At the scheduled terminal necropsy, all rats were euthanized by exsanguination under anesthesia. All rats were subjected to gross necropsy, which included an external examination of all body orifices and surfaces, and examinations of all cranial, thoracic, and abdominal organs. The brain, heart, liver, kidney, spleen, thymus, and adrenal gland in males and females, the testis, epididymis, seminal vesicle, and prostate in males, and the ovary in females were removed and weighed. Relative organ weights (mg or g/100 g of body weight) were calculated on the basis of the terminal body weight. In females, the numbers of corpora lutea and implantation sites were recorded. Samples of tissues and organs were preserved in neural phosphate-buffered 10% formaldehyde solution. The testis and epididymis were fixed in Bouin's solution. Histopathological evaluations for five rats/sex /group were performed on the tissues specified below after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin; the brain, heart, thymus, kidney, spleen, adrenal gland, small and large intestine, lung, trachea, thyroid, submandibular and mesenteric lymph node, femur bone marrow, spinal cord, sciatic nerve, tibial nerve, urinary blabber, testis, epididymis, seminal vesicle, prostate, ovary, and uterus in the control and highest dose groups, and the liver in all groups.

Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of sperm in the vaginal smear and/or a vaginal plug was considered evidence of successful mating. The day of successful mating was designated as Day 0 of pregnancy. The females were allowed to deliver spontaneously and nurse their pups until postnatal days (PNDs) 4–6. The day on which parturition was completed was designated as PND 0. Litter size and numbers of live and dead pups were recorded, and the live pups were sexed and individually weighed on PNDs 0 and 4. Dead pups were examined grossly. On PND 4, the pups were euthanized by exsanguination under anesthesia, and gross external and internal examinations were performed.

Data Analysis

The statistical analysis of pups was carried out by using the litter as the experimental unit. The body weight, body-weight gain and food consumption, precoital interval, length of gestation, numbers of implantations and live pups per litter and pup weight, delivery index, viability index, hematological and blood biochemical parameters, and organ weight were analyzed with Bartlett's test for homogeneity of variance at the 5% level of significance. When the variance was homogeneous, Dunnett's test was performed to compare the mean value in the control group with that in each DBHCB group. When the variance was heterogeneous, a Dunnett-type test was performed to

compare the mean value in the control group with that in each DBHCB group after rank conversion. Recovery in the control and highest dose groups was analyzed in the following way. Variance ratio was analyzed by an F test. If the variance ratio was equivalent, the groups were compared by a Student's t-test. If the variance was not equivalent, the Wilcoxon test was performed.

RESULTS

No deaths or DBHCB-related clinical signs of toxicity were found in male or female rats of any groups. There was no significant difference in the body weight and body-weight gain between the control and DBHCB-treated groups in males and females, including during pregnancy and lactation. No significant changes in the food consumption were found, except for a significant decrease on Days 28–29 in males and an increase on Days 31–32 in females at 250 mg/kg.

The reproductive and developmental findings in rats given DBHCB are presented in Table 1. Although one pair did not copulate in the control group, all pairs copulated and all copulated females were impregnated and delivered their pups in all DBHCB-treated groups. There was no significant difference in the copulation index, fertility index, gestation index, precoital interval, or gestation length between the control and DBHCB-treated groups. No effects of DBHCB were observed on the numbers of corpora lutea or implantations, preimplantation loss, numbers of pups delivered, live pups, or stillborn or sex ratio of live pups. There was no significant difference in the viability or body weight of pups on PNDs 0 or 4 between the control and DBHCB-treated groups. External and internal examinations revealed no morphological anomalies in the pups of any group.

Table 2 shows the hematological findings in rats given DBHCB at the end of the administration period. A significantly decreased RBC at 250 mg/kg/d and shorter APTT at 25 and 250 mg/kg/d were observed in males. The number of neutrophils was significantly increased, at 250 mg/kg/d, in males. In females, the only significant change was a lowered number of eosinophils, at 25 and 250 mg/kg/d. At the end of the recovery period, significantly increased numbers of platelets and neutrophils, as well as an increased neutrophil ratio, were observed in males at 250 mg/kg/d, in addition to a decreased lymphocyte ratio.

Table 3 presents the blood biochemical findings in rats given DBHCB at the end of the administration period. In males, significantly increased levels of ALAT at 25 mg/kg/d, as well as decreased levels of creatinine at 25 mg/kg/d and higher, were observed. Additionally, males presented decreased levels of total bilirubin, and increased levels of ALP, at 250 mg/kg/d, were observed. The levels of total protein were significantly increased at 25 mg/kg/d. A significantly increased albumin percentage and A/G ratio and decreased

