Table 7
Organ weight of male F1 adults

	DCBS (ppm)			
	0 (control)	80	600	4500
No. of male F1 adults examined	24	24	24	24
Body weight (g)	630.7 ± 74.7[a]	605.1 ± 47.7	614.2 ± 52.5	622.6 ± 51.8
Brain (g)	2.26 ± 0.10[c]	2.29 ± 0.06	2.26 ± 0.06	2.21 ± 0.09*
	0.363 ± 0.038[d]	0.380 ± 0.028	0.370 ± 0.030	0.356 ± 0.027
Pituitary gland (mg)	13.6 ± 1.4	13.9 ± 1.3	13.9 ± 1.1	14.0 ± 1.6
	2.17 ± 0.23[d]	2.30 ± 0.25	2.27 ± 0.17	2.26 ± 0.26
Thyroid[b] (mg)	24.9 ± 4.9	23.3 ± 4.7	23.8 ± 4.5	24.6 ± 4.9
	3.95 ± 0.66[d]	3.86 ± 0.78	3.88 ± 0.69	3.95 ± 0.67
Thymus (mg)	346 ± 116	269 ± 54*	331 ± 83	316 ± 62
	54.8 ± 17.0[d]	44.5 ± 8.9*	53.9 ± 12.7	50.9 ± 9.8
Liver (g)	20.80 ± 3.73	19.69 ± 2.32	21.19 ± 2.06	22.82 ± 3.37*
	3.28 ± 0.29 [d]	3.25 ± 0.19	3.46 ± 0.28	3.65 ± 0.28**
Kidney[b] (g)	3.70 ± 0.52	3.66 ± 0.23	3.69 ± 0.36	3.91 ± 0.43
	0.586 ± 0.041[d]	0.606 ± 0.042	0.602 ± 0.047	0.629 ± 0.044**
Spleen (mg)	909 ± 129	845 ± 141	847 ± 124	869 ± 162
	145 ± 16[d]	139 ± 18	138 ± 17	139 ± 17
Adrenal[b] (mg)	60.5 ± 9.8	60.3 ± 7.1	61.8 ± 7.2	61.3 ± 13.1
	9.6 ± 1.5[d]	10.0 ± 1.0	10.1 ± 1.3	9.8 ± 2.0
Testis[b] (g)	3.60 ± 0.35	3.61 ± 0.27	3.60 ± 0.27	3.78 ± 0.32
	0.575 ± 0.062[d]	0.601 ± 0.073	0.589 ± 0.066	0.610 ± 0.062
Epididymis[b] (mg)	1348 ± 138	1342 ± 67	1327 ± 111	1346 ± 118
	215 ± 24[d]	223 ± 21	217 ± 22	217 ± 19
Seminal vesicle (g)	2.30 ± 0.23	2.19 ± 0.28	2.21 ± 0.22	2.07 ± 0.26**
	0.368 ± 0.047[d]	0.364 ± 0.054	0.362 ± 0.039	0.333 ± 0.045*
Ventral prostate (mg)	838 ± 174	812 ± 181	822 ± 190	784 ± 168
	133 ± 24[d]	134 ± 28	134 ± 29	127 ± 31

[a] Values are given as the mean ± S.D.
[b] Values are represented as the total weights of the organs of both sides.
[c] Absolute organ weight.
[d] 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, $p < 0.01$.

serum hormone levels of female F1 adults between the control and DCBS-treated groups.

3.12. Sperm parameters (F0 and F1 adults)

Table 10 shows the sperm parameters in F0 and F1 adult males. No significant changes in sperm counts, percentage of motile sperm and progressively motile sperm, swimming speed and pattern, or percentage of morphologically abnormal sperm were noted in F0 adults between the control and DCBS-treated groups. A significant decrease in the mean lateral head displacement was found at 4500 ppm in F1 males.

4. Discussion

A two-generation reproductive toxicity study was performed to further evaluate the potential effects of DCBS on reproduction and development in rats.

The deaths and clinical signs observed in the present study are not thought to be attributable to the administration of DCBS, because the incidences of deaths and clinical signs were very low and inconsistent across generations, and these occurrences are not uncommon in toxicological studies.

The decreased food consumption in F0 males and females at 4500 ppm was accompanied by decreases in the body weight and body weight gain. However, lowered food consumption in F1 males at 80, 600 and 4500 ppm was occasional, inconsistent, and unaccompanied by changes in body weight or body weight gain. It seems likely that DCBS adversely affects the body weight and food consumption in F0 rats at 4500 ppm, but not in F1 rats.

Although a few F0 and F1 adults showed reproductive difficulties, necropsy and the histopathology of reproductive organs revealed no evidence of reproductive failure in these rats. Two F1 females showing abnormal estrous cycles remained in diestrus for 10–11 days, suggesting they were pseudopregnant. No significant changes in reproductive indices were noted in any

Table 8
Organ weight of female F1 adults

	DCBS (ppm)			
	0 (control)	80	600	4500
No. of female F1 adults examined	22	22	21	23
Body weight (g)	331.9 ± 32.5[a]	331.2 ± 28.5	331.3 ± 23.1	330.2 ± 30.8
Brain (g)	2.08 ± 0.08[c]	2.17 ± 0.08**	2.15 ± 0.08*	2.08 ± 0.08
	0.632 ± 0.056[d]	0.658 ± 0.056	0.651 ± 0.043	0.633 ± 0.060
Pituitary gland (mg)	15.9 ± 2.0	16.1 ± 2.4	15.8 ± 1.8	16.1 ± 1.9
	4.83 ± 0.73[d]	4.90 ± 0.79	4.78 ± 0.52	4.89 ± 0.66
Thyroid[b] (mg)	19.0 ± 3.9	18.2 ± 2.7	17.7 ± 3.5	19.4 ± 4.1
	5.72 ± 0.98[d]	5.51 ± 0.70	5.35 ± 1.08	5.89 ± 1.15
Thymus (mg)	251 ± 69	212 ± 47	261 ± 65	211 ± 63
	75.3 ± 18.4[d]	64.1 ± 14.2	79.2 ± 20.2	64.0 ± 18.7
Liver (g)	14.55 ± 1.66	14.18 ± 2.14	14.32 ± 1.49	15.83 ± 2.11
	4.39 ± 0.28[d]	4.28 ± 0.49	4.33 ± 0.41	4.81 ± 0.59**
Kidney[b] (g)	2.37 ± 0.30	2.39 ± 0.22	2.40 ± 0.21	2.53 ± 0.26
	0.713 ± 0.046[d]	0.723 ± 0.040	0.726 ± 0.063	0.771 ± 0.080**
Spleen (mg)	632 ± 73	599 ± 63	609 ± 80	639 ± 115
	191 ± 18[d]	181 ± 15	184 ± 19	194 ± 37
Adrenal[b] (mg)	70.0 ± 9.7	73.5 ± 10.9	73.4 ± 9.3	77.5 ± 8.9*
	21.2 ± 3.2[d]	22.2 ± 3.1	22.2 ± 3.0	23.6 ± 3.2*
Ovary[b] (mg)	110.6 ± 13.0	109.1 ± 16.3	108.5 ± 12.5	108.2 ± 13.4
	33.4 ± 2.9[d]	33.0 ± 4.5	32.8 ± 3.2	32.8 ± 3.3
Uterus (mg)	927 ± 191	928 ± 128	976 ± 185	949 ± 192
	280 ± 54[d]	283 ± 48	295 ± 52	288 ± 52

[a] Values are given as the mean ± S.D.
[b] Values are represented as the total weights of the organs of both sides.
[c] Absolute organ weight.
[d] 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, $p < 0.01$.

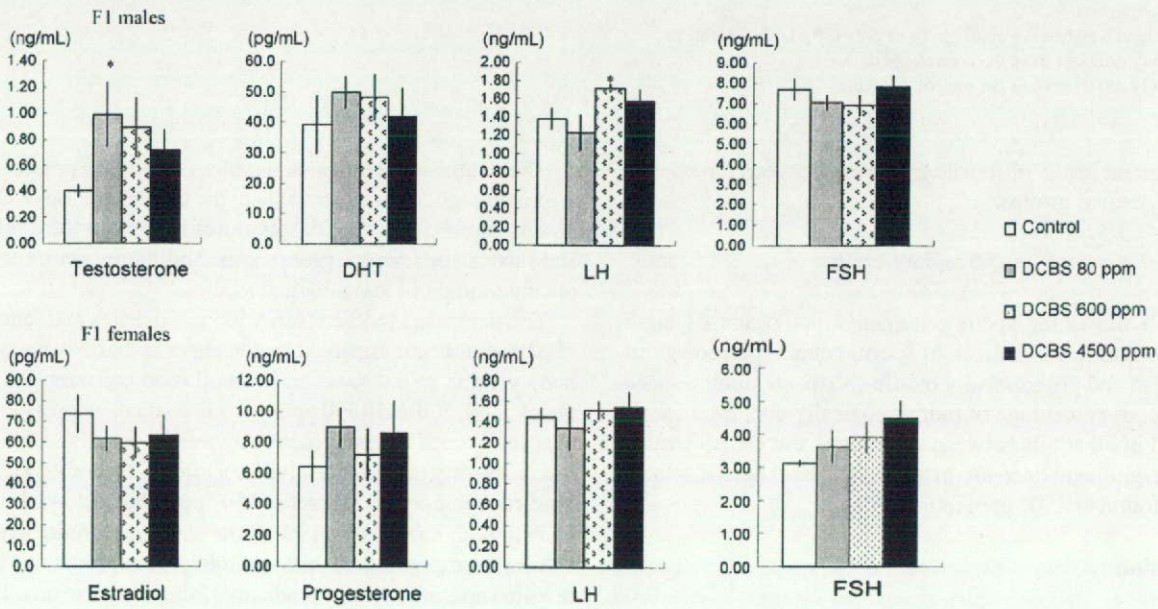

Fig. 4. Serum hormone levels in F1 males and females. The actual measurement of DHT was below the lower limit of quantification (<25.0 pg/mL) in one F1 male each in the control and 4500 ppm groups. The actual measurement of LH was below the lower limit of quantification (<0.80 ng/mL) in one F1 male and one F1 female in the 80 ppm group. Values are given as the mean ± S.E.M. *Significantly different from the control, $p < 0.05$.

