Table 1
Reproductive performance of F0 and F1 parental animals.

AAS (ppm)		0 (control)	50	500	5000
F0 generation					
No. of rats (male/female)		24/24	24/24	24/24	24/24
Copulation index (%)[a]	Males	100	95.8	91.7	100
	Females	100	100	100	100
Precoital interval (days)[b]		2.2 ± 1.0	2.5 ± 1.6	2.3 ± 1.2	2.8 ± 1.6
Fertility index (%)[c]	Males	100	91.3	100	100
	Females	100	87.5	100	100
Gestation index (%)[d]		100	100	100	100
Gestation length (days)[b]		22.3 ± 0.5	22.4 ± 0.6	22.3 ± 0.5	22.4 ± 0.5
F1 generation					
No. of rats (male/female)		24/24	24/24	23/24	24/24
Copulation index (%)[a]	Males	91.7	91.7	91.3	95.8
	Females	100	95.8	100	100
Precoital interval (days)[b]		2.7 ± 1.8	3.0 ± 2.1	3.3 ± 2.4	3.1 ± 1.3
Fertility index (%)[c]	Males	90.9	77.3	95.2	100
	Females	91.7	78.3	95.8	95.8
Gestation index (%)[d]		100	100	95.7	100
Gestation length (days)[b]		22.3 ± 0.5	22.3 ± 0.5	22.2 ± 0.4	22.2 ± 0.4

[a] Copulation index (%) = (No. of animals with successful copulation/No. of animals paired) × 100.
[b] Values are given as the mean ± S.D.
[c] Fertility index (%) = (No. of animals that impregnated a female or were pregnant/No. of animals with successful copulation) × 100.
[d] Gestation index (%) = (No. of females that delivered live pups/No. of pregnant females) × 100.

body weight of F1 males on PND 21 and of F1 females on PNDs 14 and 21 was significantly lower in the 5000 ppm group than in the control (Table 2). A similar decreasing trend was found in the body weight of male and female F2 pups around the time of weaning in the highest dose group, although no statistical significance was found.

In F1 and F2 pups, there were no significant differences in the completion rate of pinna unfolding, the age at completion of incisor eruption and eye opening, and AGD and AGD per cube root of the body weight ratio between the control and AAS-treated groups (data not shown). All male and female F1 and F2 pups in all groups achieved the surface righting reflex on PND 5, negative geotaxis reflex on PND 8 and mid-air righting reflex on PND 18, and no significant changes were found in the response time of surface righting and negative geotaxis reflex (data not shown). In F1 female animals, vaginal opening was significantly delayed at 5000 ppm (32.3 ± 1.8 days of age, compared with 30.2 ± 2.1 days of age in controls, $P \leqslant 0.01$). Body weight at the time of attainment was not significantly, but was slightly heavier in this 5000 ppm group (122.0 ± 15.7 g, compared with 115.8 ± 12.6 g in control). There were no significant differences in age at preputial separation or body weight at the time of completion in F1 males between control and AAS-treated groups (data not shown).

3.5. Necropsy, organ weight and histopathology of adults (F0 and F1)

No dose-related gross lesions were found in either F0 or F1 adults. In F0 females in the 500 and 5000 ppm groups and in F1 males and females in the 5000 ppm group, relative kidney weight was increased significantly. A significant decrease in the absolute weight of the pituitary gland was found in F0 females and in F1 males and females at 5000 ppm. In F1 females, there was also a significant decrease in the absolute thymus weight at 5000 ppm. Further, significant decreases were found in the relative weight of the seminal vesicle in 50 ppm-treated F1 males and in the absolute brain weight in 500 ppm-treated F1 females, but no dose-dependency was found in these changes (data not shown). There were no treatment-related alterations in the histopathology of male or female reproductive organs. No significant differences were found in the number of primordial follicles in the ovary of F1 females between control and 5000 ppm groups (data not shown).

3.6. Necropsy, organ weight and histopathology of weanlings (F1 and F2)

Body weight at scheduled sacrifice and absolute and relative organ weight of male and female F1 and F2 weanlings are shown in Table 3 and 4. In either generation, 5000 ppm-treated males and females had significantly lower body weights, and the absolute and relative weights of the spleen in both sexes and of the thymus in males were significantly decreased in this 5000 ppm group. A decrease in the absolute thymus weight was also observed in F1 females given 500 and 5000 ppm and in F2 females given 5000 ppm, but there were no significant changes in relative weight in F1 or F2 females. The absolute liver weight was significantly decreased in F1 and F2 males and females, accompanied with a decrease in the relative weight in F1 males and F2 females in the 5000 ppm group. The relative weights of the brain and kidney were increased significantly in F1 and F2 males and females given 5000 ppm. Further, a significant decrease in the absolute weight of the kidney, adrenal, testis, epididymis, ovary and uterus was found at 500 and/or 5000 ppm.

External and internal gross observations did not reveal any treatment-related alterations either in F1 and F2 weanlings or pups found dead during the lactation period. No dose-related changes were found in the histopathology of the liver and spleen in both sexes and of the thymus in males in either generation.

3.7. Behavioral effects (F1)

Spontaneous locomotor activity for 10 min intervals and for a total of 60 min was not significantly different between the control and AAS-treated groups in F1 males (Fig. 4). In F1 females, a significant decrease in spontaneous activity was found during the 40–50 min and 50–60 min after the start of recording in the 500 ppm group, but no significant changes were found in total activity for 60 min in this group. There were no significant differences in spontaneous locomotor activity for 10 min intervals or for a total of 60 min between the control and the other AAS-treated groups in females. In the water-filled T-maze test, pre-test swimming trials in the straight channel revealed that all male and female F1 rats in each group could swim satisfactorily, and no significant changes were observed in the elapsed time to traverse

Table 2
Developmental findings for F1 and F2 offsprings.

AAS (ppm)	0 (control)	50	500	5000
F0 parents/F1 offspring				
No. of F0 pregnant females	24	21	24	24
No. of implantations[a]	14.7 ± 3.1	14.3 ± 2.1	15.0 ± 3.3	15.1 ± 1.5
No. of litters	24	21	24	24
No. of pups delivered[a]	13.6 ± 3.1	13.5 ± 2.5	13.8 ± 3.1	14.4 ± 1.6
Delivery index (%)[a,b]	92.4 ± 8.0	94.2 ± 10.3	92.3 ± 7.8	95.4 ± 5.4
Sex ratio of pups[c]	0.509	0.493	0.476	0.487
Viability index of pups (%)[a]				
On PND 0[d]	99.5 ± 2.7	99.0 ± 2.4	99.5 ± 1.7	99.2 ± 2.3
On PND 4[e]	98.3 ± 5.0	98.0 ± 5.4	95.6 ± 20.4	99.2 ± 2.3
On PND 21[f]	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Male pup weight during lactation (g)[a]				
On PND 0	6.93 ± 0.66	6.96 ± 0.68	6.91 ± 0.48	6.90 ± 0.69
On PND 4	11.13 ± 1.88	10.84 ± 1.47	10.72 ± 0.94	10.68 ± 1.33
On PND 7	19.14 ± 2.30	18.86 ± 2.30	18.71 ± 1.51	18.49 ± 1.70
On PND 14	38.45 ± 3.57	38.32 ± 3.96	37.88 ± 2.31	36.51 ± 2.20
On PND 21	63.83 ± 5.93	62.59 ± 7.09	61.71 ± 4.94	58.67 ± 3.91**
Female pup weight during lactation (g)[a]				
On PND 0	6.66 ± 0.82	6.57 ± 0.61	6.58 ± 0.57	6.43 ± 0.63
On PND 4	10.70 ± 2.02	10.34 ± 1.25	10.22 ± 1.13	10.13 ± 1.28
On PND 7	18.40 ± 2.49	17.96 ± 2.02	17.97 ± 1.74	17.38 ± 1.79
On PND 14	37.23 ± 3.65	36.97 ± 3.30	36.59 ± 2.74	35.07 ± 2.35*
On PND 21	61.65 ± 6.05	60.03 ± 5.55	59.34 ± 5.22	56.13 ± 4.07**
F1 parents/F2 offspring				
No. of F1 parent females	22	18	23	23
No. of implantations[a]	15.0 ± 1.6	14.7 ± 1.7	14.7 ± 3.5	14.1 ± 2.2
No. of litters	22	18	22	23
No. of pups delivered[a]	13.9 ± 1.8	13.7 ± 2.4	14.0 ± 3.8	13.5 ± 2.1
Delivery index (%)[a,b]	92.7 ± 9.4	93.0 ± 11.2	90.9 ± 20.4	95.6 ± 5.7
Sex ratio of pups[c]	0.435	0.500	0.492	0.506
Viability index of pups (%)[a]				
On PND 0[d]	98.3 ± 4.5	97.6 ± 4.2	98.9 ± 3.3	99.7 ± 1.5
On PND 4[e]	97.9 ± 6.0	99.4 ± 2.4	99.4 ± 1.9	99.0 ± 2.9
On PND 21[f]	99.4 ± 2.7	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
Male pup weight during lactation (g)[a]				
On PND 0	6.97 ± 0.62	7.03 ± 0.65	6.89 ± 0.53	6.97 ± 0.75
On PND 4	10.64 ± 1.62	11.31 ± 1.22	10.95 ± 1.32	11.16 ± 1.87
On PND 7	17.97 ± 2.18	19.19 ± 1.73	18.82 ± 1.90	18.42 ± 2.39
On PND 14	36.89 ± 3.26	38.99 ± 3.14	38.28 ± 3.26	36.40 ± 3.67
On PND 21	61.07 ± 6.06	64.40 ± 5.58	63.20 ± 5.51	58.65 ± 5.90
Female pup weight during lactation (g)[a]				
On PND 0	6.46 ± 0.47	6.68 ± 0.67	6.54 ± 0.50	6.51 ± 0.63
On PND 4	9.91 ± 1.26	10.62 ± 1.18	10.30 ± 1.20	10.59 ± 1.72
On PND 7	17.15 ± 2.06	18.28 ± 1.77	17.73 ± 1.68	17.58 ± 2.34
On PND 14	35.58 ± 3.00	37.47 ± 2.74	36.65 ± 2.69	35.20 ± 3.44
On PND 21	58.47 ± 5.33	61.83 ± 4.40	60.05 ± 3.82	56.72 ± 5.39

* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.
[a] Values are given as the mean ± S.D.
[b] Delivery index (%) = (No. of pups delivered/No. of implantations) × 100.
[c] Sex ratio = total No. of male pups/total No. of pups.
[d] Viability index on PND 0 (%) = (No. of live pups on PND 0/No. of pups delivered) × 100.
[e] Viability index on PND 4 (%) = (No. of live pups on PND 4/No. of live pups on PND 0) × 100.
[f] Viability index on PND 21 (%) = (No. of live pups on PND 21/No. of live pups on PND 4 after cull) × 100.

the straight channel (Fig. 5). On days 2–4 of the T-maze test, no significant changes were observed in the elapsed time and number of errors in both sexes.