Table 1: Reproductive and developmental findings in rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of pairs	10	10	10	10
Copulation index (%)[b]	90	100	100	100
Fertility index (%)[c]	100	100	100	100
No. of pregnant females	9	10	10	10
Precoital interval (days)[a]	4.9 ± 4.4	3.4 ± 3.8	2.7 ± 1.3	2.8 ± 1.5
Gestation index (%)[d]	100	100	100	100
Gestation length (days)[a]	21.9 ± 0.4	21.9 ± 0.3	22.0 ± 0.4	22.0 ± 0.2
No. of litters	9	10	10	10
No. of corpora lutea[a]	16.1 ± 1.9	15.7 ± 1.8	15.3 ± 1.5	16.0 ± 1.9
No. of implantations[a]	15.3 ± 1.7	14.8 ± 1.5	14.1 ± 1.2	14.2 ± 3.2
Preimplantation loss (%)[a,e]	6.1 ± 4.3	6.6 ± 8.4	7.5 ± 7.0	10.9 ± 17.3
Delivery index (%)[a,f]	91.1 ± 7.2	93.8 ± 7.0	91.0 ± 13.8	96.5 ± 5.7
No. of pups delivered[a]	14.1 ± 2.2	14.0 ± 1.9	12.8 ± 2.0	14.0 ± 3.1
No. of live pups[a]	14.0 ± 2.2	13.9 ± 1.9	12.8 ± 2.0	13.9 ± 2.9
No. of stillborn[a]	0.1 ± 0.3	0.1 ± 0.3	0	0.1 ± 0.3
Sex ratio of live pups (female/total)[a]	0.53 ± 0.09	0.50 ± 0.15	0.53 ± 0.09	0.61 ± 0.16
Viability index during lactation (%)[a,g,h]				
Day 0	99.2 ± 2.4	99.3 ± 2.3	98.8 ± 3.7	98.8 ± 2.6
Day 4	100	98.8 ± 2.6	97.6 ± 4.0	97.7 ± 3.7
Male pup weight during lactation (g)[a]				
Day 0	6.5 ± 0.5	6.5 ± 0.5	6.8 ± 0.3	6.5 ± 0.4
Day 4	9.3 ± 1.1	9.4 ± 0.9	10.2 ± 0.7	9.6 ± 1.4
Female pup weight during lactation (g)[a]				
Day 0	6.0 ± 0.4	6.2 ± 0.5	6.3 ± 0.4	6.1 ± 0.4
Day 4	8.9 ± 1.0	9.0 ± 0.8	9.7 ± 0.7	9.1 ± 1.5

[a]Values are given as the mean ± SD.
[b]Copulation index (%) = (no. of females with successful copulation/no. of females paired) × 100.
[c]Fertility index (%) = (no. of females pregnant/no. of females with successful copulation) × 100.
[d]Gestation index (%) = (no. of females that delivered live pups/no. of pregnant females) × 100.
[e]Preimplantation loss (%) = ((no. of corpora lutea - no. of implantations)/no. of corpora lutea) × 100.
[f]Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.
[g]Viability index on postnatal day 0 (%) = (no. of live pups on postnatal day 0/no. of pups delivered) × 100.
[h]Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of live pups on postnatal day 0) × 100.

α2-globulin percentage were found in males at 25 and 250 mg/kg/d, as well as a decreased percentage of β-globulin at 2.5 mg/kg/d and higher. In females, the levels of total cholesterol were significantly decreased at 2.5 and 25 mg/kg/d. No significant changes in other blood biochemical parameters were noted in males and females in the DBHCB-treated groups. At the end of the recovery period, significantly increased levels of total protein, albumin, and total cholesterol and decreased creatinine levels and α2-globulin ratio were observed at 250 mg/kg/d in males. In females, parameters remained unchanged in all DBHCB-treated groups.

Table 2: Hematological findings in male and female rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of male rats	5	5	5	5
RBC (10^6/mL)	8.18 ± 0.32[a]	7.95 ± 0.31	8.07 ± 0.30	7.63 ± 0.36*
WBC (10^3/mL)	9.41 ± 1.06	8.22 ± 2.94	8.10 ± 2.37	8.94 ± 1.13
Hematocrit value (%)	45.6 ± 1.9	44.3 ± 0.9	44.7 ± 2.2	42.7 ± 1.7
Hemoglobin concentration (g/dL)	15.2 ± 0.4	14.9 ± 0.5	15.1 ± 0.9	14.2 ± 0.7
Platelet count (10^3/mL)	1063 ± 110	1145 ± 134	1202 ± 119	1205 ± 108
MCV (fL)	55.7 ± 2.3	55.8 ± 1.5	55.4 ± 0.9	55.9 ± 0.7
MCH (pg)	18.7 ± 0.7	18.7 ± 0.8	18.7 ± 0.4	18.6 ± 0.3
MCHC (g/dL)	33.5 ± 0.7	33.5 ± 0.7	33.8 ± 0.4	33.3 ± 0.4
Reticulocyte ratio (%)	2.60 ± 0.34	2.74 ± 0.57	3.00 ± 0.40	3.02 ± 0.44
PT (sec)	8.52 ± 0.42	9.50 ± 0.97	9.20 ± 0.57	8.50 ± 0.58
APTT (sec)	20.1 ± 0.8	20.9 ± 0.7	18.3 ± 1.0**	18.2 ± 0.7**
No. of female rats	5	5	5	5
RBC (10^6/mL)	6.81 ± 0.49[a]	6.90 ± 0.36	6.82 ± 0.14	6.50 ± 0.24
WBC (10^3/mL)	5.95 ± 0.96	6.19 ± 1.38	6.34 ± 1.46	5.05 ± 0.71
Hematocrit value (%)	40.2 ± 2.1	41.1 ± 1.7	39.4 ± 1.2	39.6 ± 2.3
Hemoglobin concentration (g/dL)	13.4 ± 0.7	14.0 ± 0.8	13.1 ± 0.4	13.4 ± 0.8
Platelet count (10^3/mL)	1468 ± 237	1518 ± 44	1496 ± 208	1503 ± 157
MCV (fL)	59.1 ± 2.4	59.6 ± 1.8	57.8 ± 2.1	60.9 ± 1.5
MCH (pg)	19.7 ± 0.8	20.3 ± 0.5	19.3 ± 0.7	20.5 ± 0.5
MCHC (g/dL)	33.3 ± 0.2	34.0 ± 0.6	33.4 ± 0.7	33.7 ± 0.4
Reticulocyte ratio (%)	6.48 ± 2.55	4.88 ± 1.04	4.48 ± 1.28	6.28 ± 2.55
PT (sec)	7.38 ± 0.29	7.28 ± 0.19	7.42 ± 0.27	6.94 ± 0.32
APTT (sec)	18.6 ± 1.2	19.1 ± 1.9	18.8 ± 0.3	14.7 ± 3.4

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

The organ weights of male rats given DBHCB at the end of the administration period are presented in Table 4. The absolute and relative weights of the liver were significantly higher at 25 mg/kg/d and higher. No significant changes in the weight of the reproductive organs were found. At the end of recovery period the absolute and relative weights of the liver at 250 mg/kg/d, were still significantly increased.