Table 9
Organ weight of male and female F2 weanlings

	DCBS (ppm)			
	0 (control)	80	600	4500
No. of male F2 weanlings examined	22	22	21	23
Body weight (g)	90.8 ± 8.7[a]	91.4 ± 13.1	89.1 ± 6.5	80.0 ± 7.8**
Brain (g)	1.67 ± 0.08[c]	1.69 ± 0.09	1.70 ± 0.08	1.63 ± 0.09
	1.85 ± 0.14[d]	1.87 ± 0.22	1.92 ± 0.13	2.05 ± 0.18**
Thymus (mg)	355 ± 56	325 ± 64	361 ± 47	283 ± 52**
	392 ± 58[d]	355 ± 47	406 ± 48	354 ± 55*
Liver (g)	4.08 ± 0.47	4.12 ± 0.66	4.21 ± 0.44	3.74 ± 0.54
	4.49 ± 0.23[d]	4.50 ± 0.26	4.72 ± 0.27**	4.66 ± 0.42**
Kidney[b] (g)	1.006 ± 0.102	1.009 ± 0.147	1.022 ± 0.089	0.923 ± 0.133
	1.109 ± 0.037[d]	1.105 ± 0.066	1.146 ± 0.049*	1.152 ± 0.117*
Spleen (mg)	383 ± 61	350 ± 83*	356 ± 46	286 ± 52**
	422 ± 49[d]	381 ± 53*	400 ± 50	357 ± 54**
Adrenal[b] (mg)	25.5 ± 2.8	24.1 ± 3.4	24.2 ± 3.8	22.7 ± 3.4*
	28.1 ± 2.8[d]	26.5 ± 2.6	27.2 ± 4.3	28.5 ± 4.2
Testis[b] (mg)	548 ± 106	516 ± 103	528 ± 82	525 ± 98
	602 ± 89[d]	563 ± 69	590 ± 65	653 ± 91
Epididymis[b] (mg)	79.5 ± 12.5	72.9 ± 12.3	72.7 ± 10.0	71.3 ± 11.2
	87.6 ± 10.6[d]	80.4 ± 12.6	81.7 ± 10.6	89.0 ± 10.0
Ventral prostate (mg)	50.9 ± 16.6	44.6 ± 10.4	47.0 ± 10.3	42.6 ± 12.2
	55.6 ± 15.0[d]	48.9 ± 9.9	52.7 ± 10.1	52.9 ± 13.1
No. of female F2 weanlings examined	22	22	20	23
Body weight (g)	83.6 ± 9.5	87.2 ± 10.8	82.4 ± 6.5	74.6 ± 7.9**
Brain (g)	1.62 ± 0.08	1.66 ± 0.07	1.66 ± 0.05	1.57 ± 0.07*
	1.96 ± 0.20[d]	1.92 ± 0.19	2.03 ± 0.16	2.11 ± 0.18*
Thymus (mg)	364 ± 50	326 ± 66	348 ± 68	283 ± 56**
	439 ± 63[d]	373 ± 56**	424 ± 80	379 ± 63**
Liver (g)	3.71 ± 0.47	3.87 ± 0.50	3.80 ± 0.37	3.57 ± 0.46
	4.44 ± 0.18[d]	4.44 ± 0.23	4.61 ± 0.19*	4.78 ± 0.26**
Kidney[b] (g)	0.915 ± 0.093	0.983 ± 0.137	0.960 ± 0.111	0.885 ± 0.101
	1.096 ± 0.046[d]	1.129 ± 0.085	1.164 ± 0.083**	1.187 ± 0.061**
Spleen (mg)	340 ± 63	331 ± 55	320 ± 46	274 ± 40**
	407 ± 58[d]	380 ± 46	389 ± 56	370 ± 52
Adrenal[b] (mg)	23.6 ± 2.9	23.3 ± 4.0	22.2 ± 3.3	21.6 ± 3.0
	28.4 ± 3.3[d]	26.7 ± 3.3	27.0 ± 4.0	29.0 ± 3.9
Ovary[b] (mg)	22.0 ± 3.9	22.5 ± 2.8	20.9 ± 3.1	21.4 ± 2.9
	26.6 ± 5.2[d]	26.0 ± 3.0	25.5 ± 4.4	29.0 ± 4.3
Uterus (mg)	61.8 ± 18.9	58.1 ± 11.9	50.0 ± 10.0*	46.6 ± 12.9**
	73.3 ± 17.2[d]	67.0 ± 13.5	60.7 ± 11.5*	62.3 ± 15.0*

[a] Values are given as the mean ± S.D.
[b] Values are represented as the total weights of the organs of both sides.
[c] Absolute organ weight.
[d] 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, $p < 0.01$.

generation even at the highest dose of 4500 ppm. Our previous screening test revealed that DCBS given by gavage to rats from day 14 before mating to day 3 of lactation caused significant decreases in the gestation index, numbers of corpora lutea, implantations, pups born and pups born alive, live birth index, and viability index at 400 mg/kg bw per day [10]. This dose also caused severe maternal toxicity and a total loss of pups until PND 4. No maternal or reproductive/developmental toxicity was detected at 100 mg/kg bw per day and below in our previous study. In the present feeding study, the mean daily intakes of DCBS were 416 and 417 mg/kg bw per day for the highest dose in F0 and F1 females, respectively. Consideration of these

Table 10
Sperm parameters in F0 and F1 males

	DCBS (ppm)			
	0 (control)	80	600	4500
F0 males				
No. of animals	24	23	24	24
No. of testicular sperm ($\times 10^6$)				
Per testis	184.1 ± 29.3[a]	187.7 ± 28.3	184.2 ± 32.7	180.8 ± 35.4
Per g testis	111.4 ± 13.2	110.7 ± 15.7	110.6 ± 17.1	106.1 ± 18.8
No. of epididymal sperm ($\times 10^6$)				
Per cauda	268.5 ± 47.6	276.2 ± 40.3	269.9 ± 56.8	263.7 ± 62.8
Per g cauda	856.4 ± 94.4	838.9 ± 99.4	850.3 ± 122.1	844.2 ± 191.3
Percent motile	88.1 ± 9.3	92.6 ± 8.2	93.2 ± 5.9	89.4 ± 10.2
Percent progressive	70.9 ± 17.4	77.3 ± 15.3	77.4 ± 12.1	70.5 ± 22.2
Mean path velocity (μm/s)	159.6 ± 20.8	159.8 ± 19.2	162.7 ± 22.0	156.8 ± 25.3
Straight line average velocity (μm/s)	112.1 ± 22.5	114.1 ± 20.0	116.1 ± 19.3	110.5 ± 29.2
Mean curvilinear velocity (μm/s)	365.7 ± 53.4	370.1 ± 42.5	372.3 ± 49.8	358.4 ± 56.3
Mean lateral head displacement (μm)	20.1 ± 1.1	19.9 ± 1.1	20.0 ± 1.3	19.9 ± 1.0
Mean beat cross frequency (Hz)	27.9 ± 1.5	27.4 ± 1.5	27.6 ± 2.2	28.3 ± 2.3
Mean straightness (%)[b]	69.3 ± 6.6	70.7 ± 5.7	71.0 ± 4.3	69.5 ± 8.6
Mean linearity (%)[c]	30.4 ± 2.8	30.7 ± 3.0	31.3 ± 2.5	30.6 ± 4.0
Total abnormal sperm ratio (%)	1.1 ± 0.6	1.2 ± 0.8	2.4 ± 3.5	2.0 ± 2.4
Tailless sperm (%)	1.0 ± 0.6	1.2 ± 0.8	2.2 ± 3.5	1.8 ± 2.0
F1 males				
No. of animals	24	24	24	24
No. of testicular sperm ($\times 10^6$)				
Per testis	194.5 ± 23.0[a]	181.1 ± 21.3	186.3 ± 22.5	201.0 ± 33.3
Per g testis	115.3 ± 9.5	108.4 ± 14.3	111.1 ± 11.3	113.6 ± 15.0
No. of epididymal sperm ($\times 10^6$)				
Per cauda	273.6 ± 40.0	254.0 ± 40.4	256.2 ± 46.0	250.3 ± 55.4
Per g cauda	849.9 ± 69.4	821.5 ± 106.8	827.2 ± 93.3	807.0 ± 127.5
Percent motile	92.3 ± 5.0	92.9 ± 4.0	93.3 ± 5.6	93.0 ± 7.4
Percent progressive	81.8 ± 8.1	81.8 ± 4.9	83.9 ± 6.4	82.7 ± 8.2
Mean path velocity (μm/s)	175.2 ± 9.8	171.7 ± 11.2	172.4 ± 11.4	171.3 ± 13.9
Straight line average velocity (μm/s)	126.9 ± 10.2	123.9 ± 10.3	126.0 ± 10.5	125.7 ± 12.6
Mean curvilinear velocity (μm/s)	399.5 ± 19.8	391.5 ± 28.6	395.1 ± 28.6	393.6 ± 29.8
Mean lateral head displacement (μm)	21.3 ± 0.9	20.9 ± 0.8	20.8 ± 0.8	20.5 ± 1.0*
Mean beat cross frequency (Hz)	26.4 ± 1.6	26.8 ± 1.4	26.1 ± 1.6	27.0 ± 1.8
Mean straightness (%)[b]	72.5 ± 3.3	72.1 ± 2.7	73.3 ± 2.9	73.5 ± 2.8
Mean linearity (%)[c]	32.0 ± 2.1	31.9 ± 2.0	32.1 ± 1.8	32.2 ± 1.5
Total abnormal sperm ratio (%)	1.4 ± 1.3	1.1 ± 0.8	1.2 ± 1.7	1.6 ± 1.9
Tailless sperm (%)	1.3 ± 1.2	0.9 ± 0.8	1.0 ± 1.6	1.5 ± 1.8

[a] Values are given as the mean ± S.D.
[b] Mean straightness (%) = straight line average velocity/mean path velocity × 100.
[c] Mean linearity (%) = straight line average velocity/mean curvilinear velocity × 100.
* Significantly different from the control, $p < 0.05$.

findings suggests that the highest dose of DCBS in the present study may be very close to the dose that induces severe maternal and reproductive toxicity. However, the possibility remains that the difference in the degree of toxicity may be due to differences in administration method. There are some examples showing that gavage and feed administration result with differences in the toxicokinetics of chemicals [20,21]. Further studies are needed to clarify the relationship between maternal and reproductive/developmental toxicity.

Regarding developmental parameters, lowered body weights of male and female pre-weaning F1 and F2 pups were noted at 4500 ppm. These findings indicate that the dose level of 4500 ppm used in this study was potent enough to have adverse effects on the growth of pups. It is noted that there are strong correlations between developmental landmark parameters and pup body weight data, and that pup body weight data is consistently a more sensitive indicator of the developmental status of the offspring [22,23]. Although delayed completion of incisor eruption was noted in male and female F1 pups at 80 ppm and in male and female F2 pups at 80 and 4500 ppm, the delayed completion of incisor eruption was not dose-dependent and the difference from the control value was very slight. Therefore, it is unlikely that the delay of incisor eruption observed in the present study was compound-related or toxicologically significant. There were

no significant changes in indices of pre-weaning functional development in the DCBS-treated groups. The AGD is also a developmental landmark for the differentiation of the external genitalia and is commonly used as a hormonally sensitive parameter of sexual differentiation in rodents [24]. The AGD per cube root of body weight ratio provides a more appropriate adjustment when it is necessary to normalize AGD to body weight [17]. No changes were observed in the AGD per cube root of body weight ratio at any doses of DCBS in any generation. The data on the AGD indicate a lack of effect of DCBS on AGD. These findings on pre-weaning developmental parameters suggest that DCBS adversely affects the growth of offspring, but not the pre-weaning landmarks of development or reflex ontogeny. An increase in the frequency of fetuses with internal hydrocephalus was reported in rats given *N*-cyclohexyl-2-benzothiazolesulfenamide, a structurally similar compound, during organogenesis in rats [25]. However, no significantly increased incidence of pups with anomalies was detected even at the highest dose in the present and previous studies of DCBS [10,15]. Regarding post-weaning landmarks of development, delays of preputial separation at 4500 ppm and vaginal opening at 600 and 4500 ppm were observed in the present study. Although the body weight at the age of preputial separation was not different between the control and DCBS-treated groups, a higher body weight at the age of vaginal opening was found at 600 and 4500 ppm in females. Preputial separation and vaginal opening indicate the onset of sexual maturity, and the body weight is correlated with the occurrence of these events [23]. Ashby and Lefevre [26] described that delays in preputial separation can only be interpreted with confidence when they are not accompanied by losses in body weight, or when the expected delay in preputial separation due to a loss of body weight has been exceeded. They also noted that measurement of delays in preputial separation may be of value in cases of large delays, but delays of 1–2 days are difficult to interpret with confidence [26]. In the present study, the delay of preputial separation at 4500 ppm was slight (1.5 days) and was not accompanied by a change in body weight, and the age of preputial separation was within the range of the background control data (40.3–42.8 days) for the last seven years in the laboratory performed current study. It is likely that the delay in preputial separation at 4500 ppm is related to general delays in development. In female rats, the age at vaginal opening is the most commonly measured marker of puberty, and vaginal opening is an estrogen-dependent event that results from an increase in the blood estradiol levels [27]. Although the delay of female vaginal opening at 600 and 4500 ppm was slight (1.5–1.6 days), the age of vaginal opening was over the range of the background control data (29.6–31.0 days) for the last seven years in the laboratory performed present study. In the present study at 600 and 4500 ppm, the heavier body weight was noted at the completion of vaginal opening. Therefore, the possibility that the delay in vaginal opening may have toxicological meaning is not completely ruled out. Other hormone-dependent events including estrous cyclicity and AGD, as well as serum hormone levels at the scheduled terminal necropsy were not changed in the DCBS-treated groups. Moreover, DCBS did not affect the reproductive performance. However, decreased weight of the uterus was found in F1 weanlings at 4500 ppm and F2 weanlings at 600 and 4500 ppm. It has been noted that variations in the weights of the reproductive organs, which are strongly dependent on endocrine status, can be considered a key parameter in the identification of endocrine effects [28–30]. These findings suggest that DCBS may have endocrine effects. Further studies are needed to clarify the effects of DCBS on endocrine endpoints.

