kg/day by gavage from postnatal days 4 to 21. Blood biochemical changes, including increases in the levels of AST, ALT, and ALP, 80–95% increase in the relative liver weight and histopathological changes in the liver, such as hypertrophy and single cell necrosis of hepatocytes, were observed at both doses in both sexes. In conclusion, the gender-related difference in the toxicity of DBHCB, which was observed in young rats, was markedly reduced by castration and abolished in preweaning rats.

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INTRODUCTION

2-(3′,5′-Di-*tert*-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole (CAS No. 3864-99-1; DBHCB) is an ultraviolet (UV) absorber belonging to the benzotriazole class of UV absorbers (Great Lakes Chemical Corporation, 2007). Regarding toxicity, it is reported that oral LD_{50} for DBHCB was greater than 5,000 mg/kg in rats, and DBHCB caused minimal irritation to the skin and slight irritation to the eyes in rabbits (Everlight Chemical Industrial Corporation, 2002). A 90-day feeding study of DBHCB in rats resulted in dose-dependent increases in liver weights and signs of liver toxicity at 22–800 mg/kg/day, but no detailed information is available on this study. Previously, we showed that DBHCB exerted no effects on the reproduction and development of rats in a prenatal developmental toxicity study (Ema et al., 2006) and in a combined repeated dose and reproductive/developmental toxicity screening test (Ema et al., 2008). In the latter combined study, increases in serum levels of ALP, albumin and/or A/G ratio, and in absolute and relative liver weight were found at 25 mg/kg/day and above in males, but these changes were not observed in females, even at the highest dose of 250 mg/kg/day. These findings indicate that male rats have more than a 100 times higher susceptibility to DBHCB toxicity than females.

Gender-related differences in the susceptibility of rats to toxicity have been documented for many other industrial chemicals (Hirata-Koizumi et al., 2007, 2008a; Muraoka and Itoh, 1980), environmental pollutants (Knuckles et al., 2004), insecticides (Agarwal et al., 1982; Carlson and DuBois, 1970), and pharmaceuticals (Coleman et al., 1990; Stern et al., 2007; Wang et al., 2001). For example, fluoranthene, a polycyclic aromatic hydrocarbon, showed greater effects on the kidneys of male rats, as compared to those of females, in a subchronic toxicity study (Knuckles et al., 2004). In contrast, female rats exhibited greater susceptibility to the acetylcholinesterase inhibitory effects of an organophosphorus insecticide, parathion (Agarwal et al., 1982). Such sexual variations are also reported in humans, mostly for medicines (Harris et al., 1995). Examples include the more severe adverse effects, but with greater improvement in response, of antipsychotic drugs, such as chlorpromazine and fluspirilene in women.

For such gender differences in toxic responses, sexual hormones are likely to play important roles. Agarwal et al. (1982) reported that gonadectomy abolished sex differences in acetylcholinesterase inhibition induced by parathion. Since gonadectomy increased the susceptibility of males and the administration of testosterone led to recovery from the increased sensitivity to the antiacetylcholinesterase activity of parathion, it is apparent that testosterone interferes with the effects of parathion. On the other hand, estrogen has been shown to act

as a dopamine antagonist (Harris et al., 1995), which is considered to contribute, at least in part, to sex differences in response to antipsychotic drugs. The role of sex hormones in toxicity responses seems to vary from case to case.

In the present study, in order to investigate the role of sex steroids in the mediation of gender-related differences in the susceptibility of rats to the toxicity of DBHCB, we performed a 28-day repeated-dose toxicity study of DBHCB, using male and female intact and castrated rats (castration study). Further, we determined sexual variations in DBHCB toxicity in preweaning rats, which were considered under the limited influence of sexual hormones (preweaning rat study).

MATERIALS AND METHODS

This study was performed at Shin Nippon Biomedical Laboratories, Ltd., Drug Safety Research Laboratories (SNBL DSR; Kagoshima, Japan) in 2005–2006. The experiment was approved by the Institutional Animal Care and Use Committee of SNBL DSR and was performed in accordance with the ethics criteria contained in the bylaws of the Committee of SNBL DSR.