Table 5 shows the organ weight of female rats given DBHCB at the end of the administration period. There were no significant changes in the absolute and relative weights of organs, including the reproductive organs. At the end of the recovery period, no significant changes in the absolute or relative weight of organs were observed at 250 mg/kg/d.

No changes related to the administration of DBHCB were found in the necropsy findings. Histopathological examinations revealed no test compound–related toxicological changes in the liver of males and females in all the DBHCB-treated groups. There were also no changes in the other organs, including the male and female reproductive organs, in the 250 mg/kg/d group.

Table 3: Blood biochemical findings in male and female rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of male rats	5	5	5	5
ASAT (IU/L)	116 ± 26[a]	92 ± 18	136 ± 28	121 ± 23
ALAT (IU/L)	38.8 ± 3.7	39.2 ± 2.9	58.2 ± 25.5*	48.8 ± 7.5
ALP (IU/L)	539 ± 57	476 ± 78	617 ± 178	943 ± 150**
Total bilirubin (mg/dL)	0.052 ± 0.008	0.048 ± 0.016	0.046 ± 0.013	0.024 ± 0.009**
BUN (mg/dL)	20.7 ± 1.2	19.7 ± 2.6	21.8 ± 1.9	21.3 ± 3.8
Creatinine (mg/dL)	0.312 ± 0.053	0.274 ± 0.022	0.226 ± 0.037**	0.248 ± 0.022**
Total cholesterol (mg/dL)	68.0 ± 6.9	58.4 ± 12.8	64.0 ± 7.3	61.2 ± 16.5
Glucose (mg/dL)	186 ± 14	173 ± 14	190 ± 15	198 ± 27
Total protein (g/dL)	5.60 ± 0.10	6.04 ± 0.27	6.26 ± 0.41**	5.92 ± 0.034
Albumin (%)	51.5 ± 2.3	53.3 ± 1.8	58.6 ± 2.5**	61.0 ± 1.7**
A/G ratio	1.07 ± 0.10	1.14 ± 0.09	1.42 ± 0.14**	1.57 ± 0.11**
α1-Globulin (%)	20.4 ± 2.7	20.7 ± 2.5	19.1 ± 2.9	18.1 ± 1.2
α2-Globulin (%)	9.4 ± 0.5	9.0 ± 0.3	7.8 ± 0.2**	7.6 ± 0.4**
β-Globulin (%)	14.5 ± 0.9	12.9 ± 1.0**	10.6 ± 0.6**	9.0 ± 0.3**
γ-Globulin (%)	4.2 ± 1.0	4.2 ± 0.3	4.0 ± 0.8	4.2 ± 0.8
No. of female rats	5	5	5	5
ASAT (IU/L)	130 ± 11[a]	113 ± 37	106 ± 15	104 ± 23
ALAT (IU/L)	59.0 ± 9.1	42.8 ± 7.8	49.4 ± 9.9	60.2 ± 15.3
ALP (IU/L)	215 ± 29	185 ± 71	184 ± 56	194 ± 59
Total bilirubin (mg/dL)	0.058 ± 0.016	0.074 ± 0.030	0.044 ± 0.011	0.056 ± 0.013
BUN (mg/dL)	26.1 ± 8.2	17.3 ± 5.3	19.8 ± 4.1	18.9 ± 5.0
Creatinine (mg/dL)	0.308 ± 0.044	0.290 ± 0.040	0.330 ± 0.029	0.282 ± 0.028
Total cholesterol (mg/dL)	79.6 ± 16.8	58.4 ± 3.2*	57.6 ± 13.3*	64.2 ± 12.9
Glucose (mg/dL)	109 ± 16	109 ± 13	120 ± 7	115 ± 24
Total protein (g/dL)	5.74 ± 0.31	5.60 ± 0.27	5.54 ± 0.36	5.50 ± 0.22
Albumin (%)	55.0 ± 1.8	54.2 ± 2.1	55.5 ± 0.8	55.4 ± 1.8
A/G ratio	1.23 ± 0.09	1.19 ± 0.10	1.25 ± 0.04	1.25 ± 0.09
α1-Globulin (%)	17.8 ± 2.1	19.2 ± 1.4	17.8 ± 2.2	17.6 ± 1.3
α2-Globulin (%)	8.8 ± 1.2	8.8 ± 0.9	7.9 ± 0.8	8.3 ± 0.3
β-Globulin (%)	13.5 ± 0.9	13.3 ± 0.9	13.7 ± 0.8	13.4 ± 1.0
γ-Globulin (%)	4.9 ± 1.2	4.4 ± 0.4	5.1 ± 0.5	5.3 ± 0.4

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

DISCUSSION

The present study was conducted to determine the repeated-dose and reproductive toxicity of DBHCB. The data show that the repeated oral dosing of DBHCB caused changes in the liver in males, but not in females, and no changes in the reproductive function of male and female rats.