Regarding the behavioral tests, the only significant change in the T-maze test was observed in females on day 2 of the test. Longer elapsed times at 600 and 4500 ppm and more errors at 4500 ppm were detected in females. There are behavioral functions not classically hormone-mediated and expressed by both sexes such as learning capacities, exploration activity, novelty seeking and anxiety levels that show both qualitative and quantitative differences in the two sexes [30]. The reduced activity, as well as the other effects on neuromuscular function, could be at least partially the result of lower body weight [31] and it has been found that light body weight caused worse performance in a learning task [32]. In the present study, the spontaneous activity, swimming ability in the straight channel and body weight at the time of the T-maze test was not different in F1 females between the control and DCBS-treated groups. Thus, it seems likely that DCBS may have transiently affected learning ability in the T-maze at the highest dose administered.

The changes in weight of the organs, such as the brain, thymus, kidney, and spleen that were observed at 80 and/or 600 ppm are not thought to be due to administration of DCBS, because changes occurred sporadically and not in a dose-dependent manner. The changes in the weights of the adrenal, thyroid, and male and female reproductive organs, except for the uterus, at 600 and/or 4500 ppm seem unlikely to be attributable to administration of DCBS because of inconsistent changes across ages, sexes and generations. No consistent DCBS-related effects on serum hormone levels or sperm parameters were also detected across generations. Decreased absolute weights and/or increased relative weights of the liver except for in female F0 adults, the spleen in F0 adults, and the brain and kidney in F0 and F1 adults and F1 and F2 weanlings at 4500 ppm seem to be due to secondarily lowered body weight, but not due to the direct effects of DCBS on the organs. Decreased absolute and/or relative weights of the thymus and spleen in the weanlings are supported by the results of our previous study in which atrophy of the thymus and spleen was observed at 400 mg/kg bw per day [10]. These findings may suggest that one of target systems of DCBS toxicity is the immune system in weanlings. In the present study, however, no DCBS-related histopathological changes were detected. The discrepancy in histopathological findings between the previous and present studies could be explained by a difference in the toxicokinetics of chemicals due to differences in administration method. No DCBS-related findings were found in the hematological and blood biochemical examinations. In general, the effects of DCBS on organ weights were more pronounced in weanlings than adults. These phenomena suggest that DCBS may be more toxic before weaning than after weaning, and this possibility is supported by the lowered body weight of pups during the pre-weaning period, but not post-weaning.

Table 11
Summary of relevant findings in rat two-generation reproductive toxicity study of DCBS (80, 600 and 4500 ppm)

	F0						F1						F2					
	Male			Female			Male			Female			Male			Female		
	80	600	4500	80	600	4500	80	600	4500	80	600	4500	80	600	4500	80	600	4500
Lowered body weight			+			+			+			+			+			+
Decreased food consumption			+			+												
Delayed vaginal opening											+	+						
Worse performance in water T-maze											+	+						
Reduced spleen weight									+			+			+			+
Reduced thymus weight									+			+			+			+
Reduced uterine weight												+					+	+

In conclusion, the results of the two-generation reproductive toxicity study described here provide a more comprehensive toxicity profile of DCBS than has been previously reported. Relevant findings obtained from the present rat two-generation reproductive toxicity study of DCBS are summarized in Table 11. The NOAEL in the present study is considered to be 80 ppm (5.2 mg/kg bw per day) in rats.

Acknowledgment

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ORIGINAL ARTICLE

Evaluation of reproductive and developmental toxicity of the rubber accelerator N,N-dicyclohexyl-2-benzothiazolesulfenamide in rats

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ABSTRACT **Male and female Crl:CD(SD) rats were fed a diet containing the rubber accelerator N,N-dicyclohexyl-2-benzothiazolesulfenamide (DCBS) at 0, 1500, 3000, 6000 or 10 000 p.p.m. (0, 83, 172, 343 or 551 mg/kg bw/day in males and 0, 126, 264, 476 or 707 mg/kg bw/day in females) for a total of 57 days beginning 16 days before mating in males, and a total of 61–65 days from 16 days before mating to day 21 of lactation in females. Body weight gains and food consumption were reduced in males at 6000 p.p.m. and higher and in females at 3000 p.p.m. and higher. The weights of the spleen at 6000 and 10 000 p.p.m. and of the thymus at 10 000 p.p.m. were decreased in females. No changes in estrous cyclicity, copulation index, fertility index, gestation index, delivery index, precoital interval or gestation length were observed at any dose of DCBS. Numbers of implantations at 6000 and 10 000 p.p.m. and pups delivered at 10 000 p.p.m. were reduced. There were no changes in the sex ratio or viability of pups. The body weights of male and female pups were lowered at 6000 p.p.m. and higher. Decreased weight of the spleen in weanlings was also observed in males at 1500 p.p.m. and higher and in females at 3000 p.p.m. and higher. The data indicate that DCBS possesses adverse effects on reproduction and development in rats.**

Key Words: developmental toxicity, N, N-dicyclohexyl-2-benzothiazolesulfenamide, rat, reproductive toxicity, rubber accelerator

INTRODUCTION

Sulfenamide accelerator compounds are widely used in the manufacture of automotive compartments and industrial rubber products such as tires, hoses, conveyer belts, bushings seals, gaskets and windshield wiper blades (EPA 2001). N,N-Dicyclohexyl-2-benzothiazolesulfenamide (DCBS, Fig. 1) is a sulfenamide accelerator. The annual production level of DCBS in Japan was approximately 1000 tons in 1990–1993 and 1900 tons in 2000–2003. Most of this amount was sold and handled domestically (OECD 2007). DCBS is used as an accelerator of vulcanization and is completely reacted in the vulcanizing process (OECD 2007). DCBS is regulated in Germany for use in articles that contact food, but is not regulated by the United States Food and Drug Administration for use in food contact applications (Flexsys 2000). Exposure of workers handling sulfenamide accelerator materials is likely to be highest in the area of materials packaging. During material packout at the manufacturing site, and to a lesser degree during weigh-up activities at the consumer site, there is a possibility of skin and inhalation exposure. Although consumer exposure should be minimal, the most likely route of consumer exposure is skin contact with rubber or latex articles (EPA 2001).

Only up to 6% biodegradation has been determined for DCBS in a ready biodegradability test, and a measured log Kow value of 4.8 suggests that DCBS may have a high bioaccumulation potential (OECD 2007). The possibility of such a chemical compound entering biological systems has aroused great concern regarding its toxicological potential. Generally, biological effects of chemicals should be studied in laboratory animals to investigate their possible influences on human health, and the results of animal tests of chemical toxicity relevant to humans (Clayson & Krewski 1990). However, very little information on the toxicity of DCBS has been published. The toxic effects of DCBS have been briefly summarized by the European Chemical Bureau (2000) and US EPA (2001). It was reported that the oral LD50 values were 1077–10 000 mg/kg bw in rats, the oral NOAEL for 44-day repeated dose toxicity was higher than 100 mg/kg bw/day in rats, and no effects on reproduction were observed at doses up to 400 mg/kg bw/day in rats (EPA 2001). The oral LD50 value was 8500 mg/kg bw in male mice, and repeated daily inhalation exposure of male rats for 15 days at 2 h/day and 350–400 mg/m^3 caused mucous membrane irritation (Vorobera 1969).

The Japanese Government (MHW 1998) conducted toxicity studies for DCBS, including acute toxicity, *in vitro* genotoxicity and repeat dose toxicity combined with reproductive/developmental toxicity as a part of the Safety Examination of Existing Chemical Substances and Chemical Safety Programmes. These toxicity studies are summarized in the IUCLID Data Sets (EPA 2006), OECD Screening Information Data Sets (OECD 2007) and the Hazard Assessment Sheet (CERI 2002). We previously reported the results of a screening test for repeat dose toxicity combined with a reproductive/developmental toxicity in rats, where DCBS at 400 mg/kg bw/day had a deleterious effect on reproduction and development and caused a marked decrease in the number of live pups as well as a total loss of pups by postnatal day (PND) 4 (Ema *et al.* 2007). The primary effects may be on the gestation index for dams and live birth index for pups, both of which appear to be affected at multiple points along the female reproductive process. The viability of neonatal pups may also be affected. To examine the adverse effect of dietary DCBS on survival and growth of pups, a reproductive and developmental toxicity study was performed in rats given DCBS during an extended administration period up to the weaning of pups.

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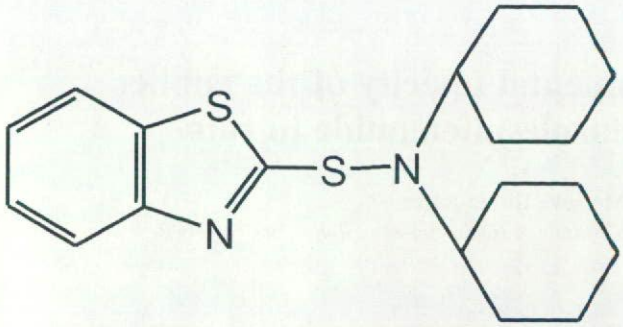

Fig. 1 Structural formula of N,N-dicyclohexyl-2-benzothiazolesulfenamide.

MATERIALS AND METHODS

This study was performed in 2005–2006 at the Safety Research Institute for Chemical Compounds (Sapporo, Japan) in compliance with *Law for the Humane Treatment and Management of Animals* (Law no. 105, October 1, 1973, revised December 22, 1999, Revised Law no. 221; revised June 22, 2005, Revised Law no. 68), *Standards Relating to the Care, Management and Refinement of Laboratory Animals* (Notification no. 88 of the Ministry of the Environment, Japan, April 28, 2006) and *Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in the Testing Facility under the Jurisdiction of the Ministry of Health, Labour and Welfare* (Notification no. 0601005 of the Health Sciences Division, Ministry of Health, Labour and Welfare, Japan, June 1, 2006).