4. Discussion

The present study was performed to provide general information concerning the effects of AAS on the integrity and performance of the male and female reproductive systems, and on the growth and development of the offspring. AAS administered via drinking water to male and female rats at 50, 500 or 5000 ppm resulted in decreased water consumption in all dose groups. This could be attributed to the astringent taste of AAS (Korea Food and Drug Administration, 2004), which would decrease the palatability of drinking water in AAS-treated groups. The change in water consumption was associated with transient decreases in food consumption in the 500 and 5000 ppm groups and in body weight in the 5000 ppm group. Nevertheless, the reproductive performance (i.e. copulation, fertility or gestation indices) was not affected up to the highest dose tested, at which average aluminium intake from food and drinking water was estimated to be 36.3–61.1 mg Al/kg bw/day. In addition, adverse effects were not found in estrous cyclicity or sperm parameters, or in the histopathology of reproductive tissues in male and female parental animals.

Previous studies demonstrated that water-soluble aluminium compounds given by oral gavage caused male reproductive toxicity, including changes in the number of spermatozoa and their motility,

Table 3
Absolute and relative organ weight of F1 male and female weanlings.

AAS (ppm)		0 (Control)	50	500	5000
Males					
No. of animals		24	20	23	24
Body weight	(g)	94.1 ± 9.1	90.8 ± 10.7	91.3 ± 9.8	80.9 ± 7.5**
Brain	(g)	1.72 ± 0.08	1.71 ± 0.07	1.70 ± 0.06	1.68 ± 0.07
	(g/100 g b.w.)	1.84 ± 0.16	1.90 ± 0.17	1.88 ± 0.16	2.09 ± 0.15**
Thymus	(mg)	392 ± 67	373 ± 72	360 ± 57	301 ± 48**
	(mg/100 g b.w.)	417 ± 61	411 ± 55	396 ± 61	372 ± 52*
Liver	(g)	4.32 ± 0.54	4.15 ± 0.55	4.12 ± 0.53	3.52 ± 0.43**
	(g/100 g b.w.)	4.58 ± 0.29	4.57 ± 0.17	4.51 ± 0.27	4.34 ± 0.25**
Kidney[a]	(g)	1.08 ± 0.13	1.04 ± 0.14	1.05 ± 0.10	0.98 ± 0.10*
	(g/100 g b.w.)	1.15 ± 0.10	1.15 ± 0.08	1.15 ± 0.06	1.21 ± 0.08*
Spleen	(mg)	421 ± 75	399 ± 66	403 ± 91	292 ± 49**
	(mg/100 g b.w.)	447 ± 64	441 ± 60	439 ± 78	361 ± 43**
Adrenal[a]	(mg)	26.4 ± 3.4	24.5 ± 2.7	25.5 ± 3.2	24.0 ± 3.4*
	(mg/100 g b.w.)	28.2 ± 3.6	27.2 ± 2.9	28.0 ± 3.0	29.8 ± 3.5
Testis[a]	(mg)	591 ± 69	571 ± 74	573 ± 72	532 ± 78*
	(mg/100 g b.w.)	628 ± 38	630 ± 41	628 ± 49	656 ± 61
Epididymis[a]	(mg)	80.7 ± 9.3	76.2 ± 10.7	78.9 ± 10.0	67.8 ± 9.9**
	(mg/100 g b.w.)	86.0 ± 8.1	84.3 ± 10.4	86.6 ± 8.3	84.2 ± 11.6
Females					
No. of animals		24	21	23	24
Body weight	(g)	87.0 ± 7.2	85.5 ± 7.6	83.3 ± 7.1	76.2 ± 7.0**
Brain	(g)	1.68 ± 0.12	1.64 ± 0.06	1.65 ± 0.06	1.62 ± 0.06
	(g/100 g b.w.)	1.93 ± 0.16	1.93 ± 0.16	1.99 ± 0.14	2.14 ± 0.16**
Thymus	(mg)	382 ± 58	365 ± 48	342 ± 51*	316 ± 41**
	(mg/100 g b.w.)	437 ± 46	429 ± 56	411 ± 54	416 ± 54
Liver	(g)	3.79 ± 0.38	3.80 ± 0.39	3.73 ± 0.42	3.28 ± 0.43**
	(g/100 g b.w.)	4.36 ± 0.38	4.45 ± 0.28	4.48 ± 0.31	4.30 ± 0.28
Kidney[a]	(g)	0.98 ± 0.10	0.97 ± 0.11	0.96 ± 0.09	0.93 ± 0.08
	(g/100 g b.w.)	1.13 ± 0.08	1.14 ± 0.06	1.15 ± 0.05	1.22 ± 0.07**
Spleen	(mg)	362 ± 63	351 ± 44	356 ± 59	272 ± 47**
	(mg/100 g b.w.)	416 ± 72	412 ± 49	428 ± 63	356 ± 46**
Adrenal[a]	(mg)	25.5 ± 3.9	23.6 ± 3.0	22.7 ± 3.0**	22.3 ± 2.6**
	(mg/100 g b.w.)	29.4 ± 4.0	27.8 ± 3.8	27.3 ± 3.5	29.4 ± 3.1
Ovary[a]	(mg)	24.6 ± 4.5	24.6 ± 4.4	23.8 ± 2.9	22.0 ± 4.0
	(mg/100 g b.w.)	28.2 ± 4.6	29.0 ± 4.6	28.9 ± 4.6	29.2 ± 6.4
Uterus	(mg)	67.3 ± 15.3	66.4 ± 21.4	64.6 ± 15.9	50.4 ± 10.9**
	(mg/100 g b.w.)	77.3 ± 16.6	77.1 ± 20.5	77.2 ± 15.8	66.2 ± 12.8

Values are given as the mean ± S.D.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.
[a] Values represent the total weights of the organs on both sides.

at much lower doses [i.e. 2.5 mg Al/kg bw/day in a 6-month exposure study in rats (Krasovskii et al., 1979) and 3.4 mg Al/kg bw/day in a 13-week exposure study in rabbits (Yousef et al., 2005)]. However, the dose-relationship demonstrated in the oral gavage studies might be significantly-inaccurate because the dietary intake of aluminium was not considered. In addition, the relevance of these oral gavage studies for human risk assessment is unclear because the toxicokinetics after a bolus dose by gavage must differ significantly from those after actual continuous exposure via the diet in humans. As for the continuous exposure studies taking into account the aluminium content in the basal diet, germinal epithelial cell degeneration and atrophy in the seminiferous tubules were observed at 75 mg Al/kg bw/day in the 26-week feeding study of SALP basic in dogs (Pettersen et al., 1990), but no such effects on male reproductive organs were detected up to 88 mg Al/kg bw/day in a similar subchronic dietary study of SALP acidic in dogs (Katz et al., 1984). Difference in outcome of these subchronic studies using dogs is considered to come from the difference in the solubility of aluminium compounds [SALP acidic is insoluble in water, and SALP basic is a mixture of 70% of a complex of SALP (sparingly soluble) and 30% of disodium phosphate (very soluble)] because it is widely assumed that insoluble aluminium compounds are less bioavailable than soluble compounds (IPCS, 2007). Considering the relationship between the solubility and bioavailability of aluminium, the present continuous exposure study using water-soluble aluminum compound could provide more reliable data on the male reproductive toxicity of aluminium.

In the present study, the preweaning body weight gain of F1 and F2 pups was depressed in the 5000 ppm group. This change could be simply attributable to decreased water consumption of dams. Since rat pups commence drinking water during the last week of the lactation period, there is also a possibility that the decreased water intake of pups inhibited their body weight gain. However, similar effects on preweaning body weight were previously reported in offspring of mice given a diet containing aluminium lactate at 500 ppm and above during the gestation and lactation periods (daily aluminium intake during lactation: 94–273 mg Al/kg bw/day)(Golub and Germann, 2001; Golub et al., 1987, 1992). In this feeding study, food consumption was decreased, but preweaning growth inhibition at 1000 ppm was greater than that of the paired fed control (Golub et al., 1987), suggesting the possibility that the fall in body weight around weaning in the present study might not be explained only by a decreased intake of water. Aluminium ingested by pups themselves and/or via maternal milk might affect preweaning growth. Maternal nursing behavior abnormality or impairment of the lactation status could be considered another possible factor.

In F1 and F2 weanlings, various organ weight changes suggestive of treatment-related effects were found, among which, decreases in the absolute weight and/or increases in the relative

Table 4
Absolute and relative organ weight of F2 male and female weanlings.