In the present study, there were no changes in the reproductive parameters regarding copulation, fertility, parturition, and nursing of their pups in rats given DBHCB beginning 28 d before mating, during pregnancy, and shortly after parturition. No changes in weight or histopathology were found in male and female reproductive organs. Moreover, the prenatal and postnatal

Table 4: Organ weights of male rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of male rats	5	5	5	5
Body weight (g)	451 ± 35[a]	463 ± 26	454 ± 37	437 ± 11
Brain (g)	2.06 ± 0.07[b]	2.09 ± 0.06	2.06 ± 0.11	2.00 ± 0.09
	0.461 ± 0.034[c]	0.453 ± 0.034	0.457 ± 0.061	0.459 ± 0.030
Heart (g)	1.41 ± 0.07[b]	1.52 ± 0.11	1.44 ± 0.16	1.42 ± 0.11
	0.314 ± 0.011[c]	0.329 ± 0.018	0.312 ± 0.018	0.325 ± 0.024
Thymus (mg)	391 ± 90[b]	401 ± 104	412 ± 174	396 ± 88
	87.4 ± 22.7[c]	86.2 ± 18.6	89.4 ± 31.5	90.8 ± 20.5
Liver (g)	14.81 ± 1.43[b]	16.46 ± 1.70	20.11 ± 3.76*	24.11 ± 2.60**
	3.28 ± 0.13[c]	3.54 ± 0.20	4.41 ± 0.55*	5.52 ± 0.66**
Kidney (g)	3.17 ± 0.25[b]	3.49 ± 0.31	3.50 ± 0.40	3.34 ± 0.18
	0.706 ± 0.082[c]	0.753 ± 0.051	0.769 ± 0.045	0.763 ± 0.045
Spleen (mg)	853 ± 82[b]	957 ± 205	908 ± 218	790 ± 62
	190 ± 19[c]	206 ± 38	199 ± 35	181 ± 17
Adrenal (mg)	61.2 ± 9.5[b]	62.1 ± 9.2	61.5 ± 6.9	50.8 ± 3.2
	13.6 ± 2.3[c]	13.4 ± 2.1	13.6 ± 2.2	11.6 ± 0.6
Testis (g)	3.23 ± 0.14[b]	3.39 ± 0.17	3.01 ± 0. 28	3.00 ± 0.25
	0.720 ± 0.062[c]	0.732 ± 0.051	0.666 ± 0.073	0.686 ± 0.058
Epididymis (g)	1.26 ± 0.07[b]	1.27 ± 0.06	1.23± 0.14	1.24 ± 0.13
	0.281 ± 0.027[c]	0.274 ± 0.010	0.271 ± 0.018	0.284 ± 0.028
Seminal vesicle (g)	1.71 ± 0.18[b]	1.69 ± 0.14	1.70 ± 0.21	1.60 ± 0.12
	0.383 ± 0.060[c]	0.365 ± 0.040	0.376 ± 0.040	0.366 ± 0.027
Prostate (g)	1.37 ± 0.099[b]	1.25 ± 0.10	1.42 ± 0.34	1.39 ± 0.19
	0.305 ± 0.035[c]	0.270 ± 0.025	0.309 ± 0.057	0.319 ± 0.038

[a]Values are given as the mean ± SD.
[b]Absolute organ weight.
[c]Relative organ weight = organ weight (g or mg)/100 g body weight.
*Significantly different from the control, $p < 0.05$.
**Significantly different from the control, $p < 0.01$.

developmental parameters regarding embryonic/fetal/neonatal survival and growth and morphological development of offspring were not affected by the administration of DBHCB. These results are consistent with the results of our previous study, in which no maternal or prenatal developmental toxicity was noted in rats given DBHCB by gavage on Days 5–19 of pregnancy at 1,000 mg/kg/d (Ema et al., 2006). These findings indicate that DBHCB has no potential for reproductive or developmental toxicity in rats.

On the hematological examination, changes in some parameters were noted in both male and female rats at higher doses. However, these changes are not considered to indicate toxicological significance because they were relatively small and were dose independent. The lowered RBC, for example, in males at 250 mg/kg/d is unlikely to represent anemia because the degree of decrease is slight and other anemic parameters, such as hematocrit, hemoglobin, MCV, MCH, MCHC, and reticulocyte count, were not affected by the administration of DBHCB. Anemia is defined clinically as the condition characterized by a hemoglobin concentration below the lower reference limit (Hall, 2007). Regarding renal function, it has been described that serum

Table 5: Organ weights of female rats given DBHCB.

DBHCB (mg/kg/day)	0 (control)	2.5	25	250
No. of female rats	5	5	5	5
Body weight (g)	282 ± 33[a]	290 ± 14	276 ± 15	283 ± 21
Brain (g)	1.96 ± 0.04[b]	1.96 ± 0.06	1.97 ± 0.09	1.94 ± 0.06
	0.706 ± 0.100[c]	0.677 ± 0.044	0.716 ± 0.068	0.688 ± 0.058
Heart (g)	1.06 ± 0.14[b]	1.00 ± 0.04	0.98 ± 0.08	1.01 ± 0.10
	0.376 ± 0.019[c]	0.346 ± 0.012	0.357 ± 0.022	0.358 ± 0.035
Thymus (mg)	219 ± 40[b]	272 ± 60	247 ± 87	253 ± 64
	77.6 ± 7.8[c]	93.6 ± 18.6	90.2 ± 32.9	90.2 ± 26.5
Liver (g)	9.89 ± 1.64[b]	8.99 ± 0.67	9.16 ± 0.69	9.69 ± 0.54
	3.51 ± 0.37[c]	3.10 ± 0.19*	3.32 ± 0.10	3.43 ± 0.20
Kidney (g)	2.16 ± 0.28[b]	2.07 ± 0.14	1.98 ± 0.21	2.03 ± 0.03
	0.770 ± 0.067[c]	0.713 ± 0.033	0.715 ± 0.057	0.721 ± 0.049
Spleen (mg)	716 ± 178[b]	713 ± 125	666 ± 172	749 ± 62
	252 ± 44[c]	246 ± 47	240 ± 52	265 ± 16
Adrenal (mg)	95.7 ± 15.2[b]	85.0 ± 10.3	85.3 ± 10.3	89.5 ± 4.0
	34.1 ± 5.23[c]	29.3 ± 3.6	30.9 ± 3.3	31.7 ± 1.6
Ovary (mg)	95.9 ± 10.4[b]	96.4 ± 6.2	95.6 ± 11.6	104.9 ± 18.8
	34.5 ± 5.9[c]	33.2 ± 1.9	34.7 ± 4.2	36.9 ± 4.5