Chemical and dosing

DCBS (CAS no. 4979-32-2) was obtained from Ouchishinko Chemical Industrial (Tokyo, Japan). DCBS in the form of off-white to tan granules is very slightly soluble in water and methanol but soluble in oil. Its melting point is 100–105°C, density is 1230 kg/m^3 and molecular weight is 347 (Flexsys 2000). DCBS (Lot no. 508001) used in this study was 99.7% pure and was kept in a sealed container under cool (1–8°C) and dark conditions. The purity and stability of the chemical were verified by analysis using high-performance liquid chromatography before and after the study. Rats were given dietary DCBS at a concentration of 0 (control), 1500, 3000, 6000 or 10 000 p.p.m. Males were fed a diet containing DCBS for a total of 57 days beginning 16 days before mating. Females were fed a diet containing DCBS for a total of 61–65 days from 16 days before mating to day 21 of lactation throughout the mating, gestation and lactation periods. Control rats were fed a basal diet only.

The dosage levels were determined based on the results of a previous study in rats that were given DCBS by gavage at 0, 6, 25, 100, or 400 mg/kg bw/day for a total of 44 days from 14 days before mating in males and a total of 40–51 days beginning 14 days before mating to day 3 of lactation throughout the mating and gestation periods in females (Ema *et al.* 2007). In that study, toxicologically significant changes were observed only at 400 mg/kg bw/day. Three of 10 females died during parturition. An increased incidence of females showing decreased locomotor activity, soil of the lower abdominal fur and reddish tears was observed. Decreased body weights were found in males and females. Decreased weight of the thymus in both sexes was noted. Decreases in the gestation index, numbers of corpora lutea, implantations, pups born and pups born alive, live birth index and viability index were detected.

Dosed diet preparations were formulated by mixing DCBS into an appropriate amount of a powdered basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) for each dietary concentration. Chemical analysis showed that DCBS in the diet was stable for at least 21 days at room temperature and the formulations were maintained in a room temperature for no more than 21 days. Generally, the diet was replaced once a week.

Animals and housing conditions

Sprague–Dawley (Crl:CD[SD]) rats were used throughout this study. Rats of this strain were chosen because they are the most commonly used in reproductive and developmental toxicity studies and historical control data are available. Male and female rats at nine weeks of age were purchased from the Tsukuba Breeding Center (Charles River Laboratories Japan, Yokohama, Japan). The rats were acclimated to the laboratory for six days prior to the start of the experiment. Male and female rats found to be in good health were selected for use. Rats (F0) were randomly distributed into five groups of six males and six females each, and all animals were assigned a unique number and tattooed on the ear prior to the start of the experiment. Animals were housed individually in suspended aluminum/stainless steel cages except during the acclimation, mating and nursing periods. From day 17 of pregnancy to the day of weaning, individual dams and litters were reared using wood chips as bedding (White Flake; Charles River Laboratories Japan,).

Animals were reared on a basal diet or a diet containing DCBS and filtered tap water *ad libitum* and maintained in an air-conditioned room at 22 ± 3°C with a humidity of 50 ± 20% and a 12-h light (8:00–20:00)/dark (20:00–8:00) cycle. The room was ventilated 10–15 times/h.

Observations

All rats were observed twice a day for clinical signs of toxicity. The body weight was recorded once a week for males and once a week during the premating period, on days 0, 7, 14 and 20 of pregnancy, and on days 0, 4, 7, 14 and 21 of lactation for females. Food consumption was recorded once a week for males, and once a week during the premating period, on days 0, 7, 14 and 20 of pregnancy and on days 0, 7, 14 and 21 of lactation for females.

Rats were euthanized by exsanguination under ether anesthesia. Males were euthanized at 17 weeks and females at 18 weeks on day 21 of lactation. The external surfaces of the rats were examined for abnormalities. The abdomen and thoracic cavities were opened and gross internal examination was performed. In females, the number of implantation sites was recorded. The brain, pituitary, thymus, thyroid, liver, kidney, spleen, adrenal gland, testis, epididymis, seminal vesicle, ventral prostate, ovary and uterus were weighed. The thyroid and seminal vesicle were weighed after fixation with 10% neutral buffered formalin.

Daily vaginal lavage samples from each female were evaluated for estrous cyclicity for two weeks of the premating period. Females with repeated 4–6 day estrous cycles were judged to be normal. 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 (day 0 of pregnancy). Copulated females were checked for signs of parturition three times a day on days 21–23 of pregnancy.

The females were allowed to deliver spontaneously and nurse their pups until PND 21. The day on which parturition was

completed by 13:00 was designated as PND 0. Total litter size and the numbers of live and dead pups were recorded. Live pups were counted, sexed, examined grossly and individually weighed on PND 0, 4, 7, 14 and 21. On PND 4, litters were randomly adjusted to eight pups comprised of four males and four females. No adjustment was made for litters with fewer than 8 pups. Selected pups were assigned a unique number and tattooed on a limb on PND 4. Unselected pups were necropsied on PND 4. Weanlings were necropsied on PND 21 and the brain, thymus, liver, spleen and uterus were weighed.

Statistical analysis

Statistical analysis of the offspring was carried out using the litter as the experimental unit.

Body weight, body weight gain, food consumption, length of estrous cycle, precoital interval, gestation length, number of implantations and pups delivered, delivery index, organ weight, organ/body weight ratio (relative organ weight) and the viability of pups were analyzed for statistical significance in the following way. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances. If the variances were equivalent, the groups were compared by one-way analysis of variance (ANOVA). If significant differences were found, Dunnett's multiple comparison test was performed. If the groups did not have equivalent variances, the Kruskal–Wallis test was used to assess the overall effects. Whenever significant differences were noted, pairwise comparisons were made by Mann–Whitney *U*-test. The incidence of females with normal estrous cycles, copulation index, fertility index, gestation index and neonatal sex ratio was analyzed by the χ^2 test or Fisher's exact test.

The 0.05 level of probability was used as the criterion for significance.

RESULTS

Clinical observations, body weight and food consumption (F0 males and females)

No deaths were found in F0 males and females. In males, there were no compound related clinical signs of toxicity at any doses. Hematuria and soil of perigenital fur were each observed at 10 000 p.p.m. in one female.

Table 1 shows body weight gain in F0 males and females during dosing. In males, body weight gain on days 0–7 of the dosing period at 6000 p.p.m. and higher was significantly lowered. In females,

Table 1 Body weight gains of F0 parental male and female rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	0 (Control)	1500	3000	6000	10 000
No. males	6	6	6	6	6
Initial body weight (g)†	367 ± 7	367 ± 6	366 ± 7	366 ± 8	366 ± 7
Body weight gain during dosing period (g)†					
Days 0–7	48.0 ± 10.4	36.8 ± 14.5	36.3 ± 4.8	26.7 ± 8.5**	25.2 ± 6.5**
Days 7–14	38.2 ± 9.2	33.7 ± 13.4	34.5 ± 8.7	35.2 ± 5.6	29.5 ± 5.5
Days 14–21	21.7 ± 9.2	27.3 ± 7.1	24.3 ± 5.2	23.0 ± 12.6	21.8 ± 3.1
Days 21–28	26.8 ± 10.2	25.7 ± 8.3	22.3 ± 10.5	23.5 ± 6.7	25.2 ± 5.0
Days 28–35	21.2 ± 7.7	20.8 ± 6.5	28.5 ± 12.6	24.0 ± 3.7	19.2 ± 4.1
Days 35–42	14.8 ± 6.9	15.3 ± 6.5	20.3 ± 6.9	17.3 ± 6.3	20.5 ± 5.2
Days 42–49	13.8 ± 8.3	19.5 ± 4.2	13.5 ± 2.7	19.8 ± 5.5	17.2 ± 2.9
Days 49–56	14.8 ± 7.6	19.5 ± 6.3	16.7 ± 5.0	20.5 ± 6.2	17.0 ± 3.5
No. females	6	6	6	6	6
Initial body weight (g)†	238 ± 6	239 ± 7	237 ± 5	238 ± 6	237 ± 7
Body weight gain during premating period (g)†					
Days 0–7	6.5 ± 7.7	8.8 ± 8.8	6.8 ± 4.6	−6.5 ± 9.7*	−19.3 ± 9.3**
Days 7–14	15.7 ± 8.5	16.3 ± 6.9	14.2 ± 5.9	12.2 ± 8.0	13.0 ± 8.7
Body weight gain during pregnancy (g)†					
Days 0–7	45.3 ± 6.5	42.5 ± 4.2	32.8 ± 5.4*	31.2 ± 8.6*	19.5 ± 12.0**
Days 7–14	38.3 ± 6.0	35.7 ± 5.4	36.8 ± 7.0	35.2 ± 6.2	31.2 ± 8.6
Days 14–20	76.7 ± 14.6	68.3 ± 4.3	75.8 ± 12.4	68.7 ± 7.7	62.5 ± 12.2
Body weight gain during lactation (g)†					
Days 0–4	28.0 ± 15.7	10.8 ± 24.3	28.0 ± 15.7	8.2 ± 8.7	−2.5 ± 14.6*
Days 4–7	6.5 ± 2.7	12.0 ± 9.0	10.0 ± 10.2	5.3 ± 7.3	0.5 ± 10.9
Days 7–14	1.3 ± 10.7	10.2 ± 7.3	4.2 ± 8.1	14.2 ± 11.1	6.0 ± 12.5‡
Days 14–21	−19.0 ± 14.7	−31.7 ± 9.9	−17.0 ± 8.3	−8.8 ± 9.8	2.6 ± 14.3‡*

*Significantly different from the control, $P < 0.05$; **significantly different from the control. $P < 0.01$.

†Values are given as mean ± SD; ‡data were obtained from five females because one female was excluded (total litter loss on day 9 of lactation).

body weight gains were decreased on days 0–7 of the premating period at 6000 p.p.m. and higher, on days 0–7 of pregnancy at 3000 p.p.m. and higher, and on days 0–4 of lactation at 10 000 p.p.m. Body weight gain on days 14–21 of lactation was significantly increased at 10 000 p.p.m.

In F0 males, food consumption was significantly decreased during the first week at 6000 p.p.m. and higher and during the second week at 10 000 p.p.m. In F0 females, food consumption was significantly decreased throughout the premating, pregnancy and lactation periods at 6000 and 10 000 p.p.m., except on days 7–14 and 14–20 of pregnancy at 6000 p.p.m. A tendency towards decreased food consumption was observed on days 0–7 of pregnancy at 3000 p.p.m.

The mean daily intakes of DCBS were 83, 172, 343 and 551 mg/kg bw in F0 males, and 126, 264, 476 and 707 mg/kg bw in F0 females for 1500, 3000, 6000 and 10 000 p.p.m., respectively.

Estrous cyclicity (F0 females)

All F0 females showed normal estrous cycles in all groups, and the length of the estrous cycles was not significantly different between the control and DCBS-treated groups.

Reproductive and developmental effects (F0 parents/F1 offspring)

The reproductive and developmental parameters for F0 parents/F1 offspring are presented in Table 2. In F0 parent animals in all groups, all pairs copulated, all male and female rats were fertile and all females delivered live pups. All rats of all groups mated within four days. There were no significant differences between control and DCBS-treated groups in copulation index, fertility index, gestation index, precoital interval, gestation length, delivery index, sex ratio of F1 pups, or viability of F1 pups during lactation. Significantly lower numbers of implantations at 6000 and 10 000 p.p.m. and pups delivered at 10 000 p.p.m. were observed. Body weights of male pups were significantly lowered on PND 4, 7 and 21 at 6000 p.p.m. and on PND 7, 14 and 21 at 10 000 p.p.m. In female pups, significantly lower body weights were observed on PND 7, 14 and 21 at 6000 p.p.m. and higher. No malformed pups were detected in any groups.