AAS (ppm)		0 (Control)	50	500	5000
Males					
No. of animals		22	18	22	23
Body weight	(g)	89.9 ± 7.5	94.1 ± 8.3	91.7 ± 7.9	82.9 ± 10.2*
Brain	(g)	1.70 ± 0.06	1.73 ± 0.06	1.70 ± 0.06	1.67 ± 0.08
	(g/100 g b.w.)	1.90 ± 0.17	1.85 ± 0.14	1.86 ± 0.12	2.04 ± 0.23*
Thymus	(mg)	375 ± 69	379 ± 50	365 ± 50	296 ± 53**
	(mg/100 g b.w.)	417 ± 65	404 ± 53	399 ± 46	359 ± 58**
Liver	(g)	4.12 ± 0.55	4.49 ± 0.53	4.34 ± 0.44	3.69 ± 0.48*
	(g/100 g b.w.)	4.57 ± 0.32	4.77 ± 0.34	4.73 ± 0.19	4.46 ± 0.20
Kidney[a]	(g)	1.02 ± 0.10	1.08 ± 0.10	1.03 ± 0.10	1.01 ± 0.13
	(g/100 g b.w.)	1.13 ± 0.08	1.15 ± 0.08	1.13 ± 0.07	1.22 ± 0.07**
Spleen	(mg)	390 ± 86	387 ± 48	393 ± 40	292 ± 52**
	(mg/100 g b.w.)	435 ± 93	413 ± 54	430 ± 41	352 ± 46**
Adrenal[a]	(mg)	26.0 ± 3.8	25.2 ± 3.5	25.5 ± 3.3	24.7 ± 4.3
	(mg/100 g b.w.)	29.0 ± 4.1	26.7 ± 3.3	28.0 ± 3.9	29.8 ± 3.0
Testis[a]	(mg)	546 ± 83	571 ± 83	572 ± 70	515 ± 67
	(mg/100 g b.w.)	607 ± 72	605 ± 59	623 ± 51	624 ± 65
Epididymis[a]	(mg)	74.8 ± 7.5	76.1 ± 10.6	75.1 ± 9.9	68.7 ± 9.1
	(mg/100 g b.w.)	83.7 ± 10.2	80.7 ± 7.5	82.0 ± 9.0	83.6 ± 11.5
Females					
No. of animals		22	18	22	23
Body weight	(g)	85.3 ± 7.2	87.6 ± 6.5	84.0 ± 4.9	77.2 ± 5.7**
Brain	(g)	1.65 ± 0.05	1.65 ± 0.06	1.64 ± 0.06	1.62 ± 0.06
	(% of body weight)	1.95 ± 0.16	1.89 ± 0.13	1.95 ± 0.08	2.11 ± 0.15**
Thymus	(mg)	367 ± 68	354 ± 60	352 ± 47	300 ± 40**
	(mg/100 g b.w.)	432 ± 84	405 ± 66	419 ± 48	390 ± 55
Liver	(g)	3.93 ± 0.41	3.97 ± 0.37	3.80 ± 0.33	3.33 ± 0.34**
	(g/100 g b.w.)	4.61 ± 0.25	4.54 ± 0.19	4.52 ± 0.25	4.31 ± 0.31**
Kidney[a]	(g)	0.96 ± 0.08	0.97 ± 0.09	0.94 ± 0.06	0.93 ± 0.08
	(g/100 g b.w.)	1.13 ± 0.07	1.11 ± 0.07	1.12 ± 0.05	1.21 ± 0.07**
Spleen	(mg)	355 ± 53	330 ± 33	349 ± 52	276 ± 35**
	(mg/100 g b.w.)	416 ± 51	378 ± 35*	415 ± 59	358 ± 42**
Adrenal[a]	(mg)	23.3 ± 2.3	23.2 ± 2.3	23.2 ± 3.4	23.2 ± 2.4
	(mg/100 g b.w.)	27.4 ± 2.7	26.6 ± 2.9	27.5 ± 3.5	30.0 ± 3.1*
Ovary[a]	(mg)	24.6 ± 3.0	24.9 ± 4.0	24.2 ± 4.1	20.4 ± 3.2**
	(mg/100 g b.w.)	29.1 ± 4.3	28.5 ± 4.0	29.0 ± 5.5	26.7 ± 4.9
Uterus	(mg)	71.0 ± 55.7	66.8 ± 16.5	58.5 ± 11.8	53.5 ± 11.1*
	(mg/100 g b.w.)	82.3 ± 59.4	76.0 ± 15.4	69.6 ± 12.4	69.5 ± 14.8

Values are given as the mean ± S.D.
* Significantly different from the control, $P < 0.05$.
** Significantly different from the control, $P < 0.01$.
[a] Values represent the total weights of the organs of both sides.

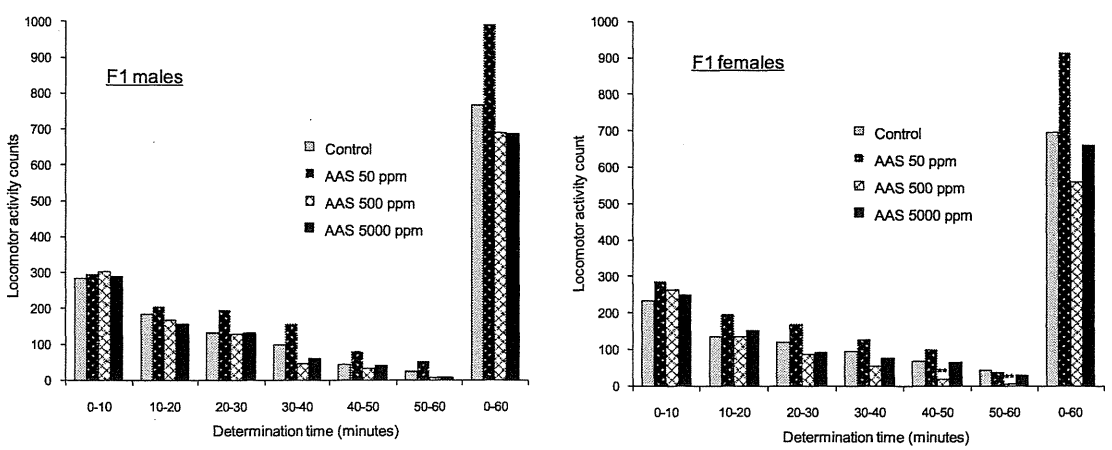

Fig. 4. Locomotor activity count in F1 parental rats. Data are presented as the mean of 10 animals/sex/group. **Significantly different from the control, $P < 0.01$.

weight of the brain, kidney, adrenal, testis, epididymis, ovary, and uterus are considered to be secondary changes that occur with the fall of body weight. On the other hand, decreases in the absolute and relative weights of the liver and spleen in both sexes and in the absolute and relative thymus weights in males in the 5000 ppm group could not be explained only by the fall of body weight. In these organs, no histopathological changes were detected, and further, there were no changes in the weights of these organs in F0 or F1 adults. Previously, Golub et al. (1987) demonstrated that gestational and lactational exposure of mice to excessive dietary aluminium (1000 ppm as aluminium lactate) markedly decreased the spleen weight of offspring at weaning age.

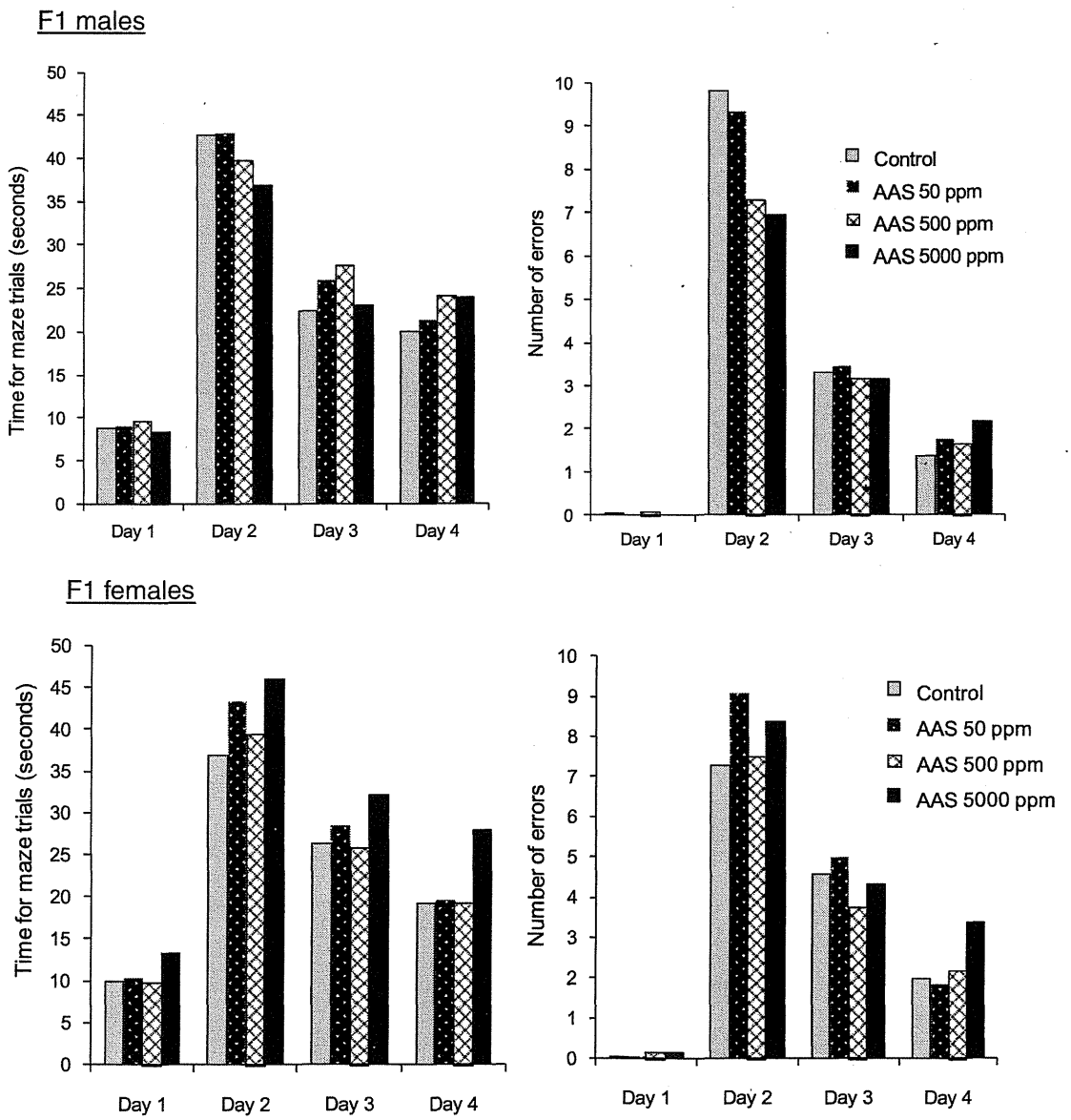

Fig. 5. Performance in a water-filled multiple T-maze in F1 parental rats. Data are presented as the mean of 10 animals/sex/group. There were no statistically significant differences between the control and AAS-treated groups.

Decreased concentrations of interleukin-2, interferon-γ and tumor necrosis factor-α in spleen cells were reported in mice exposed to a similar level of aluminum lactate via the diet from conception through 6 months of age (Golub et al., 1993). Although the association between such immunosuppressive effects and decreased spleen weight of weanlings is unclear, aluminium might have a certain effect on the developing spleen. Further study would be required to clarify the effects of developmental aluminium exposure on splenic function, including immune function. In the above-mentioned study conducted by Golub et al. (1987), dietary aluminium exposure did not affect the liver and thymus weight of weanlings significantly; therefore, the decreased weight of these organs observed in the present study might have resulted from reduced water consumption rather than ingested aluminium because water is essential for organ growth.