[a]Values are given as the mean ± SD.
[b]Absolute organ weight.
[c]Relative organ weight = organ weight (g or mg)/100 g body weight.
*Significantly different from the control, $p < 0.05$.

creatinine levels parallel changes in BUN caused by alterations in renal blood flow, renal function, or urinary outflow (Hall, 2007). The changes in creatinine levels in male rats at 25 mg/kg/d and higher are not thought to have toxicological significance because there were no changes in BUN or histopathological alterations of the kidney in the DBHCB-treated groups. In male rats, changes in some blood biochemical parameters suggestive of liver toxicity were observed at higher doses. The increased levels of total protein and albumin suggest an acceleration of protein synthesis in the liver, and these phenomena are supported by the increased weight of the liver at higher doses. These changes were noted only in males, indicating a sex difference in the toxicity of DBHCB.

The no observed adverse effect level (NOAEL) for repeated-dose toxicity of DBHCB is considered to be 2.5 mg/kg/d in male rats, based on the increased levels of albumin and weight of the liver, and 250 mg/kg/d, the highest dose used in the present study, in female rats. Our findings indicate that male rats have more than a 100-fold greater susceptibility to DBHCB toxicity than female rats. Previously, we showed sex differences in toxicity in the 28-d and 52-week repeated-dose toxicity studies of a structurally similar compound, 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole (HDBB), which is also used as a UV absorber (Hirata-Koizumi et al., 2007, 2008a). In the 28-d repeated-dose toxicity study, using rats given HDBB by gavage at 0, 0.5, 2.5,

12.5, or 62.5 mg/kg/d, adverse effects on the liver and heart were noted at all doses in males and at 12.5 mg/kg and higher in females (Hirata-Koizumi et al., 2007). In the 52-week repeated-dose toxicity study with rats given HDBB by gavage at 0, 0.1, 0.5, or 2.5 mg/kg/d in males and 0, 0.5, 2.5, or 12.5 mg/kg/d in females, toxic effects were observed in the liver at 0.5 mg/kg/d and higher in males and 12.5 mg/kg/d in females (Hirata-Koizumi et al., 2008a).

It has been recognized that there are sex differences in the toxicity of chemical compounds in rats. A recent subchronic toxicity study showed that fluoranthene, a polycyclic aromatic hydrocarbon, had greater effects on males than females, especially in the kidney, in F344 rats (Knuckles et al., 2004). On the other hand, female rats exhibited a higher susceptibility to hypothermic effects and inhibition of hypothalamic cholinesterase by the carbamate cholinesterase inhibitor, rivastigmine (Wang et al., 2001). These findings suggest that sexual hormones may play an important role in sex differences in toxicity. It has already been shown that orchidectomy resulted in the complete ablation of the above-mentioned sex differences in hypothalamic cholinesterase inhibition induced by rivastigmine (Wang et al., 2001). Testosterone is likely to interfere with the effects of rivastigmine, because testosterone decreases cholinesterase inhibition in gonadectomized males and females. More recently, we showed that castration markedly reduced sex differences in the toxicity of HDBB in male and female rats (Hirata-Koizumi et al., 2008b). We also reported no sex differences in susceptibility to the toxic effects of HDBB in preweaning rats (Hirata-Koizumi et al., 2008c). It is important to investigate the role of sex steroids in the mediation of sex differences in susceptibility to DBHCB toxicity and to determine the toxic effects of DBHCB in preweaning rats. A repeated-dose toxicity study of DBHCB is currently in progress, using castrated and preweaning male and female rats.

To date, there has been no available data for human exposure to this chemical. Actual human exposure to DBHCB may be very low because it was not detected in polyethylenterephthalate bottles in Brazil (Monteiro et al., 1998) or polyethylene products in Japan (Kawamura et al., 1997). Consideration of these findings and the results of the present study together suggest that the human risk of adverse effects from DBHCB exposure is very low.

CONCLUSIONS

In conclusion, the administration of DBHCB during premating, mating, and pregnancy, as well as shortly after parturition, caused no changes in the reproductive function of male and female rats. DBHCB produced increases in the liver weight, albumin levels, and A/G ratio at 25 mg/kg/d and higher, as well as ALP levels at 250 mg/kg/d in males, but no change in females. These findings indicate a sex difference in the toxicity of DBHCB in rats.

ACKNOWLEDGMENT

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

REFERENCES

Chemical Land21 (2005). Benzotriazole anti-UV 327. Available online at: http://www.chemicalland21.com/specialtychem/finechem/BENZOTRIAZOLE%20ANTI%20UV%20327.htm. Accessed on October 4, 2007.