Necropsy and organ weights (F0 males and females)

Atrophy of the thymus was found in two females at 10 000 p.p.m. No compound-related gross lesions of the reproductive organs were noted in F0 males and females. In males, significantly increased relative weights of the liver and kidney were observed at 10 000 p.p.m.

The organ weights of F0 females are shown in Table 3. The body weight at the scheduled terminal sacrifice was significantly lowered at 6000 and 10 000 p.p.m. The absolute weight of the ovary was significantly lowered at 10 000 p.p.m. Significantly increased relative weights were found for the pituitary at 3000 p.p.m., the liver at 6000 p.p.m., and the brain, kidney and adrenal gland at 10 000 p.p.m. The absolute and relative weights of the thymus at 10 000 p.p.m. and the spleen at 6000 p.p.m. and higher were significantly decreased.

Necropsy and organ weights (F1 weanlings)

No compound related gross lesions were observed in F1 weanlings.

The organ weights of F1 male weanlings are presented in Table 4. The body weight at the scheduled sacrifice was significantly reduced at 6000 and 10 000 p.p.m. The absolute weights of the brain at 6000 and 10 000 p.p.m. and the liver at 10 000 p.p.m. were also significantly reduced. The relative weights of the liver at 1500 and 6000 p.p.m. and of the brain at 10 000 p.p.m. were significantly increased. Significantly decreased absolute and relative weights of the spleen, except for the relative weight at 3000 p.p.m., were noted at 1500 p.p.m. and higher.

The organ weights of F1 female weanlings are presented in Table 5. The body weight at the scheduled sacrifice was significantly reduced at 6000 p.p.m. and higher. Significantly reduced absolute weights of the brain at 6000 and 10 000 p.p.m., the liver at 10 000 p.p.m., and the uterus at 3000 p.p.m. and 10 000 p.p.m. were also observed. The relative weight of the brain was significantly increased at 10 000 p.p.m. The absolute and relative weights of the spleen were significantly reduced at 3000 p.p.m. and higher.

DISCUSSION

This study was designed to assess the effects of DCBS on continuous parameters such as body weight and food consumption, as well as endpoints for reproductive and developmental toxicity.

Significant decreases in body weight gain and food consumption were observed at 6000 p.p.m. and higher in F0 males and females. In females at 3000 p.p.m., body weight gain was significantly decreased during early pregnancy. Food consumption also decreased, but not significantly. The data indicate that changes in body weight gain were associated with changes in food consumption and that DCBS adversely affects body weight gain and food consumption at 6000 p.p.m. in male rats and 3000 p.p.m. in female rats. The higher relative weights of the liver and kidney at the highest dose in F0 males seem to be due to secondary effects of lowered body weight rather than direct effects of DCBS on the organs. More pronounced effects on organ weights were noted in females. Lower absolute and relative weights of the thymus at 10 000 p.p.m. and spleen at 6000 p.p.m. and higher were detected. In our previous study, histopathological examination revealed atrophy of the thymus and spleen at 400 mg/kg bw/day (Ema *et al.* 2007). Other changes in female organ weight such as the relative weights of the brain, pituitary, liver, kidney and adrenal gland, as well as the absolute weight of the ovary are unlikely to be due to the toxic effects of DCBS because the degree of changes was relatively small, no dose-dependency was shown and no changes were noted in absolute or relative weight. These findings suggest that the immune system may be a target of DCBS toxicity, and that female rats have a higher susceptibility to the toxicity of DCBS than male rats. These findings are consistent with our previous study (Ema *et al.* 2007). The higher susceptibility to DCBS toxicity in females may be explained by the stress of pregnancy and lactation. DCBS is likely to be not reproductively toxic in male rats because DCBS caused neither pathological changes in male reproductive organs nor changes in male reproductive parameters.

In our previous study, DCBS given by gavage to rats at 400 mg/kg bw/day from 14 days before mating to day 3 of lactation caused significant decreases in the gestation index, number of corpora lutea, implantations, pups born and pups born alive, live birth index and viability index (Ema *et al.* 2007). This dose also caused severe maternal toxicity and a total loss of pups by PND 4. No maternal or reproductive/developmental toxicity was detected at 100 mg/kg bw/day in our previous study. In the present study, no serious reproductive difficulties were noted even at the highest dose of 10 000 p.p.m., and necropsy of the reproductive organs revealed no evidence of reproductive failure. Although decreased numbers of implantations and pups delivered were noted at the highest dose, the viability of pups until weaning was not significantly decreased. In the present feeding study, the mean daily intakes of DCBS at the

Table 2 Reproductive and developmental findings for F0 parents/F1 offspring of rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. pairs	6	6	6	6	6
Copulation index[b]					
Male/female (%)	100/100	100/100	100/100	100/100	100/100
Pre-coital interval (days)[a]	2.2 ± 0.8	2.3 ± 1.2	3.2 ± 0.8	3.0 ± 0.9	2.7 ± 1.2
Fertility index[c]					
Male/female (%)	100/100	100/100	100/100	100/100	100/100
Gestation index (%)[d]	100	100	100	100	100
Gestation length (days)[a]	22.2 ± 0.4	22.2 ± 0.4	22.2 ± 0.4	22.0 ± 0.0	22.2 ± 0.4
No. implantations[a]	16.0 ± 1.8	15.0 ± 0.9	16.3 ± 1.2	13.5 ± 2.0*	12.8 ± 1.2**
Delivery index (%)[a,e]	95.8 ± 8.0	96.7 ± 3.7	95.8 ± 5.3	95.6 ± 8.1	86.7 ± 21.1
No. pups delivered[a]	15.3 ± 2.2	14.5 ± 1.0	15.7 ± 1.8	13.0 ± 2.6	11.2 ± 3.1*
Sex ratio of F1 pups[f]	0.467	0.448	0.564	0.526	0.463
Viability index (%)[a]					
PND 0[g]	100 ± 0	100 ± 0	100 ± 0	100 ± 0	91.2 ± 12.9
PND 4[h]	99.1 ± 2.3	97.9 ± 3.3	95.9 ± 5.3	90.6 ± 12.2	72.1 ± 40.8
PND 21[i]	97.9 ± 5.1	97.9 ± 5.1	100.0 ± 0.0	89.6 ± 25.5	83.3 ± 40.8
Male pup body weight during lactation (g)[a]					
PND 0	6.8 ± 0.4	6.7 ± 0.7	6.3 ± 0.4	6.2 ± 0.6	6.5 ± 0.7
PND 4	10.6 ± 0.9	10.3 ± 0.8	9.6 ± 0.6	9.1 ± 0.7**	9.1 ± 2.2[j]
PND 7	18.7 ± 1.3	17.7 ± 1.3	17.6 ± 1.3	14.5 ± 2.2**	13.3 ± 3.7[j]**
PND 14	39.2 ± 3.0	36.2 ± 3.0	37.3 ± 2.9	33.0 ± 4.0	26.3 ± 7.2[j]**
PND 21	67.0 ± 4.6	61.1 ± 6.1	62.8 ± 3.2	55.7 ± 7.6*	44.1 ± 9.9[j]**
Female pup body weight during lactation (g)[a]					
PND 0	6.4 ± 0.4	6.4 ± 0.5	6.0 ± 0.3	5.8 ± 0.6	6.2 ± 0.5
PND 4	10.1 ± 1.1	9.9 ± 0.7	9.0 ± 0.6	8.7 ± 0.7	8.5 ± 1.9
PND 7	18.2 ± 2.0	17.4 ± 0.7	16.0 ± 1.2	13.8 ± 1.3**	11.7 ± 4.2*
PND 14	38.6 ± 3.5	36.1 ± 2.1	35.0 ± 2.4	31.5 ± 4.9*	25.3 ± 7.2[k]**
PND 21	65.1 ± 5.2	60.1 ± 3.7	58.2 ± 3.3	53.5 ± 9.0*	42.5 ± 9.9[k]**

*Significantly different from the control, $P < 0.05$; **significantly different from the control. $P < 0.01$.

[a]Values are given as mean ± SD; [b]copulation index (%) (number of animals with successful copulation/number of animals paired) × 100; [c]fertility index (%) (number of animals that impregnated a female or were pregnant/number of animals with successful copulation) × 100; [d]gestation index (%) (number of females that delivered live pups/number of pregnant females) × 100; [e]delivery index (%) (number of pups delivered/number of implantations) × 100; [f]sex ratio (total number of male pups/total number of pups delivered); [g]viability index on PND 0 (number of live pups on PND 0/number of pups delivered) × 100; [h]viability index on PND 4 (number of live pups on PND 4/number of live pups on PND 0) × 100; [i]viability index on PND 21 (number of live pups on PND 21/number of live pups selected on PND 4) × 100; [j]data were obtained from five litters because one female experienced total male litter loss by day 4 of lactation; and [k]data were obtained from five litters because one female experienced total litter loss by day 9 of lactation.

PND, post natal day.

highest dose were 551 and 707 mg/kg bw in F0 males and females, respectively. One possible explanation for the discrepancy in the degree of reproductive and developmental toxicity between the present and previous studies may be the difference in administration method. Some studies have shown that gavage and feed administration result in different toxicokinetics for various chemicals (Yuan *et al.* 1994, 1995). Further studies are needed to clarify the difference in DCBS toxicokinetics between gavage and feed administrations.

Regarding the development of offspring, decreases in the numbers of implantations and pups delivered and lowered body weights of male and female pups were noted at 6000 p.p.m. and higher. These findings indicate that the dose level of 6000 p.p.m. used in this study was potent enough to adversely affect the survival and growth of pups. Reduced weight of the spleen was also observed in male and female weanlings. These findings also suggest that the immune system may be a target of DCBS toxicity. Other changes in the weights of organs, such as the brain and liver in male weanlings and the brain, liver and uterus in female weanlings are unlikely to be due to the toxic effects of DCBS because the degree of changes was relatively small, no dose-dependency was shown, no changes were noted in the absolute or relative weight, and also

Table 3 Absolute and relative organ weights of F0 female rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. females	6	6	6	6	5
Body weight (g)†	331 ± 18	316 ± 16	320 ± 11	306 ± 14*	274 ± 20**
Brain (g)†	2.10 ± 0.05‡	2.11 ± 0.08	2.10 ± 0.05	2.06 ± 0.10	2.06 ± 0.03
	0.63 ± 0.03§	0.67 ± 0.04	0.66 ± 0.02	0.67 ± 0.04	0.76 ± 0.05**
Pituitary (mg)†	13.3 ± 1.6‡	13.4 ± 2.4	15.4 ± 0.9	13.9 ± 1.9	12.9 ± 2.6
	4.03 ± 0.44§	4.24 ± 0.65	4.81 ± 0.32*	4.53 ± 0.46	4.70 ± 0.66
Thyroid (mg)†	18.3 ± 3.6‡	17.6 ± 3.5	17.7 ± 4.3	18.8 ± 2.7	17.5 ± 3.6
	5.52 ± 0.87§	5.55 ± 0.99	5.51 ± 1.18	6.15 ± 0.94	6.39 ± 1.02
Thymus (mg)†	255 ± 47‡	205 ± 63	237 ± 45	186 ± 89	116 ± 60**
	77.1 ± 14.4§	65.0 ± 19.6	74.2 ± 13.1	60.1 ± 26.5	41.7 ± 19.9*
Liver (g)†	13.03 ± 0.83‡	12.51 ± 0.71	13.42 ± 1.18	13.69 ± 0.68	12.18 ± 1.60
	3.94 ± 0.21§	3.97 ± 0.23	4.20 ± 0.27	4.48 ± 0.09**	4.46 ± 0.59
Kidney (g)†	2.34 ± 0.16‡	2.38 ± 0.13	2.35 ± 0.10	2.20 ± 0.12	2.51 ± 0.41
	0.71 ± 0.04§	0.75 ± 0.05	0.74 ± 0.04	0.72 ± 0.03	0.92 ± 0.18**
Spleen (mg)†	682 ± 74‡	589 ± 68	600 ± 89	493 ± 24**	459 ± 46**
	206 ± 20§	187 ± 19	188 ± 31	161 ± 5**	168 ± 15**
Adrenal (mg)†	75.5 ± 11.0‡	81.8 ± 12.9	77.0 ± 8.8	72.0 ± 8.8	88.2 ± 8.3
	22.9 ± 3.2§	26.0 ± 3.9	24.1 ± 2.7	23.5 ± 2.8	32.4 ± 3.8**
Ovary (mg)†	109 ± 18‡	113 ± 17	101 ± 5	101 ± 10	75 ± 23**
	32.9 ± 3.8§	36.1 ± 6.8	31.6 ± 2.4	32.9 ± 3.9	27.1 ± 6.4
Uterus (mg)†	513 ± 68‡	465 ± 73	489 ± 101	414 ± 71	369 ± 183
	156 ± 24§	148 ± 26	153 ± 32	135 ± 22	132 ± 56

*Significantly different from the control, $P < 0.05$; **significantly different from the control, $P < 0.01$.
†Values are given as the mean ± S.D; ‡absolute organ weight; §relative organ weight (organ weight [g or mg]/100 g body weight).