Vaginal opening is the initial sign of the estrogenic rise that accompanies the first ovulation followed by estrous cyclicity as the initial sign of the central drive of ovarian activity (Ramirez and Sawyer, 1965; Rasier et al., 2006); it is widely used as a marker of female puberty. In the present study, vaginal opening was slightly delayed (mean = 2.1 days) in F1 females at 5000 ppm and the age at completion was outside the normal range for this strain of rat in the laboratory in which the study was conducted (historical control data for the last seven years: 29.6–31.0 days). Although it is well known that decreased body weight can result in non-specific delays in puberty, the body weight at the time of vaginal opening was slightly heavier in the 5000 ppm group than in the control in the present study. Delayed age at vaginal opening is known to be caused by fetal and/or postnatal exposure to various chemicals disrupting steroid functions or hypothalamic–pituitary functions (Goldman et al., 2000; Rasier et al., 2006). The putative/suggested mechanism includes blockage of the response of estrogen-dependent tissues to the ovarian steroid hormone (lindane) (Cooper et al., 1989), inhibition of steroid synthesis (ketoconazole and fadrozole) (Marty et al., 1999) and decreased gonadotropin levels (luteinizing hormone-releasing hormone

antagonist, Org30276) (Meijs-Roelofs et al., 1990). In the present study, rats with delayed vaginal opening progressed to showing normal reproductive capacity and outcome. In addition, no effects were found on AGD, estrous cyclicity or on the weight and histopathology of reproductive organs in weanlings and adults. It seems unlikely that aluminium has a clear impact on hormonal events. In order to clarify the etiology of this slight delay in female sexual maturation, further studies are required.

Our previous two-generation study of aluminium sulfate administered via drinking water to rats gave the same results regarding parental toxicity and reproductive/developmental toxicity as the present study (Hirata-Koizumi et al., 2011); reduced water consumption in all 120, 600 and 3000 ppm groups (respective calculated aluminium intake: 2.96–4.72, 8.06–14.0, 31.2–55.6 mg Al/kg bw/day), and decreased body weight of parental animals, inhibition of preweaning body weight gain, decreased liver and spleen weight of weanlings, and a slight delay of vaginal opening in the highest dose group. In this two-generation study of aluminium sulfate, as well as in the present study of AAS, no treatment-related changes were found in reflex ontogeny, spontaneous locomotor activity or performance in a water-filled multiple T-maze, indicating that previous findings of developmental neurobehavioral effects were possibly related to the toxic effects of aluminium given at higher doses than those given in these two-generation studies. Some developmental effects observed in these two-generation studies could be considered to come from ionized aluminium in drinking water, but there is also a possibility that these are secondary effects due to decreased water consumption. In order to reach more definitive conclusions, further study including paired-comparison data is required to assess the effects of decreased water intake in the absence of AAS or aluminium sulfate exposure. Conservative evaluation of the present data led to the conclusion that the no observed adverse effect level of AAS for two-generation reproductive/developmental toxicity in rats is 500 ppm (5.35 mg Al/kg bw/day) primarily based on the effect on preweaning body weight gain.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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A chemical category approach of genotoxicity studies for branched alkylphenols

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A chemical category is a group of chemicals whose toxicological properties are expected to be similar or follow a regular pattern as a result of structural similarity. The category approach is beneficial for decreasing in the resource of risk assessment for huge amount of unevaluated existing chemicals, and also in the use of all kinds of animal tests including even *in vivo* genotoxicity tests from a point of view of the animal welfare. The present paper reports the results of *in vivo* micronucleus tests of *o-sec*-butylphenol (CAS : 89-72-5) and 2-isopropyl-5-methylphenol (CAS : 89-83-8) and discusses genotoxic potential of seven alkylphenols, *o-sec*-butylphenol, 2-isopropyl-5-methylphenol, *p-sec*-butylphenol (CAS : 99-71-8), 2-*tert*-butylphenol (CAS : 88-18-6), 2, 4-di-*tert*-butylphenol (CAS : 96-76-4), 4-*tert*-butylphenol (CAS : 98-54-4) and 6-*tert*-butyl-*m*-cresole (CAS : 88-60-8) by the category approach. Based on the negative results of *in vivo* micronucleus tests, it can be concluded that these category chemicals are not likely clastogenic *in vivo*. Further *in vivo* micronucleus assays on untested substances may not be required by using the category approach, but further supporting information such as physicochemical profiles and (Q) SAR predictions may be necessary to strengthen the rationale for the category approach.

Keywords : category approach, alkylphenol, genotoxicity

Introduction

A chemical category is a group of chemicals whose toxicological properties are expected to be similar or follow a regular pattern as a result of structural similarity. A category approach is used in many chemical programmes such as the OECD High Production Volume (HPV) programme[1], the US HPV Challenge programme[2] and the EU Existing Substances programme[3]. The overall data set can allow the estimation of the hazard for the untested endpoints. Data gap filing can be done from one or more tested chemicals to an untested chemical. The category approach is effective for hazard identification and hazard estimation, and it is beneficial for decreasing in the resource of risk assessment for huge amount of unevaluated existing chemicals, and also in the use of all kinds of animal tests including even *in vivo* genotoxicity tests from a point of view of the animal welfare.

Structurally similar alkylphenols shown in Table1 are listed in the most recent OECD HPV List of chemicals to be investigated for environment and human health effects[4] and were selected as target substances for the Safety Examination of Existing Chemicals in Japan in order to obtain reliable information in compliance with the OECD Test Guidelines and in accordance with the principles for GLP[5]. Of these chemicals, 4-*tert*-butylphenol (CAS : 98-54-4) and 6-*tert*-butyl-*m*-cresole (CAS : 88-60-8) were already assessed under the OECD HPV programme[6]. In the OECD HPV programme, screening information data sets (SIDS) for at least two different genotoxic endpoints have been required for the initial assessment[7], and the Ames assays and *in vitro* chromosome aberration assays for these chemicals were performed.

Table 1 shows summary results of genotoxicity studies of the branched alkylphenols. All the chemicals showed negative results in the Ames assays with and

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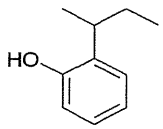
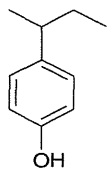
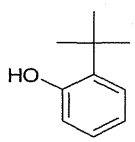
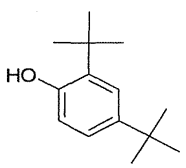
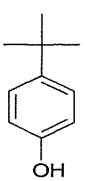
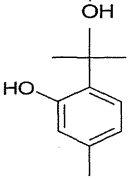
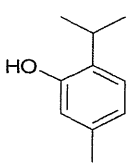
Table 1 Summary results of genotoxicity studies of branched alkylphenols conducted under the Safety Examination of Existing Chemicals in Japan

Substance name (CAS No.)	Structure	S9	Ames	In vitro Chromosome Aberration		In vivo micronucleus
				[C][a]	[P][b]	
o-sec-Butylphenol (89-72-5)		w/o	−	+	−	[current study]
		with	−	+	−	
p-sec-Butylphenol (99-71-8)		w/o	−	±	−	
		with	−	±	−	
2-*tert*-Butylphenol (88-18-6)		w/o	−	−	−	−[e]
		with	−	+	+	
2,4-di-*tert*-Butylphenol (96-76-4)		w/o	−	−	−	
		with	−	+	−	
4-*tert*-Butylphenol[c] (98-54-4)		w/o	−	−	+	−
		with	−	+	+	
6-*tert*-Butyl-*m*-cresole[c] (88-60-8)		w/o	−	−	−	−[d]
		with	−	+	−	
2-Isopropyl-5-methylphenol (89-83-8)		w/o	−	−	−	[current study]
		with	−	+	−	

[a] [C] : Clastogenicity ; [b] [P] : Polyploidy ; [c] The initial assessment of the chemical was already assessed under the OECD HPV programme ; [d] The in vivo micronucleus test was carried out by the Chemicals Evaluation and Research Institute, Japan. ; [e] The result form the German Chemical Society-Advisory Committee on Existing Chemicals of Environmental Relevance (Beratergremium fur Umweltrevante Alstoffe : BUA)[21]

without metabolic activation. On the other hand, an equivocal result on *p-sec*-butylphenol (CAS: 99-71-8) and positive results on the other six chemicals were observed for clastogenicity in the *in vitro* chromosome aberration assays with and/or without metabolic activation. Polyploidy was also observed for 2-*tert*-butylphenol (CAS : 88-18-6) and 4-*tert*-butylphenol with and/or without metabolic activation. However, *in vivo* micronucleus tests on 4-*tert*-butylphenol (not publically available) and 6-*tert*-butyl-*m*-cresole[6] showed negative results, and these findings seem to suggest that these branched alkylphenols can be non-genotoxic *in vivo* although *in*

vitro genotoxicity is equivocal.

These branched alkylphenols are widely used as antioxidants in rubbers, plastics, foods and oils to inhibit or slow oxidative process, and they are also used as intermediates for synthesis of resins, plasticizers, surface-active agents, perfumes and other products[8]. Consumer exposure to these branched alkylphenols can occur through the use of products containing these chemicals. The similarities in structure, use and *in vitro* genotoxicity seem to support grouping these chemicals into one category. To ascertain robustness of the chemical category of these branched alkylphenols on genotoxicity, additional *in vivo* micronucleus tests were assessed on 2-isopropyl-5-methylphenol (CAS: 89-83-8) as a target of isopropyl substitutions and *o-sec*-butylphenol (CAS: 89-72-5) as a target of *sec*-butyl substitutions. The present paper reports the results of *in vivo* micronucleus tests of 2-isopropyl-5-methylphenol and *o-sec*-butylphenol and discusses genotoxic potential of these chemicals by the category approach.