Ema, M., Fukunishi, K., Matsumoto, M., Hirose, A., Kamata, E. (2006). Evaluation of developmental toxicity of ultraviolet absorber 2-(3′,5′-di-tert-butyl-2′-hydroxyphenyl)-5-chlorobenzotriazole in rats. *Drug. Chem. Toxicol.* 29:215–225.

Everlight Chemical Industrial Corporation (2002). EVERSORB 75. Safety data sheet. Available online at: http://www.hansonco.net/EVERSORB-75-MSDS.pdf. Accessed on October 4, 2007.

FDA (U.S. Food and Drug Administration) (2000). Definitions of food types and conditions of use for food contact substances. Available online at: http://www.cfsan.fda.gov/~rdb/opa-fcn3.html. Accessed on October 4, 2007.

FDA (U.S. Food and Drug Administration) (2005a). Inventory of effective premarket notifications for food contact substances. Available online at: http://www.cfsan.fda.gov/~dms/opa-fcn.html. Accessed on October 4, 2007.

FDA (U.S. Food and Drug Administration) (2005b). Inventory of premarket notification limitations, specifications, and use for food contact substances. Available online at: http://www.cfsan.fda.gov/~dms/opa-fcn2.html. Accessed on October 4, 2007.

Hall, R. L. (2007). Clinical pathology of laboratory animals. In Gad, S. C., ed. *Animal Models in Toxicology*, 2nd ed. Boca Raton, Florida, USA: CRC Press (Taylor & Francis Group), pp. 787–830.

Hirata-Koizumi, M., Watari, N., Mukai, D., Imai, T., Hirose, A., Kamata, E., Ema, M. (2007). A 28-day repeated dose toxicity study of ultraviolet absorber 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole in rats. *Drug Chem. Toxicol.* 30: 327–341.

Hirata-Koizumi, M., Ogata, H., Imai, T., Hirose, A., Kamata, E., Ema, M. (2008a). A 52-week repeated dose toxicity study of ultraviolet absorber 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole in rats. *Drug Chem. Toxicol.* 31: 81–96.

Hirata-Koizumi, M., Matsuyama, T., Imai, T., Hirose, A., Kamata, E., Ema, M. (2008b). Gonadal influence on the toxicity of 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole in rats. *Drug Chem. Toxicol.* 31: 115–126.

Hirata-Koizumi, M., Matsuyama, T., Imai, T., Hirose, A., Kamata, E., Ema, M. (2008c). Lack of gender-related difference in the toxicity of 2-(2′-hydroxy-3′,5′-di-*tert*-butylphenyl)benzotriazole in preweaning rats. *Drug Chem. Toxicol.* 31: 275–287.

Kawamura, Y., Miura, M., Sugita, T., Tamada, T. (1997). Residue and release of antioxidants and ultraviolet stabilizers in polyethylene products in contact with foodstuffs. *Shokuhinn eiseigaku Zasshi* 38:27–33.

Kawamura, Y., Ogawa, Y., Nishimura, T., Kikuchi, Y., Nishikawa, J., Nishihara, T., Tanamoto, K. (2003). Estrogenic activities of UV stabilizers used in feed contact plastics and benzophenone derivatives tested by the yeast two-hybrid assay. *J. Health Sci.* 49:205–212.

Knuckles, M. E., Inyang, F., Ramesh, A. (2004). Acute and subchronic oral toxicity of fluoranthene in F-344 rats. *Ecotoxicol. Environ. Saf.* 59:102–108.

METI (Ministry of Economy, Trade and Industry, Japan) (2006). Research results on the actual production and import volume of chemicals in 2005 (in Japanese). Available online at: http://www.meti.go.jp/policy/chemical_management/kasinhou/kanshikagakubusshitsu/kanpo-1–20061010.pdf. Accessed on September 12, 2007.

Miller, D., Wheals, B. B., Beresford, N., Sumpter, J. P. (2001). Estrogenic activity of phenolic additives determined by an *in vitro* yeast bioassay. *Environ. Health Perspect.* 109:133–138.

Monteiro, M., Rubio, C. N., Reyes, F. G. R. (1998). A GC/MS method for detecting UV stabilizers in polyethyleneterephthalate bottles. *J. High Resol. Chromatogr.* 21:317–320.

OECD (Organization for Economic Co-operation and Development) (1996). OECD Guideline for the Testing of Chemicals, 422. Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test.

Tenkazai.com (2007). Market trend of Resin additives "Light stabilizer." Available online at: http://www.tenkazai.com/market.html. Accessed on October 4, 2007.

Wang, R. H., Schorer-Apelbaum, D., Weinstock, M. (2001). Testosterone mediates sex difference in hypothermia and cholinesterase inhibition by rivastigmine. *Eur. J. Pharmacol.* 433:73–79.

Drug and Chemical Toxicology, 31:473–486, 2008

ISSN: 0148-0545 print / 1525-6014 online
DOI: 10.1080/01480540802390627

informa
healthcare

Reproductive and Developmental Toxicity Screening Study of 4-Aminophenol in Rats

Tomoaki Harada,[1] Eisuke Kimura,[2] Mutsuko Hirata-Koizumi,[1] Akihiko Hirose,[1] Eiichi Kamata,[1] and Makoto Ema[1]

[1]Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, Tokyo, Japan
[2]Mitsubishi Chemical Safety Institute Ltd., Uto, Japan