Table 4 Absolute and relative organ weights for F1 male weanlings of rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. males	6	6	6	6	5
Body weight (g)†	67.1 ± 6.7	62.5 ± 4.5	63.8 ± 4.2	55.3 ± 8.9*	43.8 ± 10.6**
Brain (g)†	1.70 ± 0.05‡	1.63 ± 0.12	1.59 ± 0.04	1.51 ± 0.05**	1.45 ± 0.11**
	2.55 ± 0.21§	2.61 ± 0.24	2.50 ± 0.15	2.78 ± 0.42	3.44 ± 0.74*
Thymus (mg)†	257 ± 44‡	219 ± 33	265 ± 45	246 ± 36	190 ± 65
	382 ± 50§	351 ± 57	415 ± 59	449 ± 60	424 ± 50
Liver (g)†	2.56 ± 0.35‡	2.65 ± 0.29	2.69 ± 0.38	2.37 ± 0.38	1.72 ± 0.49**
	3.80 ± 0.17§	4.22 ± 0.20*	4.20 ± 0.37	4.30 ± 0.33*	3.90 ± 0.22
Spleen (mg)†	372 ± 63‡	276 ± 53**	296 ± 32*	250 ± 45**	148 ± 36**
	556 ± 84§	442 ± 80*	466 ± 56	452 ± 32*	337 ± 31**

*Significantly different from the control, $P < 0.05$; **significantly different from the control, $P < 0.01$.
†Values are given as mean ± S.D; ‡absolute organ weight; §relative organ weight (organ weight [g or mg]/100 g body weight).

because the changes seem to be secondary effects of the lowered body weight. In the present study, external and internal morphological examinations of offspring were performed, but no skeletal examinations were conducted. To accurately evaluate prenatal developmental toxicity including teratogenicity, it is necessary to interrupt pregnancy 12–24 h before the expected term either by hysterectomy or the necropsy of maternal animals (Wilson 1965).

The adverse effects of DCBS on reproduction and development noted in the present feeding study are almost consistent with the findings of our previous gavage study (Ema *et al.* 2007), which showed decreased numbers of implantations and pups delivered and decreased body weight of the pups at higher doses. These endpoints appear to be affected at multiple points of the female reproductive and developmental process. The decreased number of implantations

Table 5 Absolute and relative organ weights for F1 female weanlings of rats given N,N-dicyclohexyl-2-benzothiazolesulfenamide

Dose (p.p.m.)	Control	1500	3000	6000	10 000
No. females	6	6	6	6	5
Body weight (g)†	65.7 ± 7.2	61.1 ± 3.4	59.9 ± 4.6	54.0 ± 9.6*	42.8 ± 9.6**
Brain (g)†	1.60 ± 0.09‡	1.56 ± 0.07	1.53 ± 0.03	1.50 ± 0.05*	1.37 ± 0.08**
	2.46 ± 0.25§	2.56 ± 0.16	2.57 ± 0.18	2.84 ± 0.39	3.34 ± 0.78**
Thymus (mg)†	272 ± 46‡	253 ± 33	252 ± 27	243 ± 51	216 ± 82
	415 ± 56§	415 ± 57	422 ± 37	456 ± 101	491 ± 92
Liver (g)†	2.58 ± 0.31‡	2.47 ± 0.27	2.42 ± 0.42	2.27 ± 0.43	1.71 ± 0.49**
	3.93 ± 0.14§	4.03 ± 0.22	4.02 ± 0.41	4.19 ± 0.13	3.96 ± 0.29
Spleen (mg)†	360 ± 57‡	296 ± 16	267 ± 60*	247 ± 50**	163 ± 59**
	548 ± 66§	484 ± 9	442 ± 72*	456 ± 37*	371 ± 58**
Uterus (mg)†	44.7 ± 6.6‡	41.3 ± 6.1	35.7 ± 2.1*	42.0 ± 6.9	32.4 ± 4.8**
	68.9 ± 14.0 Temp.§	67.7 ± 9.8	60.0 ± 7.4	78.5 ± 10.8	77.3 ± 10.3

*Significantly different from the control, $P < 0.05$; ** significantly different from the control, $P < 0.01$.
†Values are given as the mean ± S.D; ‡absolute organ weight; §relative organ weight (organ weight [g or mg]/100 g body weight).

was the most striking effect in the present study. In our previous study, a decreased number of corpora lutea was noted in female rats given DCBS (Ema *et al.* 2007). Therefore, it is likely that the decreased number of implantations can be attributed to the decreased number of corpora lutea. The present study does not provide complete information on all aspects of reproduction and development due to the relatively small numbers of animals in the dose groups and selectivity of the endpoints. To further evaluate the reproductive and developmental toxicity of DCBS in rats, a two-generation reproductive toxicity study should be performed.

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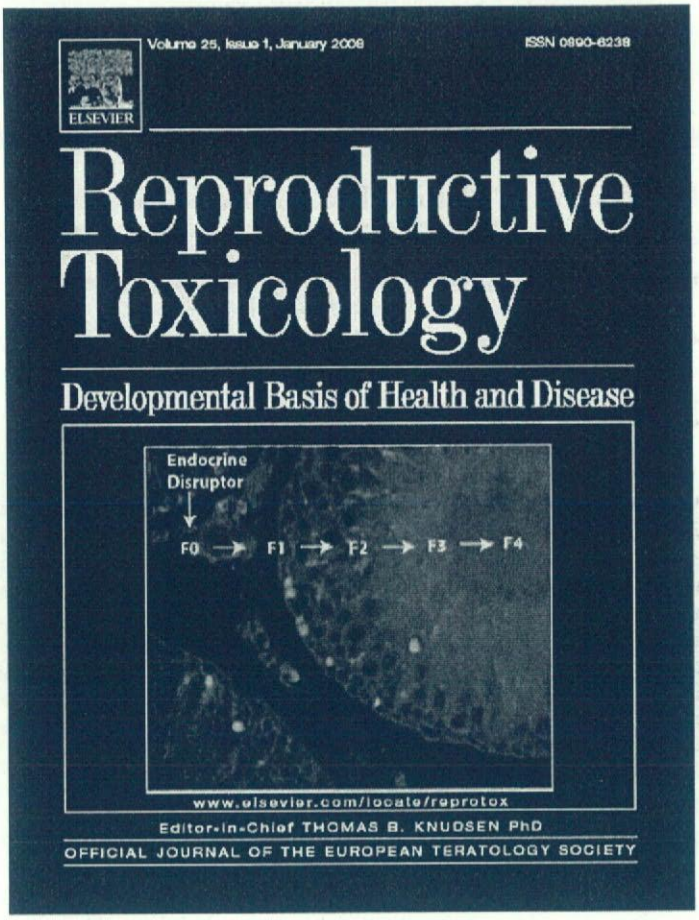

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Evaluation of developmental neurotoxicity of polysorbate 80 in rats

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Abstract

The developmental neurotoxicity of polysorbate 80 (PS80) was evaluated in rats. Crl:CD(SD) rats were given drinking water containing PS80 at 0, 0.018, 0.13, 1.0, or 7.5% (0, 0.035, 0.245, 1.864, or 16.783 ml/kg bw/day) on day 0 of pregnancy through day 21 after delivery. Pregnant rats were allowed to deliver spontaneously. Potential adverse effects of pre- and post-natal exposure on the development and function of the nervous system in offspring of rats given PS80 were examined. Maternal body weight was lowered at 7.5%. Number of pups born was lowered at 7.5%. There were no compound-related effects on locomotor activity of offspring on postnatal days (PNDs) 14–15, 17–18, 20–21 and 33–37. No compound-related changes were found in developmental landmarks, sexual maturation, or reflex responses. Although decreased rate of avoidance responses was noted on PNDs 23–27 in male and female offspring at 7.5%, no compound-related changes were found in performance in the conditioned avoidance response on PNDs 60–67. Histopathological examinations of the brain revealed no toxicological changes. Lowered body weight was observed in male and female offspring at 7.5%. The NOAEL in this study was considered to be 1.0% (1.864 ml/mg/kg bw/day).

Keywords: Polysorbate 80; Tween 80; Developmental neurotoxicity; Behavior; Developmental landmarks; Rat

1. Introduction

Polysorbate 80 (PS80, CAS No. 9005-65-6, polyoxyethylene (20) sorbitan monooleate, commercially also known as Tween® 80) is a mixture of polyoxyethylene ethers of mixed partial oleic acid esters of sorbitol anhydrides and related compounds [1]. PS80 is very soluble in water and soluble in ethanol. PS80 is widely used in biochemical applications including, solubilizing proteins, isolating nuclei from cells in cell culture, growing tubercule bacilli, and emulsifying and dispersing substances in medicinal and food products [2]. PS80 is often used in foods as an emulsifier in ice cream, frozen custard, ice milk, fruit sherbet, and nonstandardized frozen desserts. PS80 is also used in yeast-defoamer formulations and as a solubilizing and depersing agent in pickles and pickle products [1]. Exposure of the general population to PS80 is mainly through its use as a food additive.

Several reports on neurobehavioral toxicity of PS80 are available. Varma et al. [3] reported that PS80 caused a decreased locomotor activity and hyperthermia at 2 ml/kg, and exhibition of paralytic activity at 10 ml/kg after oral administration, and decreased locomotor activity, depression and potentiation of the penobarbitone sleeping time at 2 ml/kg after intraperitoneal administration in mice. They concluded that intraperitoneal doses generally showed more pronounced effects than oral doses, and PS80 did not show any neuropharmacological effects in a dose not more than 1 ml/kg when given either intraperitoneally or orally [3]. PS80 also caused behavioral and neurochemical changes in cats after intraperitoneal administration [4,5]. Intraperitoneal injection of 0.1% saline solution of PS80 in a volume of 3 ml/kg three times every 12 h decreased the carbachol-induced growing response and increased the content of 5-hydroxyindoleacetic acid in the hypothalamus in cats [4]. As for the developmental neurotoxicity of PS80, Brubaker et al. [6] reported that locomotor activity was enhanced in pre-weaning male offspring of rats received drinking water containing PS80 at 1.25 ml/l (0.125%) during the pre-mating, mating, pregnancy and lactation periods. However, their study did not provide enough information on all aspects of developmental neurotoxicity due to the use of one dose group and the

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selection of endpoints. Only pre-weaning locomotor activity in male offspring was determined, and no other parameters were evaluated in their study. The present study was therefore conducted to further evaluate the developmental neurotoxicity of PS80, including locomotor activity, in rats using a study design similar to the OECD Draft Proposal for New Guideline 426, Developmental Neurotoxicity Study [7].