Materials and Methods

In vivo micronucleus test in mouse bone marrow

The test was performed according to the Guideline for Screening Mutagenicity Testing of Chemicals, Japan and OECD TG 474[9] and in compliance with GLP requirements[5]. 2-Isopropyl-5-methylphenol (Purity> 98%; Lot No.CAN1119) was obtained from Wako Pure Chemical Industries, Ltd., Japan and cyclophosphamide (CAS No.50-18-0; Lot No.73H0846) obtained from Sigma Chemical Co. was used as a positive control. Crj: BDF1 mice, 8-weeks of age, from Charles River Laboratories, Japan were used after more than 1 week acclimatization. Mice were housed in a temperature-and humidity-controlled room (23±1℃; 55±5%) with a light-dark (12 h-12 h) cycle. In a dose finding study, 5 male and 5 female mice were singly given 2-isopropyl-5-methylphenol by gavage at 500, 750, 1000, 1250, 1750 or 2000 mg/kg bw, in which deaths were observed at 1500 and 1750 mg/kg bw in females while no death was observed in males for four days (data not shown). Subsequently, a single dose of 2-isopropyl-5-methylphenol at 1500-2000 mg/kg bw caused deaths in males in a preliminary study (data not shown); therefore, 1250 mg/kg bw was set as the highest dose in the main test. Sampling time was set at 24 h after administration according to the preliminary study, in which no differences were observed in a sampling time of 24, 48, or 72 h.

Mice (5/sex/dose) were received single oral gavage administration of 2-isopropyl-5-methylphenol at 0 (control: olive oil), 156.3, 312.5, 625, or 1250 mg/kg bw. Positive control mice (5/sex) received single oral doses of cyclophosphamide at 50 mg/kg bw. All groups of mice were killed 24 h after treatment. Bone marrow samples were prepared according to the method of Schmid[10,11] for the control, 312.5, 625, or 1250 mg/kg bw groups. Samples were stained with 0.04 mg/mL acridine orange. According to the method of Hayashi et al.[12], the incidence of micronuclei was determined. Two thousand polychromatic erythrocytes (PCE) for each animal were observed for the incidence of micronucleated erythrocytes, and the proportion of PCE among the total erythrocyte population was also determined from a sample of 500 total erythrocytes for each animal.

Data were analyzed using the Fisher's exact test with Bonferroni correction for group mean comparisons[13]. Dose-dependent increases of the number of micronucleated polychromatic erythrocyte per total number of PCE (MNPCE) was detected using the Cochran-Armitage test[14]. Proportion of PCE among the total erythrocyte population was analyzed by the t-test with Bonferroni correction.

In vivo micronucleus test in rat bone marrow

The test was performed according to OECD TG 474[9] and Guideline for Genotoxicity Tests on Drugs[15], and in compliance with GLP requirements[5]. *o-sec*-Butylphenol (Purity 99.15%) was obtained from Honshu Chemical Industry, Japan and cyclophosphamide monohydrate (CAS No.6055-199-2; Lot No.036K1225) obtained from Sigma-Aldrich Co. was used as a positive control. Crl: CD (SD) rats, 7-weeks of age, from Charles River Laboratories, Japan were used after one week of acclimatization. Rats were housed in a temperature-and humidity-controlled room (21.8-22.9℃; 46.6-62.2%) with a light-dark (12 h-12 h) cycle. The animals were given commercial food and water *ad libitum*. In a dose finding study, 3 male and 3 female rats were given *o-sec*-butylphenol by gavage at 150, 300, 600, 1200 mg/kg bw once a day for two days (24 h interval), in which deaths were observed at 1200 mg/kg bw/day and clinical changes were observed at 600 mg/kg bw/day in both sex (data not shown). Therefore, 600 mg/kg bw was set as the highest dose.

Table 2 Results of the micronucleus test in mice after gavage dose of 2-isopropyl-5-methylphenol (CAS: 89-83-8)

Dose	Number of mice	MNPCE (%)[a]	PCE/ (PCE+NCE) (%)[b]
Male			
0 mg/kg (Solvent control: olive oil)	5	0.12 ± 0.08[c]	48.6 ± 8.6
312.5 mg/kg	5	0.20 ± 0.10	55.7 ± 5.4
625 mg/kg	5	0.19 ± 0.16	48.2 ± 12.3
1250 mg/kg	5	0.15 ± 0.12	53.6 ± 10.5
50 mg/kg (Positive control: CP)	5	1.57 ± 0.70*	45.6 ± 13.1
Female			
0 mg/kg (Solvent control: olive oil)	5	0.17 ± 0.14	63.8 ± 4.8
312.5 mg/kg	5	0.14 ± 0.07	60.6 ± 8.0
625 mg/kg	5	0.15 ± 0.09	62.8 ± 4.8
1250 mg/kg	5	0.11 ± 0.04	64.2 ± 8.2
50 mg/kg (Positive control: CP)	5	1.43 ± 0.35*	54.9 ± 6.2

PCE: Polychromatic erythrocyte, MNPCE: Micronucleated PCE, NCE: Normochromatic erythrocyte, CP: Cyclophosphamide
*: Significantly different from the solvent control ($P<0.01$)
[a]: Number of micronucleated polychromatic erythrocytes/ total number of polychromatic erythrocytes observed.
[b]: Number of polychromatic erythrocytes/ total number of erythrocytes observed.
[c]: Values are given as mean ± S. D.

All rats were weighed prior to dosing and preparation of bone marrow samples. Clinical signs of toxicity were observed at 1 and 3 h after treatment, and prior to dosing and preparation of bone marrow samples. Rats (5/sex/dose) were received oral gavage administration of *o-sec*-butylphenol twice with 24 h intervals at 0 (control: corn oil), 75, 150, 300 or 600 mg/kg bw. Positive control rats (5/sex) received two oral doses of cyclophosphamide (24 h intervals) at 20 mg/kg bw/day.

All groups of rats were killed 24 h after last treatment. One femur was removed from each rat, and bone marrow cells were flushed out with 10% neutral buffer formalin. Excess serum was removed by centrifugation. Bone marrow samples were stained with 0.05 w/v% acridine orange. According to the method of Hayashi et al.[12], the incidence of micronuclei was determined. Two thousand PCE for each animal were observed for the incidence of micronucleated erythrocytes, and the proportion of PCE among the total erythrocyte population was also determined from a sample of 1000 total erythrocytes for each animal.

Data were analyzed using the Kastenbaum and Bowman's method[16] for group mean comparisons. Dose-dependent increases of the MNPCE were detected using the Cochran-Armitage test[17]. Body weight and proportion of PCE among the total erythrocyte population were analyzed by the MiTOX® (Mitsui Engineering & Shipbuilding Co., Ltd).

Results

In vivo micronucleus test in mouse bone marrow after gavage dose

Table 2 shows a result of the micronucleus test in mice after gavage doses of 2-isopropyl-5-methylphenol. There were no deaths at any doses of 2-isopropyl-5-methylphenol although signs of toxicity were observed at 1250 mg/kg bw. A frequency of MNPCE was not significantly increased in males and females up to the dose of 1250 mg/kg bw while a frequency of MNPCE was significantly increased in the positive controls in both sexes. Proportion of PCE among the total erythrocyte populations was not changed in any dosing groups.

In vivo micronucleus test in rat bone marrow after gavage dose

Table 3 shows a result of the micronucleus test in rats after gavage doses of *o-sec*-butylphenol. One male showed diarrhea, and two males showed ataxic gait and a decrease in locomotor activity at 600 mg/kg bw/day. Four females showed ataxic gait and three of them also showed a decrease in locomotor activity at 600 mg/kg bw/day. One female in the 300 mg/kg bw/day group died before the sampling due to the incorrect administration. Body weights were not statistically changed in both sexes at any doses. A frequency of MNPCE was not changed in females at any doses. On the other hand, gavage dose of *o-sec*-butylphenol significantly increased a frequency of MNPCE compared to the solvent control

Table 3 Results of the micronucleus test in rats after gavage dose of *o-sec*-butylphenol (CAS: 89-72-5)

Dose	Number of mice	MNPCE (%)[a]	PCE/(PCE+NCE) (%)[b]
Male			
0 mg/kg (Solvent control: corn oil	5	0.06 ± 0.08[c]	51.0 ± 5.2
150 mg/kg	5	0.10 ± 0.05	54.7 ± 3.4
300 mg/kg	5	0.14 ± 0.09	52.9 ± 5.1
600 mg/kg	5	0.20 ± 0.05*[d]	56.6 ± 1.4
20 mg/kg (Positive control: CP)	5	5.45 ± 1.25*	44.7 ± 5.1
Female			
0 mg/kg (Solvent control: corn oil)	5	0.11 ± 0.05	55.0 ± 5.7
150 mg/kg	5	0.13 ± 0.08	57.0 ± 3.5
300 mg/kg	4[e]	0.10 ± 0.07	53.8 ± 5.8
600 mg/kg	5	0.11 ± 0.04	52.7 ± 3.0
20 mg/kg (Positive control: CP)	5	3.19 ± 1.30*	25.8 ± 4.0*

PCE: Polychromatic erythrocytes, MNPCE: Micronucleated PCE, NCE: Normochromatic erythrocyte, CP: Cyclophosphamide
*: Significantly different from the solvent control ($P<0.05$)
[a]: Number of micronucleated polychromatic erythrocytes/ total number of polychromatic erythrocytes observed.
[b]: Number of polychromatic erythrocytes/ total number of erythrocytes observed.
[c]: Values are given as mean ± S. D.
[d]: The frequency of MNPCE (0.20 ± 0.05%) was within background control data from 2001 to 2007 of the laboratory (Mean ± 3SD=0.13 ± 0.24%; n=449).
[e]: One female in the 300 mg/kg bw/day group died before the sampling due to the incorrect administration.

at 600 mg/kg bw/day in males. Proportion of PCE among the total erythrocyte populations was not changed.

Discussion

Equivocal results on *in vitro* genotoxicity of branched alkylphenols were obtained in the previous studies. Müller and Sofuni[18] indicated that some chemicals produce chromosome aberration *in vitro* but do not produce positive results in Ames assays. The clastogenic response of such chemicals is often associated with high cytotoxicity[19], high osmolality and pH extremes[20]. There are also chemicals that show positive results in the *in vitro* chromosome aberration tests but negative in the rodent micronucleus tests. The numerical proportions of positive results in the Ames assays, *in vitro* chromosome aberration assays and *in vivo* micronucleus assays were reported to be 7.7% (23/298), 28.9% (77/266) and 6.7% (19/283), respectively in pharmaceutical chemicals[18]. To ascertain if genotoxic potential of branched alkylphenols can be expressed in animals, additional *in vivo* micronucleus tests were performed on 2-isopropyl-5-methylphenol and *o-sec*-butylphenol.