Twelve male and female rats per group were given 4-aminophenol (PAP) by gavage at 0, 20, 100, or 500 mg/kg/day. Males were dosed for a total of 49 days, beginning 14 days before mating. Females were dosed for a total of 40–60 days, from 14 days before mating to Day 3 of lactation throughout the mating and gestation periods. Four males and 2 females died at 500 mg/kg/day, and all surviving males and females showed brown urine at 100 mg/kg/day and above. Body-weight gain was lower in males and females at 500 mg/kg/day, and food consumption was decreased in males at 500 mg/kg/day and in females at 100 and 500 mg/kg/day. Absolute and relative weights of the testes and epididymides were decreased at 500 mg/kg/day. Histopathological examinations revealed decreased spermatocyte and spermatid levels in the testis, debris of germ cell in the epididymis lumen, basophilic tubules in the kidney, and deposits of hemosiderin in the red pulp and extramedullary hematopoiesis in the spleen in males at 500 mg/kg/day. Longer gestation period, decreased delivery index, and lower body weight of pups on postnatal day (PND) 0 and increased number of stillborns at 500 mg/kg/day were also observed. At this dose, the viability of pups on PND 4 was decreased markedly. No adverse effects on reproduction or development were detected at 20 and 100 mg/kg/day. These findings indicate that PAP is general and reproductive/developmental toxic, but is unlikely to be teratogenic, in rats.

Keywords Reproductive and developmental toxicity, 4-Aminophenol, Dye, Testicular toxicity, Neonatal death, Rat.

Address correspondence to Makoto Ema, Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; E-mail: ema@nihs.go.jp

INTRODUCTION

4-aminophenol (CAS No. 123-30-8; PAP) has numerous applications, and more than 500 tons a year is produced in the United States (Scorecard, 2005) and a further 100 tons is produced in Japan (NITE, 2004). PAP is used industrially as a dye for textiles, fur, and feathers as a photographic developer (Haz-Map, 2007) and is generally used in hair dyes with other aminophenols. In pharmaceutics, PAP is known as a breakdown product of acetaminophen (paracetamol), which is widely used as an antipyretic and analgesic over-the-counter drug (Gemborys and Mudge, 1981; Newton et al., 1982). It has caused some anxiety that humans have been exposed to PAP in occupational surroundings, from environmental contamination and from consumer products.

The possibility of chemical compounds entering the biological system has aroused great concern about their toxic potential. It is generally assumed that the biological effects produced by chemical compounds should be studied in laboratory animals to investigate their possible influences on human health, and the results of toxicity studies of chemicals in animals are relevant to humans (Clayson and Krewski, 1990). However, little information on the toxicity of PAP, except for its nephrotoxicity, has been published. The Material Safety Data Sheet (MSDS) of this compound (Mallinckrodt Inc., 2003) noted that PAP is harmful if swallowed, inhaled, or absorbed through the skin and may cause methemoglobinemia. It is reported that the oral LD_{50} value of PAP was determined as 671 mg/kg in rats, and PAP caused mild conjunctival reaction and mild skin irritation in rabbits (Lloyd et al., 1977). PAP is also noted to be a fairly potent methemoglobin-producing agent in mice (Smith et el., 1967). In rats, no increase in the level of methemoglobin has been reported, other than slight reductions in total erythrocytes and hemoglobin in females fed a diet containing PAP at 0.7% for 13 weeks (Burnett et al., 1989).

There are many studies available concerning PAP-induced nephrotoxicity. The therapeutic dosage of acetaminophen was not toxic, but large overdoses produced an acute nephrotoxicity in rats (Newton et al., 1983). The toxic potential of PAP as a nephrotoxicant was stronger than acetaminophen in F344 rats (Newton et al., 1982, 1983, 1985). The nephropathy was also noted in male and female Sprague-Dawley (SD) rats given dietary PAP at 0.7% for 13 or 27 weeks (Burnett et al., 1989). The mechanism of PAP's nephrotoxicity has been investigated thoroughly, and its toxicity is known to be site-specific for the S3 segment of the proximal tubule (Green et al., 1969; Calder et al., 1971; Kiese et al., 1975; Newton et al., 1982; Gartland et al., 1989).

Although the areas of reproductive and developmental toxicology are becoming increasingly important parts of the overall toxicology profile for chemicals, only a few reports are available on the developmental toxicity of this compound. PAP was teratogenic in hamsters administered on Day 8 of pregnancy by intraperitoneal and intravenous injection, but not by gavage at

200 mg/kg/day (Rutkowski and Ferm, 1982). No teratogenicity was found in rats given PAP at up to 250 mg/kg/day by oral application on Days 6 to 15 of pregnancy (Spengler et al., 1986). Increased postimplantation loss, reduced fetal weight, and reduced ossification and increased skeletal variations in fetuses were observed at a dose that also induced lower maternal weight in rats given dietary PAP at 0.7% (equivalent to about 520 mg/kg/day) during the 13-week premating and pregnancy period (Burnett et al., 1989). The above toxicology reports on PAP can be regarded as not totally adequate for the toxicological assessment for PAP, because these studies were non–Good Laboratory Practice (GLP) studies or did not fully comply with a specific testing guideline (Klimisch et al., 1997; OECD, 2005). Therefore, PAP was selected as a target substance for the Safety Examination of Existing Chemicals in Japan to obtain reliable information on the possible effects on the reproduction and development in compliance with the OECD Test Guideline and in accordance with the principles for GLP. The present paper reports the results of reproductive/developmental toxicity screening tests of PAP in rats.

MATERIALS AND METHODS

This study was performed in compliance with OECD guideline 421 of the Reproduction/Developmental Toxicity Screening Test (OECD, 1995) and in accordance with the principles for GLP (OECD, 1998; EA, MHW, and MITI, 1988) and Guidance for Animal Care and Use of Mitsubishi Chemical Safety Institute Ltd. (Uto, Japan).