Animals and Housing Conditions

Eleven-week-old male and 10-week-old female Crl: CD (SD) rats were purchased from Hino Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan) and individually housed in stainless steel cages suspended over a cage board. After a 7 day acclimation, females were cohabited overnight with 1 male each. Females with vaginal plugs were regarded as pregnant, and this day was designated as Day 0 of gestation. Twelve pregnant rats were assigned each for the castration and preweaning rat study. On Day 20 of gestation, pregnant females were transferred to aluminum cages with wooden chips as bedding (White Flake; Charles River Laboratories Japan, Inc.) and allowed to deliver spontaneously and rear their pups. The day of birth was defined as postnatal day (PND) 0. The sex of pups was determined on PND 0.

In the castration study, after weaning on PND 21, male and female pups found to be in good health were selected, and half of them were castrated under ether anesthesia on PNDs 25–29. Intact or castrated animals were randomly divided into DBHCB-treated and control groups of 6 males and 6 females each. They were subjected to treatment at 6 weeks of age.

In the preweaning rat study, the litters were adjusted randomly to 4 males and 4 females on PND 3. Four litters were selected and assigned to each of three dose groups, including control groups, by stratified random sampling based on body weight; the initial number of pups for treatment was 16/sex/group.

Animals were maintained in an air-conditioned room at 21.7–23.1°C, with relative humidity of 44–67%, a 12-h light/dark cycle, and ventilation with 15

air changes/hour. A basal diet (CE-2; CLEA Japan, Inc., Tokyo, Japan) and water, which met the drinking water standard under the Water Works Law of Japan, were provided *ad libitum*.

Chemicals and Doses

DBHCB was obtained from Musashino Chemical Laboratory, Ltd. (Kitaibaraki, Japan). The DBHCB (Lot no. 05004IX3) used in this study was 99.9% pure, based on high-performance liquid chromatography (HPLC) analysis, and was kept in a dark place at room temperature under airtight conditions. The test article was suspended in 5w/v% gum Arabic solution, and administered to the animals by gastric intubation. Control rats received the vehicle alone. Dosing solutions were prepared at least once a week and kept in a cool and dark place under airtight conditions until dosing. The stability of the formulations under these conditions had been confirmed for up to 14 days in the previous combined repeated-dose and reproductive/developmental toxicity screening test (Ema et al., 2008).

In the previous combined study, male and female rats were given DBHCB by gavage for 55–69 days at 0, 2.5, 25, or 250mg/kg/day. Increases in absolute and relative liver weight and in serum levels of alkaline phosphatase (ALP) and albumin and/or A/G ratio were observed at 25mg/kg/day and above in males, but no changes in these parameters were found in females. Taking into account these previous results, the dose levels of DBHCB in the present study were set as 250mg/kg/day for the castration study and 250 or 500mg/kg/day for the preweaning rat study. The daily application volume (10mL/kg body weight) was calculated according to the latest body weight.

Experimental Design

Castration Study

Male and female young intact and castrated rats were given DBHCB once-daily at 0 or 250mg/kg by gavage for 28 days. A Teflon gastric tube for rats (RZ-2; CLEA Japan, Inc., Tokyo, Japan), attached to a disposable syringe, was used for dosing.

All animals were observed daily before and 1–2h after dosing for clinical signs of toxicity. Body weight was measured on Days 0, 4, 7, 11, 14, 18, 21, 25, and 28 of the dosing period, and food consumption was recorded twice a week.

On the day after the last dosing, all animals were euthanized by exsanguination under deep ether anesthesia, and the body surface, organs, and tissues were examined macroscopically. The liver was then removed, weighed, and fixed in 10% neutral-buffered formalin. Paraffin sections for microscopic examination were routinely prepared and stained with hematoxylin-eosin. Histopathological examination of the liver was conducted for all animals.

Preweaning Rat Study

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

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

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

Data Analysis

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

RESULTS

Castration Study

Intact Rats

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

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

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

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

Values are expressed as the mean ± SD (g).
*Significantly different from the respective control, $p < 0.05$.
**Significantly different from the respective control, $p < 0.01$.

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

Castrated Rats

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

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

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

[a]Values are expressed as the mean ± SD (g).
[b]Values are expressed as the mean ± SD (g/100g body weight).
*Significantly different from the respective control, $p < 0.05$.
**Significantly different from the respective control, $p < 0.01$.

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

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

Values represent the number of animals with findings.
+: Very slight.
++: Slight.
+++: Moderate.
**Significantly different from the control, $p < 0.01$.

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

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

Preweaning Rat Study

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Values represent the number of animals with findings.
+: Very slight.
++: Slight.
+++: Moderate.
*Significantly different from the control, $p<0.05$.

DISCUSSION

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

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