After gavage doses of 2-isopropyl-5-methylphenol, a frequency of MNPCE was not significantly increased in males and females up to 1250 mg/kg bw while a frequency of MNPCE was significantly increased in the positive controls in both sexes. Proportion of PCE among the total erythrocyte populations was not changed; indicating inhibition of bone marrow cell proliferation was not induced under the test conditions. These results indicate that 2-isopropyl-5-methylphenol does not induce genotoxic effects *in vivo*.

After gavage doses of *o-sec*-butylphenol, a frequency of MNPCE was not changed in females at any doses. In contrast, dose of *o-sec*-butylphenol significantly increased a frequency of MNPCE compared to the solvent control at 600 mg/kg bw/day in males. However, the frequency of MNPCE (0.20 ± 0.05%) was within background control data from 2001 to 2007 of the laboratory (Mean ± 3SD=0.13 ± 0.24%; n=449). Therefore, the increase in MNPCE was considered to be due to low MNPCE in the control group. Proportion of PCE among the total erythrocyte populations was not changed; indicating inhibition of bone marrow cell proliferation was not induced under the test conditions. These results indicate that *o-sec*-butylphenol does not induce genotoxic effects *in vivo*.

The previous assessments under the HPV programme also showed that gavage doses of 6-*tert*-butyl-m-cresole up to 125 mg/kg bw, the maximum tolerated dose, did not induce micronucleus in bone marrow cells nor suppress their proliferation in ICR mice[6], and 4-*tert*-butylphenol did not induce micronucleus in bone

Table 4 The category approach on genotoxicity of alkylphenols

Substance name	Molecular weight	Log Kow[a]	Ames	In vitro Chromosome Aberration	In vivo micronucleus
o-sec-Butylphenol	150.22	3.27	negative	positive	negative (current study)
p-sec-Butylphenol	150.22	3.08	negative	equivocal	negative (read across)
2-*tert*-Butylphenol	150.22	3.31	negative	positive	negative[b]
2, 4-di-*tert*-Butylphenol	206.32	5.19	negative	positive	negative (read across)
4-*tert*-Butylphenol	150.22	2.4-3.4	negative	positive	negative
6-*tert*-Butyl-*m*-cresole	164.24	4.11	negative	positive	negative[c]
2-Isopropyl-5-methylphenol	150.22	3.3	negative	positive	negative (current study)

[a] Data from NITE (2010)[31] and OECD (2010)[6].
[b] Data from BUA (2003)[21].
[c] Data from OECD (2010)[6].

marrow cells nor suppress their proliferation at up to the maximum tolerated dose of 50 mg/kg bw in ICR mice (not publically available). In addition, the German Chemical Society-Advisory Committee on Existing Chemicals of Environmental Relevance (Beratergremium fur Umweltrevante Alstoffe: BUA) also stated that 2-*tert*-butylphenol does not induce any micronuclei in the bone marrow of mice at toxic dosages *in vivo*, while it is non-mutagenic in bacteria but is clastogenic *in vitro* in mammalian cells[21].

There are numerous reasons why activity shown *in vitro* may not be observed *in vivo*; for example, lack of absorption, inability of the active metabolite to reach DNA, rapid detoxication and elimination[22]. There are only a few data available on toxicokinetics for the whole body of these branched alkylphenols, but no direct information in the target cells of bone marrow. 4-*tert*-Butylphenol was rapidly excreted as glucouronide and sulfate conjugates in urine and feces in rats[23,24]. In workers handling 4-*tert*-butylphenol, most of the chemical was excreted within 24 hours, and metabolites in the urine was correlated with exposure levels of the chemical[25]. 2-Isopropyl-5-methylphenol is readily absorbed from the intestine and excreted rapidly as glucouronide and sulfate conjugates in humans, dogs, rabbits and rats[26-28]. After a single dose of 2-isopropyl-5-methylphenol, peak plasma concentrations were reached after 2 hours and eliminations half-life was 10.2 hours in humans. Sulphate and glucronide conjugates of 2-isopropyl-5-methylphenol, but not free 2-isopropyl-5-methylphenol, were corrected in urine.

The physicochemical properties and chemical structure can be used to make some predictions regarding the ADME of substances. A range of Log Kow of these category chemicals is 2.4-5.19 (Table 4), which suggests that the substances could readily absorbed and distributed in physiological fluids[29]. The alkylphenols are expected to have slightly higher acid dissociation constants (pKa) than phenol (pKa 10.0 at 25℃); therefore, will not be ionized significantly at physiological pH's[30]. Alkylphenols which contains phenol moieties are likely to undergo Phase II conjugation and systemic exposure to unchanged substance may be limited[29]. Based on available data, the rapid conjugation and excretion of these chemicals may explain why genotoxicity was not observed *in vivo* although *in vitro* clastogenicity was increased with S9 mix. However, there is a possibility that active metabolites did not reach the target cells of born marrow at high concentration and could react to chromosomes in hepatic cells. An *in vivo* genotoxic assay for hepatic cells may be useful for further evaluation.

In the present paper, we showed that 2-isopropyl-5-methylphenol and *o-sec*-butylphenol were not clastogenic *in vivo* under the test conditions, and existing data also showed that 6-*tert*-butyl-*m*-cresole, 4-*tert*-butylphenol and 2-*tert*-butylphenol were not clastogenic *in vivo*[6,21]. Based on the weight of evidence, it can be concluded that these branched alkylphenols are not genotoxic *in vivo* (Table 4). The use of the category approach is useful to identify common or trend properties of members of the category and to use measured data to similar untested chemicals without further testing to fill data gap. In conclusion, further *in vivo* micronucleus assays on *p-sec*-butylphenol and 2,4-di-*tert*-butylphenol may not be required by using the category approach, but further supporting information such as physicochemical profiles and (Q)

SAR predictions may be necessary to strengthen the rationale for the category approach.

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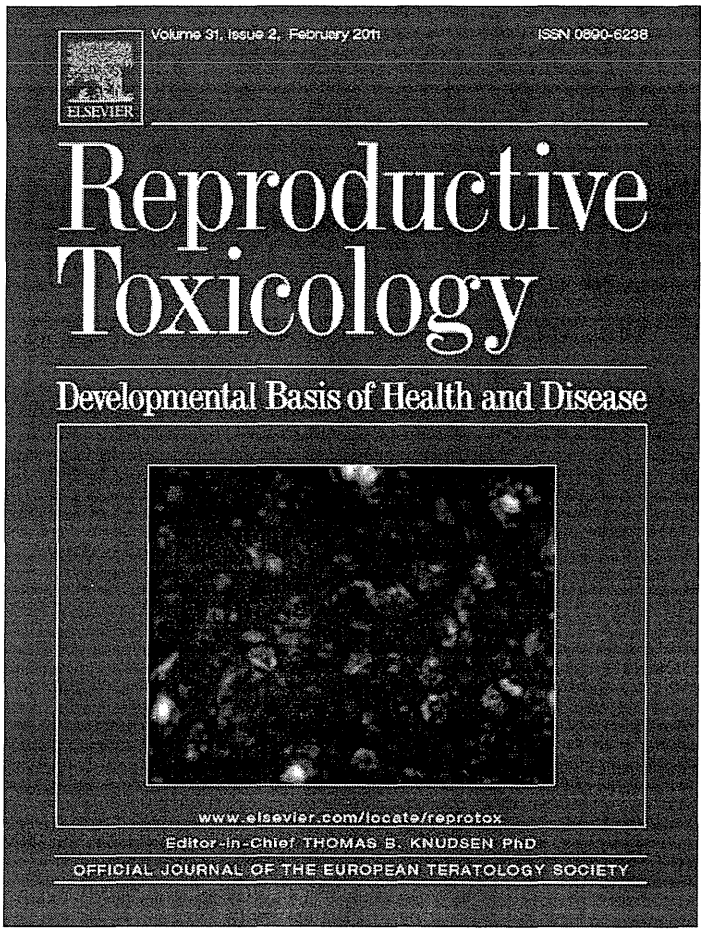

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Two-generation reproductive toxicity study of aluminium sulfate in rats

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ABSTRACT

In a two-generation reproductive toxicity study, male and female rats were given aluminium sulfate (AS) in drinking water at 0, 120, 600 or 3000 ppm. AS reduced water consumption in all treatment groups, and body weight was transiently decreased in the 3000 ppm group. In the F1 and F2 pups, preweaning body weight gain was inhibited at 3000 ppm, and the liver and spleen weight was decreased at weaning. At this dose, vaginal opening was slightly delayed. There were no compound-related changes in other reproductive/developmental parameters, including developmental neurobehavioral endpoints. The data indicated that the NOAEL of AS in this two-generation study is 600 ppm for parental systemic toxicity and reproductive/developmental toxicity. The total ingested dose of aluminium from drinking water and food (standard rat diet, containing 25–29 ppm of aluminium) combined for this 600 ppm group was calculated to be 8.06 mg Al/kg bw/day.

1. Introduction

Aluminium is the most abundant metal on Earth and constitutes 8.13% of the crust [1]. It is released into the environment largely by natural processes, but also due to anthropogenic activities [2]. People engaging in certain occupations, such as welding, aluminium soldering and production of abrasives, could be exposed to aluminium-containing dust particles by inhalation [3–5]; however, aluminium exposure by the general population is considered to occur mainly through food ingestion [1] although the use of aluminium-containing antacids and buffered analgesics may result in much higher aluminium intake [6,7]. While aluminium is inherently contained in most foodstuffs, its salts are artificially added to various food products (acidity regulator, raising agent, anti-caking agent, etc.) [8]. Use of aluminium and aluminium compounds in the processing, packaging and storage of food products is also a significant factor in the increased aluminium levels in foods [8]. On the other hand, aluminium salts are widely used as flocculants in the treatment of drinking water to reduce organic matter, color, turbidity and microorganism levels [9], which may lead to increased aluminium intake by the general public. Total dietary exposure to aluminium, including exposure via drinking water, has been assessed using a duplicate diet, total diet or market basket approach in a number of countries [8]. Based on these data, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) estimates that the mean total dietary exposure of the adult population ranges from 14 to 280 mg Al/week [8].