Animals

International Genetic Standard (Crj:CD(SD)IGS) rats were used throughout this study. This strain was chosen because it is most commonly used in studies on toxicity, including reproductive and developmental toxicity, and historical control data are available. Males and females at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, Inc. (Yokohama, Japan). The rats were acclimated to the laboratory for 13 days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Vaginal smears of each female were recorded, and only females showing a 4-day estrous cycle were used in the experiment. Male and female rats were distributed on a random basis into four groups of 12 males and 12 females each. Rats were housed individually, except during the acclimation, mating, and nursing periods. From Day 0 of pregnancy to the day of sacrifice, individual dams and litters were reared by using wooden chips (White Flake; Charles River Japan, Inc.) as bedding.

Animals were reared on a sterilized basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water *ad libitum* and maintained in an

air-conditioned room at 25 ± 2°C, with a relative humidity of 55 ± 5%, a 12-h light-dark cycle, and ventilation with 10–20 air changes per hour.

Chemicals and Dosing

PAP was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). PAP, a white or pale-yellow crystalline powder, is slightly soluble in water, alcohol, and ether, but is insoluble in chloroform and benzene. The melting point of PAP is 189.6–190.2°C, and it has a molecular weight of 109.13 (HSDB, 1996). The purity and stability of the chemical were verified by analysis before the study. The purity of PAP (Lot No. 044K0101) used in this study was 99.0%, and it was kept in the dark at room temperature. Rats were dosed once-daily by gastric intubation with PAP at a dose of 0 (control), 20, 100, or 500 mg/kg. The dosage levels were determined based on the results of a previous 28-day repeated-dose toxicity study in rats given PAP by gavage at 0 (vehicle), 4, 20, 100, or 500 mg/kg/day (JECDB, 1995). At 500 mg/kg/day, the death in 1 male with renal necrosis, decreases in body-weight gain, food consumption, erythrocyte count, hematocrit value (Ht) and hemoglobin content (Hb), increased relative weight of the liver and spleen, basophilic tubules in the kidney, and brown urine were observed. At 100 mg/kg/day, brown urine and basophilic tubules in the kidney were also observed. No toxicological effects were detected at 4 and 20 mg/kg/day.

PAP was suspended in 0.5% (w/v) carboxymethylcellulose-Na solution. Males were dosed for a total of 49 days from 14 days before mating. Females were dosed for a total of 40–60 days, beginning 14 days before mating to Day 3 of lactation throughout the mating and gestation period. The volume of each dose was adjusted to 10 mL/kg body weight based on the latest body-weight measurement during the premating and mating period in males and females or the body weight on Day 0 of pregnancy in females after copulation. Control rats were given 0.5% (w/v) carboxymethylcellulose-Na solution. The stability of formulations in the dark at room temperature has been confirmed for up to 6 h. The formulations were prepared just before use and were used within 6 h.

Observations

All rats were observed daily for clinical signs of toxicity. Body weight was recorded twice a week in males, and twice a week during the premating and mating periods, on Days 0, 7, 14, and 20 of pregnancy and on Days 0 and 4 of lactation in females. Food consumption was determined twice a week during the premating period in males, and twice a week during the premating period, on Days 1, 7, 14, and 20 of pregnancy, and on Days 1 and 4 of lactation in females.

Rats were euthanized by exsanguination under anesthesia on the day after the last administration in males and on Day 4 of lactation in females.

The external surfaces of the rats were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. In males, the testes and epididymides were weighed. In females, the numbers of corpora lutea and implantation sites and weight of the ovaries were recorded. The testis and epididymis were fixed with Bouin's solution and preserved in 10% neutral buffered formalin, and the ovary was stored in 10% neutral buffered formalin. Histopathological evaluations were performed on hematoxylin-eosin-stained tissue sections of the testis, epididymis, and ovary in the control and highest dose groups, and the liver, spleen, and kidney were examined for gross alterations. The testis and epididymis of the 100-mg/kg/day group were also examined, since test-substance-related changes were found in the highest group.

Daily vaginal lavage samples of each female were evaluated for estrus cycle throughout the premating period. Each female rat was mated overnight with a single male rat of the same dosage group until copulation occurred or the mating period (2 weeks) had elapsed. During the mating period, daily vaginal smears were examined for the presence of sperm. The presence of the sperm in the vaginal smear and/or a vaginal plug was considered evidence of successful mating, and this day was designated as Day 0 of pregnancy.

Once insemination was confirmed, the females were checked for signs of parturition before noon from Day 20 of pregnancy. The day on which parturition was completed by 12:00 was designated as postnatal day (PND) 0. The females were allowed to deliver spontaneously and nurse their pups until PND 4. Litter size and numbers of live and dead pups were recorded. Pups were sexed, examined grossly, and weighed individually on PNDs 0 and 4. On PND 4, the pups were euthanized by exsanguination under anesthesia and gross internal examinations were performed.

Data Analysis

The statistical analysis of pups was carried out by using the litter as the experimental unit. The body weight, body-weight gain, food consumption, count of estrus, precoital interval, gestation length, weight of the organs, relative organ weight, numbers of corpora lutea, implantations and live and dead pups, total number of pups, and weight of live pups were analyzed by using Bartlett's test for homogeneity of variance at the 5% level of significance. If homogeneous, the data were analyzed by using Dunnett's multiple comparison test to compare the mean of the control group with that of each dosage group. If not, the PAP-treated groups were compared with that of the control group by using Steel's multiple comparison test. The implantation, delivery index, viability index, and rate of stillborn pups were analyzed by using Wilcoxon's rank sum test. The copulation, fertility and gestation indexes, sex ratio of pups, and females showing abnormal estrus cycles were analyzed by

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