2. Materials and methods

This study was performed in accordance with the principles for Good Laboratory Practice [8]. This study was conducted also in compliance with the "Law of Humane Treatment and Management of Animals" [9] and "Guidance for Animal Care and Use" of Ina Research Inc. and in accordance with the protocol reviewed by the Institutional Animal Care and Use Committee of Ina Research Inc. fully accredited by AAALAC International [Accredited Unit No. 001107].

2.1. Animals and housing conditions

Crl:CD(SD) rats were used throughout this study. Rats of this strain were chosen because they are the most commonly used in reproductive and developmental toxicity studies and historical control data are available. Male rats at 10 weeks of age and female rats at 9 weeks of age were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). The males and females were acclimated to the laboratory for 7 days, prior to the start of the experiment, and rats found to be in good health were selected for use. Vaginal smears of each female rat were recorded, and rats showing regular estrous cycles were used in the experiment. Animals were reared on a basal diet (NMF; Oriental Yeast Co. Ltd., Tokyo, Japan) and water *ad libitum*, and were maintained in an air-conditioned room at 21.0–25.0 °C, with a relative humidity of 40–70%, a 12-h light (7:00–19:00)/dark (19:00–7:00) cycle, and ventilation of 16 air changes/h. Virgin female rats were mated overnight with male rats. The day when sperm was detected in the vaginal smear was considered to be day 0 of pregnancy. The pregnant rats, weighing 215–324 g, were distributed into five groups of 22 females to equalize the body weights among groups. 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 on sterilized wooden chips (Sun Flake: Charles River Laboratories Japan, Inc.).

2.2. Chemical and dosing

Polysorbate 80 (PS80) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The PS80 used in this study was a technical grade (Lot no. EWP7301/code no. 162-21771, saponification value: 49.3, hydroxyl value: 70.1), and was kept in a dark and cool place. The stability of the PS80 was verified by analysis before and after the study. Rats were given PS80 in their drinking water at a concentration of 0 (control), 0.018, 0.13, 1.0, or 7.5% on day 0 of pregnancy through day 21 after delivery. The dosage levels were determined based on the results of our previous dose-finding study, in which decreased body weight gain and food and water consumption at 10.0% and higher, slight decrease in the body weight gain and food consumption at 7.5%, and no adverse effects at 5.0% and below were observed in female rats given PS80 in their drinking water for 14 days (data not shown). Dosed water preparations were formulated by mixing and dissolved PS80 into an appropriate amount of distilled water (Otsuka Pharmaceutical Factory, Inc., Naruto, Japan) for each water concentration. Rats were given PS80 at a constant water concentration. The control rats were given only water. The stability of formulations at room temperature has been confirmed for up to 7 days. During use, formulations were maintained at room temperature for not more than 5 days, and were 100.4–108.6% of the target concentration.

2.3. Observations of dams

All pregnant rats were observed daily for clinical signs of toxicity. Maternal body weight and water consumption were recorded daily, and food consumption was recorded every 3 or 4 days. Female rats were checked for signs of parturition before and after noon from days 20 to 25 of pregnancy to determine the time of delivery. The day on which parturition was completed by 16:00 was designated as day 0 after delivery. The females were allowed to deliver spontaneously and nurse their pups until day 21 after delivery. Parental female rats were euthanized by exsanguination under isoflurane anesthesia on day 21 after delivery. The external surfaces of rats were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. For each female, the number of uterine implantation sites was recorded, and the weights of the brain, liver, kidney, spleen, and adrenal were determined.

2.4. Observations of offspring

The day of birth was designated as postnatal day (PND) 0. On PND 0, total litter size and the numbers of live and dead pups were recorded, and pups were counted, sexed, and examined grossly on PND 0. All pups were observed daily for clinical signs of toxicity, and individually weighed on PNDs 0, 4, 7, 14 and 21. On PND 4, each of the litters was randomly adjusted to eight pups comprising of four males (1m, 2m, 3m and 4m) and four females (1f, 2f, 3f and 4f). Litters of less than eight pups were not used in the experiment. All pups were observed daily for pinna unfolding beginning on PND 2, fur appearance and incisor eruption beginning on PND 8, and eye opening beginning on PND 12. Body weights of pups were recorded on the day of completion of these developmental landmarks. Pups were weaned on PND 21.

2.4.1. Functional/behavioral observations during the pre-weaning period

One male (1m) and one female (1f) pup selected from all dams in each group was evaluated for surface righting reflex on PND 5, and negative geotaxis reflex on PND 8. Locomotor activity of offspring (1m and 1f) on PNDs 14–15, 17–18, and 20–21 at 20:00, 2:00, 8:00 and 14:00 was determined in the open field. Subject rats were placed individually in a box (26 cm in length and width, and 20 cm height) in a 3 × 3 matrix, consisting of a black acrylic plate, with a camera directly overhead and were allowed to explore freely for 10 min. The distance traveled by each monitored rat was recorded with video-based tracking software (BigBrother, Actimetrics, Inc., Wilmette, IL). Locomotor activity was determined under white noise (60 dB) to attenuate external sound, and light at 166–300 lx during the diurnal period and an infrared lamp during the nocturnal period.

2.4.2. Functional/behavioral observations during the adolescent and young adult periods

All remaining male (2m, 3m and 4m) and female (2f, 3f and 4f) pups of each dam were observed daily for clinical signs of toxicity, and individually weighed on PNDs 28, 35, 42, 49, 56 and 70.

Male (2m) and female (2f) pups selected from all dams in each group were evaluated for pupillary reflex, Preyer's reflex, pain response and mid-air righting on PNDs 23–26 and 62–64, and locomotor activity was determined on PNDs 33–37 and 60–66. An open-field with a box (39 cm in length and width, and 30 cm height) in a 2 × 2 matrix was used to evaluate locomotor activity in post-weaning offspring. Other procedures for the determination of locomotor activity were the same as described above for pre-weaning pups. Offspring (2m and 2f) were observed daily for male preputial separation beginning on PND 35 or female vaginal opening beginning on PND 25. The body weight of the respective rats was recorded on the day of preputial separation or vaginal opening.

Conditioned avoidance response was determined on PNDs 23–27 in male (3m) and female (3f) pups of half of the dams in each group, and on PNDs 60–67 in male (3m) and female (3f) pups of the other half of the dams in each group. The shuttle box (40 cm in length, 20 cm width, and 20 cm height), which consisted of transparent acrylic plastic panels, was divided into two equal compartments by a roller (40 and 55 mm in diameter for pups on PNDs 23–27 and PNDs 60–67, respectively). A rat placed in one compartment could get over the roller and cross to the other side. The grid floor of each compartment consisted of stainless steel rods spaced at 10 mm (for pups on PNDs 23–27) or 13 mm (for pups on PNDs 60–67) center to center. An electric shock could be delivered through the grid floor of the occupied compartment from a shock generator/scrambler (MU Co., Chino, Japan). A subject rat was given 2 min to adapt to the shuttle box after its introduction into one compartment. The trial began with a warning buzzer

(2000 Hz, 25 dB) as the conditioned stimulus (CS) for 5 s. A rat crossing to the opposite side of the shuttle box during the buzzer period would successfully avoid the electric shock (3 mA) that followed the buzzer. If the rat had not yet crossed to the opposite compartment of the shuttle box after the 5 s buzzer period, an electric shock was applied for 10 s, as the unconditioned stimulus. A 30 s intertrial interval preceded the next presentation of the CS. Each rat was tested for 60 min a day for three consecutive days. The rate of successfully conditioned responses for every 10 and 60 min was calculated.

One male (4m) and one female (4f) pups selected from each dam were maintained as reserve animals for replacements or additional tests.

2.4.3. Necropsy of offspring

Humane sacrifice was performed on PND 22 for pups (1m and 1f), on PND 70 for pups (2m, 3m, 2f and 3f), and on PNDs 103–126 for pups (4m and 4f) of each dam. The external surfaces of pups were examined. The abdomen and thoracic cavities were opened, and a gross internal examination was performed. For histopathological examinations, half of pups in each group (10–11/sex/group) killed on PNDs 22 or 70 were perfused with heparinized phosphate-buffered solution and paraformaldehyde-phosphate buffered solution, and the brain, spinal cord in the thoracic and lumbar regions, and sciatic nerve were removed and stored in 10% neutral buffered formalin. Histopathological evaluations were performed on the cerebrum, cerebellum, medulla oblongata, pons, spinal cord and sciatic nerve of male and female pups in the control and highest dose groups after fixation, paraffin embedding, and sectioning and staining with hematoxylin and eosin. The remaining pups (8–11/sex/group) killed on PNDs 22 or 70 were subjected to weighing of the brain, liver, kidney, spleen and adrenal.

2.5. Statistical analysis

Statistical analysis of offspring before weaning was carried out using the litter as the experimental unit. The initial body weight, body weight gain and food and water consumptions of maternal rats, numbers of implantations and pups per litter, organ weight, pup weight, day of completion of developmental landmarks, latency of reflex response, distance traveled by pups, rate of avoidance response were analyzed with Bartlett's test [10] for homogeneity of variance at the 5% level of significance. If it was homogeneous, the data were analyzed using Dunnett's multiple comparison test [11] to compare the mean of the control group with that of each dosage group, and if it was not homogeneous, the mean rank of the PS80-treated groups was compared with that of the control group with the Dunnett-type test which was used for the gestation length, delivery index, incidence of pups with malformations, viability index of pups and rate of pups that completed reflex responses to compare the mean rank of groups treated with PS80 and the control group. The fecundity index and gestation index were analyzed by chi-square test. The 0.05 level of probability was used as the criterion for significance.

3. Results

3.1. Findings in dams

No feces were found in one female on days 20–21 of pregnancy and on day 0 of lactation at 7.5%. During lactation, loose stools in 18 females on days 2–21, and scattering of offspring on days 0–1, scant or no feces on days 1–2, reddish brown soiled perianal region on days 12 and 20 and death of all offspring on day 2 in one female each were observed at 7.5%. No clinical signs of toxicity were noted at 1.0% and below (data not shown).

The body weights of maternal rats during pregnancy and lactation are shown in Fig. 1. A significantly lower body weight was observed on days 3, 10, 12–20 of pregnancy and days 0, 2–18 of lactation at 7.5%. In this group, body weight gain was also significantly decreased during pregnancy.