In humans, aluminium is regarded as a primary cause of dialysis encephalopathy syndrome, in which various neurological symptoms, such as speech difficulty, myoclonus and dementia, have been observed in patients on chronic hemodialysis [10,11]. For more general exposure, it is suspected that oral aluminium exposure via foods and drinking water may be associated with the risk of Alzheimer's disease and cognitive impairment, but this hypothesis remains controversial [12–14]. The neurotoxicological properties of aluminium have been clearly shown in laboratory animals, and the observed effects include encephalopathy, impairments of cognitive and motor function and neurofibrillary degeneration [15–18]. In animals, aluminium compounds also affect male reproductive systems [19–23], and developmental toxicity, including effects on the developing nervous system, has been reported after maternal exposure [24–32].

Concerning the adverse effects of aluminium on human health, its reference values in food and drinking water should be established based on appropriate toxicological data; however, the available data are insufficient to assess its health effects. As human data, there have been a number of epidemiological studies about

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the neurological effects of aluminium exposure via drinking water, but these studies did not account for aluminium intake from food, which is the most important route of exposure. Epidemiological studies on dietary aluminium exposure are preliminary at this time [8]. As for animal studies, most have focused on the specific endpoints or mechanisms of action, and the dosage is insufficient for dose–response assessment. In addition, considering the low oral bioavailability of aluminium [33,34] and actual human exposure via food and drinking water, many available study results from administration by gavage as well as by the parenteral route are not appropriate to evaluate the risk. In the WHO guidelines for drinking water quality, it was concluded that a health-based guideline value cannot be derived because of limitations in the animal data as a model for humans and the uncertainty surrounding human data [9]. JECFA clearly stated the need for further data on the bioavailability and developmental and multigenerational toxicity while it established a provisional tolerable weekly intake (PTWI) for aluminium of 1 mg/kg bw in food based on the available toxicological information [8].

In the present study, a two-generation reproductive toxicity study was conducted for aluminium sulfate (AS). AS is a water-soluble salt of aluminium, and is primarily used as a flocculant for water purification, paper sizing agent, fire extinguisher materials, etc. [35,36]. The present study was conducted according to OECD test guidelines under GLP. The selected route of administration is via drinking water because it is relevant to human exposure. As for the reproductive toxicity of aluminium, oral exposure studies evaluating sufficient endpoints in both sexes as well as multigenerational studies have not been reported yet; therefore, the data presented would provide useful information to assess the risk to human health from aluminium exposure.

2. Materials and methods

This study was conducted in 2008–2009 at the Safety Research Institute for Chemical Compounds Co., Ltd. (Sapporo, Japan). The study design complied with the OECD guideline 416 "Two-generation reproduction toxicity study" [37], and the Japanese guidelines for the designation of food additives and for revision of standards for the use of food additives [38]. All procedures involving the use and care of animals were performed in accordance with the principles for Good Laboratory Practice [39,40] and applicable animal welfare regulations ["Act on Welfare and Management of Animals" [41,42], "Standards Relating to the Care, Management of Laboratory Animals and Relief of Pain" [43] 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" [44]].

2.1. Chemical and dosing

AS (CAS No. 10043-01-3) was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). The AS (Lot No. 007X1828) used in this study was 98.5% pure, and was kept in a sealed container under cool and dark conditions. The test article was dissolved in ion-exchanged water, and served as drinking water to the animals. Control rats were given the ion-exchanged water alone as drinking water. Before the start of the study, the stability of AS in ion-exchanged water at concentrations of 0.1, 0.6 and 15 mg/mL was confirmed after at least 4-day storage at room temperature following 6-day refrigerated storage; therefore, dosing solutions were prepared at least once every 6 days and kept in a cool place until serving. Fresh drinking water was served at least once every 4 days. During the study, the concentrations of AS in drinking water were analyzed in the first and last preparations and once every 3 months, and confirmed to be 97.5–106.3% of the target by high performance liquid chromatography. AS contained in the drinking water for the control group was less than the quantitation limit (5 μg/mL).

Prior to the present two-generation reproductive toxicity study, a dose-finding study was performed in male and female rats given drinking water containing AS at 0, 1000, 3000, 10,000 or 30,000 ppm. In that study, males were dosed for 7 weeks, beginning 14 days before mating, and females were dosed for 6–8 weeks beginning 14 days before mating to day 4 of lactation throughout the mating and gestation period. In the highest dose group, animals were euthanized at the end of the 2nd week of administration because of a marked decrease in body weight as a result of water avoidance. Water consumption also decreased in all other treatment groups. Decreased food consumption and body weight were observed at 3000 ppm and above. At autopsy, thickening of the limiting ridge in the stomach, and atrophy of the thymus and spleen were detected at 10,000 ppm. The relative weights of the liver, thymus and spleen were decreased in females in 3000 and 10,000 ppm groups. Although there were no changes in any reproductive parameters, the body weights of pups on postnatal day (PND) 4 were decreased at 10,000 ppm. Taking into account the results of this dose-finding study, the dose levels of AS in the present study were set as 120, 600 or 3000 ppm.

2.2. Animals and housing conditions

Crl:CD(SD) rats (4 weeks old) were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). This strain was chosen because they are the most commonly used in reproductive and developmental toxicity studies, and historical control data are available. The animals were acclimated to the laboratory for 7 days, and subjected to treatment at 5 weeks of age. They were carefully observed during the acclimation period, and male and female rats found to be in good health were selected for use. The rats were distributed into four groups of 24 males and 24 females each by stratified random sampling based on body weight, and all animals were assigned a unique number and the ear was tattooed prior to the start of the experiment.

Throughout the study, animals were maintained in an air-conditioned room at 21–25 °C, with a relative humidity of 36–59%, a 12-h light/dark cycle (8:00–20:00) and ventilation at 10–15 times/h. They were housed individually, except for the acclimation, mating and nursing periods, in suspended wire-mesh cages. From day 17 of gestation to day 21 after delivery, the wire-mesh floor of the cage was replaced with a stainless-steel tray, and individual dams and litters were reared using wood chips as bedding (White Flake; Charles River Laboratories Japan, Inc., Yokohama, Japan). All animals were fed *ad libitum* with a standard rat diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan), but were supplied with different drinking water solutions, as mentioned above, through two generations. Aluminium concentration in the standard diet, analyzed by atomic absorption spectrometry for each lot of diet, ranged from 25 ppm to 29 ppm.

2.3. Experimental design

Twenty-four F0 rats (5-week-old males and females)/sex/group were exposed to AS in drinking water at 0, 120, 600 or 3000 ppm. After 10-week administration of AS, each female rat was mated with a male rat of the same dosage group, and pregnant females were allowed to deliver spontaneously and nurse their pups. Administration of AS was continued throughout the mating, gestation and lactation periods. F0 parental male rats were necropsied after the parturition of paired females. F0 females were necropsied after weaning of their pups.

For the second (F1) generation, 24 male and 24 female weanlings in each group were selected as F1 parents on PNDs 21–25 to equalize the mean body weights among groups as much as possible. One male and 1 female F1 weanlings were selected from each of litters born during the 5 days including the day of the largest number of F0 parturition, and if the number of litters was insufficient, a second weanling pup in the litter was selected with care to prevent litter effects. The day on which F1 parental animals were selected was designated as day 0 of dosing for the F1 generation. F1-selected rats were given drinking water with the respective formulation, and were mated, allowed to deliver and nurse their F2 pups, and necropsied in the same manner as described for F0 rats. Unselected F1 weanlings and all F2 weanlings were necropsied on PND 26.

2.4. Mating procedures

Each female was mated with a single male of the same dosage group until successful copulation occurred or the mating period of 2 weeks had elapsed. For F1 matings, cohabitation of siblings was avoided. During the mating period, vaginal smears were examined daily for the presence of sperm, and the presence of sperm in the vaginal smear and/or a vaginal plug were considered as evidence of successful mating. The day of successful mating was designated as day 0 of gestation. Females that did not mate successfully during the 2-week mating period were cohabited with another male from the same group who had been proven to copulate with limits of not less than 7 days.

2.5. Parental data

Throughout the study, all parental animals were observed for clinical signs of toxicity at least twice a day. The body weight and food consumption were measured weekly. For females exhibiting evidence of successful mating, body weight and food consumption were recorded on gestational days 0, 7, 14 and 20 of gestation and days 0, 7, 14 and 21 of lactation (and additionally day 4 of lactation for body weight). Water consumption was recorded twice a week, and on days 0, 4, 7, 11, 14, 17 and 20 of gestation and days 0, 4, 7, 11, 14, 17, 19 and 21 of lactation. The intake of test substance was calculated based upon mean values for body weight and water consumption in each group.

For each female, daily vaginal lavage samples were evaluated for estrous cyclicity throughout the last 2 weeks of the premating period and during cohabitation until evidence of copulation was detected. Females having repeated 4–6 day estrous cycles were judged to have normal estrous cycles.

2.6. Litter data

Once insemination was confirmed, female rats were checked at least three times daily on days 21–25 of gestation to determine the time of delivery. The females were allowed to deliver spontaneously and nurse their pups until PND 21 (the day of weaning). The day on which dams held their pups under the abdomen in the nest by 13:00 was designated as day 0 of lactation or PND 0. On PND 0, all live and dead pups were counted, and live pups were sexed and examined grossly. They were observed daily for clinical signs of toxicity, and the body weight of live pups was recorded on PNDs 0, 4, 7, 14 and 21. On PND 4, litters were randomly adjusted to eight pups of four males and four females. No adjustment was made for litters of fewer than eight pups. Pups were assigned a unique number and limb tattooed on PND 4.

2.7. Developmental landmarks

All F1 and F2 live pups were observed for pinna unfolding from PND 1 to PND 4. Body weight was recorded daily during this period. The anogenital distance (AGD) was measured using calipers on PND 4 in all F1 and F2 pups, and the normalized value of AGD to body weight, AGD/cube root of the body weight ratio, was calculated. One male and one female F1 and F2 pup selected from each dam were evaluated for incisor eruption beginning on PND 8 and eye opening beginning on PND 12, and continued until each pup fulfilled the criteria. The body weight of the respective F1 and F2 pups was recorded on the day the criteria were fulfilled. Surface righting reflex, negative geotaxis and mid-air righting reflex were assessed on PND 5, 8 and 18, respectively, for one male and one female F1 and F2 pup selected from each dam. All F1 offspring selected as F1 parents were observed daily for male preputial separation beginning on PND 35 or female vaginal opening beginning on PND 25 until completion. The body weight of the respective F1 rats was recorded on the day of completion of these pubertal landmarks.