A significant decrease in food consumption on all measuring days during pregnancy and lactation was noted at 7.5%. No

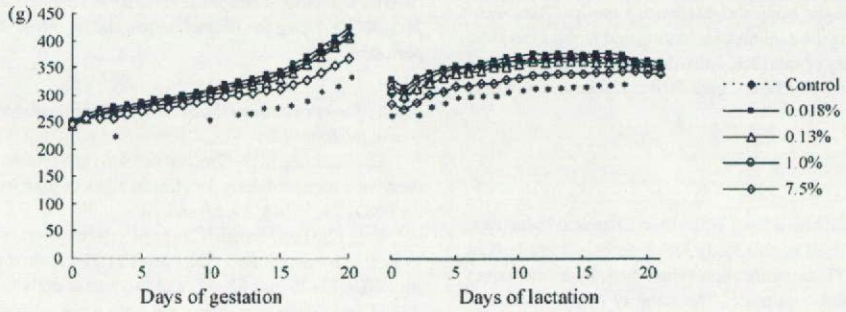

Fig. 1. Body weight of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

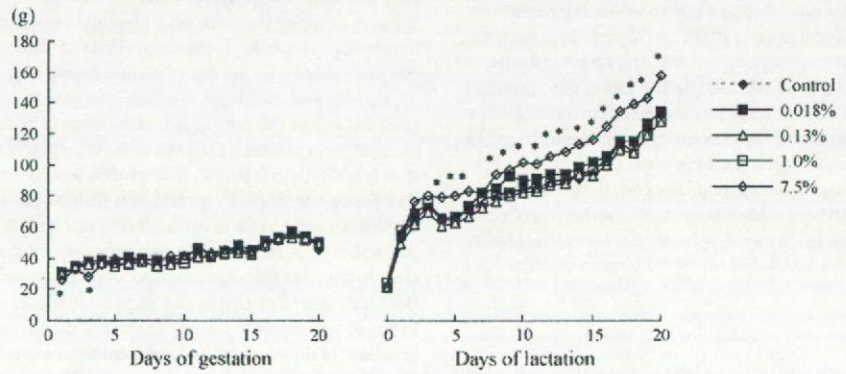

Fig. 2. Water consumption of maternal rats given polysorbate 80 during pregnancy and lactation. *Significantly different from the control, $p < 0.05$.

significant changes in food consumption were found at 1.0% and below (data not shown).

The water consumption of maternal rats during pregnancy and lactation are shown in Fig. 2. At 7.5%, water consumption was significantly decreased on days 1 and 3 of pregnancy and increased on days 5–7 and 9–21 of lactation. No significant changes in water consumption were found at 1.0% and below. The least water consumption was observed on day 0 of lactation and the most water consumption was observed on day 21 of lactation in all groups.

The average daily intakes of PS80 during pregnancy were 0.024, 0.171, 1.314 and 10.576 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The average daily intake of PS80 during lactation were 0.046, 0.321, 2.521 and 23.908 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The average daily intakes of PS80 throughout the administration period were 0.035, 0.245, 1.864 and 16.783 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively. The least intake of PS80 was noted on day 0 of lactation and the most intake of PS80 was noted on day 21 of lactation in all groups. The ranges of average daily intakes of PS80 based on each day of the administration period were 0.012–0.071, 0.093–0.499, 0.837–3.765 and 5.869–36.431 ml/kg bw for 0.018, 0.13, 1.0 and 7.5%, respectively.

At necropsy of dams, dilatation of the cecum in seven females and a significant increase in the relative weight, but not in the absolute weight, of the kidney, were observed at 7.5%. No changes in gross pathology or in absolute and relative weights were detected in any organs at 1.0% and below (data not shown).

3.2. Reproductive/developmental findings

The reproductive findings in maternal rats are presented in Table 1. One female at 7.5% showed total litter loss by day 2 of lactation, one female at 0.018 and 1.0% and two females at 0.13% were not impregnated, and one pregnant female at 1.0%, died on day 22 of pregnancy; however, no significant differences were noted in the fecundity index, gestation index and length of gestation between control and PS80-treated groups.

The developmental findings are shown in Table 2. The number of pups born was significantly reduced at 7.5%. There were no significant effects of treatment of PS80 on the numbers of implantations, pups born alive and dead pups, delivery index and sex ratio of pups and the viability index of pups before weaning. A fetus with acaudate and anal atresia was observed at 0.018%, but no fetuses with external malformations were found in other groups. Although no significant changes in the body weight of male and female pups were observed on PNDs 0, 4 and 7 in PS80-treated groups, significantly reduced body weights were noted on PNDs 14 and 21 at 7.5%. No PS80-related clinical signs of toxicity were found during the pre-weaning period.

The body weights after weaning in male and female offspring of rats given PS80 during pregnancy and lactation are shown in Table 3. At 7.5%, a significantly reduced body weight was noted on PNDs 28, 35, 42, 49 and 56 in males and on PNDs 28 and 35 in females.

One male at 1.0% died on PND 23; however, there were no compound-related clinical signs of toxicity or adverse effects on the survival rate in male and female weaned rats (data not shown).

3.3. Developmental landmarks in offspring

Physical development in male and female pups is presented in Table 4. There was no significant difference in the age of male and female pups that displayed pinna unfolding, fur appearance, incisor eruption, or eye opening. Body weight at the age of fur appearance in males and eye opening in both sexes was significantly reduced at 7.5%.

Data on sexual development in male and female pups is shown in Table 5. No significant differences in age at preputial separation in males or vaginal opening in females, or body weight at the age of preputial separation or vaginal opening were found between control and PS80-treated groups.

Examination of reflex ontogeny revealed no significant difference between control and PS80-treated groups in the latency of response, i.e., the time taken by the subject to complete reflex response, or the incidence of pups completing reflex response. All male and female pups in all groups, except for one male pup at 1.0%, showed completion of the reflex response when testing surface righting reflex on PND 5 and negative geotaxis reflex on

Table 1
Reproductive findings in rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of females copulated	22	22	22	22	22
No. of pregnant females	22	21	20	21	22
No. of non-pregnant females	0	1	2	1	0
Fecundity index (%)[a]	100	95.5	90.9	95.5	100
No. of deaths during pregnancy	0	0	0	1	0
Gestation length (days)[b]	21.9 ± 0.4	21.8 ± 0.4	21.9 ± 0.4	21.9 ± 0.4	21.7 ± 0.5
No. of females with live born	22	21	20	20	22
Gestation index (%)[c]	100	100	100	95.2	100
No. of females with totally litter loss	0	0	0	0	1

[a] Fecundity index (%) = (no. of pregnant females/no. of females confirmed mating) × 100.
[b] Values are given as the mean ± S.D.
[c] Gestation index (%) = (no. of females with live pups born/no. of pregnant females) × 100.

Table 2
Developmental findings in rats given polysorbate 80 during pregnancy and lactation

	Polysorbate 80 (%)				
	0 (control)	0.018	0.13	1.0	7.5
No. of litters	22	21	20	21[a]	22
No. of implantations[b]	16.1 ± 1.7	15.4 ± 2.7	15.1 ± 1.6	15.8 ± 1.4	15.1 ± 2.1
Total no. of pups born[b]	15.4 ± 1.8	14.8 ± 2.4	14.2 ± 2.1	14.6 ± 1.2	13.9 ± 1.7*
No. of pups born alive[b]	14.7 ± 1.8	14.6 ± 2.3	13.7 ± 2.0	14.5 ± 1.2	13.6 ± 2.0
No. of dead pups[b]	0.7 ± 1.2	0.1 ± 0.4	0.5 ± 0.7	0.2 ± 0.7	0.3 ± 0.8
Delivery index (%)[c]	95.8	96.0	94.1	92.9	92.8
Proportion of male pups (%)[d]	54.2	49.2	53.6	47.7	44.7
Viability index before weaning (%)					
Postnatal day 0[e]	95.5	99.1	96.6	99.0	97.6
Postnatal day 4[f]	98.6	98.2	98.5	98.7	93.8
Postnatal day 21[g]	100	100	100	100	100
Body weight of male pups before weaning (g)[b]					
Postnatal day 0	6.4 ± 0.5	6.5 ± 0.5	6.5 ± 0.5	6.5 ± 0.4	6.1 ± 0.8
Postnatal day 4	10.1 ± 1.0	10.6 ± 1.3	10.4 ± 1.3	10.4 ± 0.8	9.8 ± 1.1
Postnatal day 7	16.9 ± 1.4	17.6 ± 1.5	17.1 ± 1.9	17.4 ± 1.1	16.2 ± 1.8
Postnatal day 14	35.3 ± 1.6	36.5 ± 2.4	34.8 ± 2.5	35.6 ± 1.8	32.4 ± 3.3*
Postnatal day 21	58.0 ± 3.6	59.0 ± 4.1	57.0 ± 3.5	57.1 ± 3.0	50.4 ± 5.1*
Body weight of female pups before weaning (g)[b]					
Postnatal day 0	6.0 ± 0.5	6.2 ± 0.5	6.2 ± 0.5	6.1 ± 0.3	5.8 ± 0.7
Postnatal day 4	9.6 ± 1.0	10.2 ± 1.1	9.9 ± 1.3	9.9 ± 0.8	9.5 ± 1.0
Postnatal day 7	16.1 ± 1.4	17.0 ± 1.6	16.3 ± 1.9	16.6 ± 1.1	15.7 ± 1.8
Postnatal day 14	34.0 ± 1.9	35.2 ± 2.3	33.5 ± 2.3	34.4 ± 1.4	31.5 ± 3.1*
Postnatal day 21	55.0 ± 3.7	56.4 ± 4.0	54.5 ± 3.1	55.1 ± 2.0	49.2 ± 4.7*
External examination of pups[h]					
No. of pups (litters) examined	339 (22)	310 (21)	284 (20)	278 (19)	306 (22)
No. of pups with malformations	0	1[i]	0	0	0

[a] One female, who delivered four live pups on day 23 of pregnancy, was euthanized on day 0 after delivery, and her data were excluded.
[b] Values are expressed as the mean ± S.D.
[c] Delivery index (%) = (no. of pups born/no. of implantations) × 100.
[d] Proportion of male pups = (no. of male pups/total no. of pups) × 100.
[e] Viability index on postnatal day 0 (%) = (no. of pups born alive/total no. of pups born) × 100.
[f] Viability index on postnatal day 4 (%) = (no. of live pups on postnatal day 4/no. of pup born alive) × 100.
[g] Viability index on postnatal day 21 (%) = (no. of live pups on postnatal day 21/no. of live on postnatal day 4 after cull) × 100.
[h] External examinations were performed on all pups born on postnatal day 0.
[i] One live pup had acaudate and anal atresia.
* Significantly different from the control, $p < 0.05$.

PND 8. As for the sensory function of offspring, all male and female pups in all groups completed pupillary reflex, Preyer's reflex, pain response and mid-air righting reflex when tested on PNDs 23–26 and 62–64 (data not shown).

3.4. Locomotor activity in offspring

Locomotor activity of male and female pups during the pre-weaning period is presented in Fig. 3. No significant differences in the distance traveled by male and female pups during the nocturnal period (20:00 and 2:00) and diurnal period (8:00 and 14:00) were found between control and PS80-treated groups when locomotor activity was determined on PNDs 14–15. Although a significantly higher activity was observed in male pups in the 1.0% group at 2:00 on PND 18, no significant changes in activity were noted in males and females at any other test time on PNDs 17–18. There were no significant differences between control and PS80-treated groups in locomotor activity of male and female offspring at any test time on PNDs 20–21.

After weaning, no significant differences in the distance traveled by male and female offspring were detected at any test time when activity was determined on PNDs 33–37 and PNDs 60–66 (data not shown).

3.5. Conditioned avoidance response in offspring

The rate of successfully conditioned responses for every 10 min test period on PNDs 23–27 is presented in Fig. 4. On the first day of the test, the rate of successful responses for 60 min was lower in males and females at 7.5%, and a significantly decreased rate was noted in males during the last 10 min and in females during the first and third 10 min test periods. However, there were no significant changes in the rate of successful responses in any 10 min test periods in males and females of any PS80-treated groups on the second- and third-day of the test. No significant changes in the total rate of successfully responses for 60 min were found in male and female pups in any PS80-treated groups on any test days.