2.8. Behavioral test

Spontaneous locomotor activity was measured at 4 weeks of age in 10 male and 10 female F1 rats randomly selected from each group, using a multi-channel activity monitoring system (SUPERMEX; Muromachi Kikai Co., Ltd., Tokyo, Japan). Rats were placed individually in transparent polycarbonate cages [285 (W) mm × 450 (D) mm × 210 (H) mm, CL-0108-1; CLEA Japan, Inc., Tokyo, Japan], which were placed under an infrared sensor that detects thermal radiation from animals, and spontaneous motor activity was determined at 10-min intervals and for 60 min.

A test in a water-filled multiple *T*-maze was conducted in 10 male and 10 female F1 rats selected from each group at 6 weeks of age. The apparatus was similar to that described by Biel [45]. The water temperature of the maze was kept 20.5–22 °C. As a preliminary swimming ability test, each rat was allowed to swim three times in a straight channel on the day before the maze trial, and then tested in the maze with three trials per day for the next three consecutive days. The elapsed time between entry into the water at the starting point and touching the goal ramp, and the number of errors were recorded. To prevent the exhaustion of the rats, no animal was allowed to remain in the water for more than 3 min in any trial.

2.9. Termination/necropsy (adults)

All surviving parental male rats were euthanized by exsanguination under ether anesthesia after the parturition of paired females. All female rats showing successful reproductive performance were evaluated for estrous cycle stage by examination of the vaginal smear after weaning of pups, and euthanized at the proestrous stage by exsanguination under ether anesthesia. Females that did not copulate or had not completed parturition and dams with total litter loss were euthanized in the same way around the same time as females with successful reproduction. For all parental animals, the external surfaces were examined. The abdomen and thoracic cavity were opened, and gross internal examination was performed. Major organs were removed and the number of uterine implantation sites was recorded for each female. The testis and epididymis were fixed with Bouin's solution and preserved in 70% ethanol, and the other organs were stored in 10% neutral-buffered formalin. The brain, pituitary, thyroids, thymus, liver, kidneys, spleen, adrenals, testes, epididymides, seminal vesicles (with coagulating glands and their fluids), ventral prostate, uterus and ovaries were weighed before fixation. The thyroid and seminal vesicle were weighed after fixation.

Histopathological evaluations were performed in all animals of the control and highest dose groups, in females with abnormal estrous cycles, abnormal delivery or totally dead pups, in males and females without evidence of copulation or insemination, and in all animals with grossly abnormal reproductive organs. Of these animals, the testes, epididymides, seminal vesicles, ventral prostate, coagulating gland, ovaries, uterus and vagina, which were fixed as mentioned above, were embedded in paraffin by a routine procedure. They were sectioned, stained with hematoxylin–eosin and examined histopathologically under a light microscope. If treatment-related histopathological changes were found in the highest dose group, were the same tissues from the next lower dose group then examined.

In 10 F1 females, randomly selected from the control and highest dose groups, the number of primordial follicles was counted as follows. The right ovary, fixed in 10% neutral-buffered formalin, was dehydrated and then embedded in paraffin in longitudinal orientation by routine procedures. Sections were cut serially at 5 μm and every 20th section was serially mounted on a slide and stained with hematoxylin and eosin. About 40 sections per ovary were used to determine the primordial follicles.

2.10. Termination/necropsy (pups)

Following the adjustment of litter size on PND4, culled pups were euthanized by inhalation of carbon dioxide and subjected to a gross external and internal observation. Grossly abnormal organs/tissues were removed and stored in 10% neutral-buffered formalin. All pups found dead before weaning were necropsied immediately, and the whole body was stored in 10% neutral-buffered formalin.

F1 weanlings not selected to become parents and all F2 weanlings were euthanized and necropsied on PND 26, as described for adults. For one male and one female F1 and F2 weanlings selected from each dam, the brain, thymus, liver, kidneys, spleen, adrenals, testes, epididymides, ventral prostate, uterus and ovaries were removed and the organ weights were measured. Major organs, including the weighed organs, were stored in 10% neutral-buffered formalin.

Since test substance-related organ weight changes were found in the liver and spleen of the highest dose group, they were histopathologically examined for 10 male and 10 female F1 and F2 weanlings in the control and highest dose groups. The examined animals were randomly selected from animals whose organs were stored. If treatment-related histopathological changes were observed in the highest dose group, were the same tissues from the next lower dose group then examined. For the histopathological examination, paraffin sections were routinely prepared and stained with hematoxylin and eosin.

2.11. Sperm parameters

Sperm parameters were determined for all F0 and F1 male adults on the day of the scheduled sacrifice. The right testis was used to count testicular homogenization-resistant spermatid heads. The right epididymal cauda was weighed and used for sperm analysis. For sperm motility, the percentage of motile sperm and progressively motile sperm, and the swimming speed and pattern were determined using a computer-assisted cell motion analyzer (TOX IVOS; Hamilton Thorne Bioscience, Beverly, MA, USA). After recording sperm motion, the cauda epididymal fluid was diluted and the sperm were enumerated with a hemacytometer under a light microscope. Sperm count per gram of epididymal tissue was obtained by dividing the total count by the gram weight of the cauda epididymis. The sperm was stained with eosin and mounted on a slide glass. Two hundred sperm in each sample were examined under a light microscope, and the percentage of morphologically abnormal sperm was calculated.

2.12. Statistical analysis

Parametric data, such as body weight, food and water consumption, length of the estrous cycle and gestation, precoital interval, the number of implantations and pups born, delivery index, reflex response time, age at sexual maturation, parameters of behavioral tests, organ weight and sperm parameters, were analyzed by Bartlett's test for homogeneity of distribution. For preweaning pups, body weight, AGD, viability, and age at the completion of developmental landmarks were similarly analyzed using the litter as the experimental unit. When homogeneity was recognized, one-way analysis of variance was performed. If a significant difference was detected, Dunnett's test was conducted for comparisons between control and individual treatment groups. Data without homogeneity were analyzed using the Kruskal–Wallis rank sum test. If significant differences were found, the Mann Whitney's *U* test was conducted for comparison between the control and each dosage group. The incidence of parental animals with clinical signs, and autopsy and histopathological findings, the incidence of females with normal estrous cycles, incidence of weanlings with histopathological findings, copulation, fertility and gestation index, neonatal sex ratio and completion rate of negative geotaxis were compared between the AS and control group using Fisher's exact test. The incidence of pups with clinical signs or autopsy findings per litter, the completion rate of pinna unfolding in each litter, and the success rate of surface and mid-air righting reflex were analyzed by the Wilcoxon rank sum test. The number of primordial follicles in the control and highest dose groups was compared by Student's *t*-test because the homogeneity of variance was indicated by the *F*-test. All of these statistical analyses were conducted using the 5% level of probability as the criterion for significance.

3. Results

3.1. Clinical observations, water consumption, food consumption and body weight during the pre-mating, mating, gestation and lactation periods (F0 and F1)

In the 120 ppm group, one F1 male was found dead at 9 weeks of dosing. In this animal, soiling of periocular and perinasal fur and decreased locomotor activity were observed before death. At

autopsy, various changes, including accumulation of ascitic and pleural fluid and dark purple discoloration of the liver and kidneys, were found. In the 600 ppm group, a subcutaneous mass was observed in the abdominal region of one F0 female from the beginning of 5 weeks of dosing, and this animal was found dead at 2 weeks of gestation. One F1 male at 3000 ppm was also found dead at 12 weeks of dosing without any clinical signs of toxicity. In these two animals, no abnormality was found on gross internal examination. No significant difference was seen between control and AS-treated groups in the incidence of clinical signs of toxicity in either male or female F0 and F1 rats (data not shown).

Water consumption, food consumption and the body weight of F0 parental animals are shown in Figs. 1–3, respectively. In F0 males and females of all AS-treated groups, water consumption was significantly lower than in controls almost throughout the dosing period. In F0 males, there were significant decrease in food consumption in the first week of dosing at 600 and 3000 ppm, and during week 8 and weeks 13–14 of dosing at 3000 ppm. Food consumption of F0 females showed a significantly lower value during week 1 of dosing at 3000 ppm and during week 3 of lactation at 600 and 3000 ppm. The body weight of F0 males and females was significantly lowered in the first 2 or 3 weeks of dosing at 3000 ppm.

Figs. 4–6 show the water and food consumption, and body weight of F1 parental animals, respectively. Water consumption was significantly decreased through the dosing period in 600 ppm and 3000 ppm treated males, and during weeks 3–6, week 8 and week 10 of dosing in 120 ppm treated males. In F1 females, significant reductions in water consumption were found almost throughout the dosing period at 3000 ppm, during week 10 of dosing and week 3 of lactation at 600 ppm, and during weeks 9–10 of dosing at 120 ppm. Food consumption was significantly decreased during week 10 of dosing in F1 males of the 600 and 3000 ppm groups, and during week 3 of lactation in F1 females of the same groups. There was also a transient significant increase in food consumption during week 6 of dosing in F1 females of the 120 ppm group. The body weight of F1 males and females exhibited no significant differences between the control and AS-treated groups, except that F1 females of the 120 ppm group had significantly higher body weight during weeks 6–8 of dosing.

Based on water consumption and body weight, daily AS intakes during the premating and postmating periods in males and during the premating, gestation and lactation periods in females were calculated for each of the AS-treated groups. Calculated mean AS intakes during the whole of these period were 8.6, 41.0 and 188 mg/kg bw/day in F0 males, 14.4, 71.5 and 316 mg/kg bw/day in F0 females, 10.7, 50.2 and 232 mg/kg bw/day in F1 males, and 15.3, 74.2 and 338 mg/kg bw/day in F1 females, in the 120, 600 and 3000 ppm groups, respectively. The total ingested dose of aluminium from drinking water and food combined was estimated from the water and food consumption and body weight. Average aluminium intake was 1.62, 2.96, 8.06 and 31.2 mg Al/kg bw/day in F0 males, 2.29, 4.50, 13.5 and 52.0 mg Al/kg bw/day in F0 females, 1.93, 3.55, 9.78 and 38.5 mg Al/kg bw/day in F1 males, and 2.35, 4.72, 14.0 and 55.6 mg Al/kg bw/day in F1 females for control through high-dose groups.

3.2. Reproductive effects (F0 parents/E1 offspring and F1 parents/F2 offspring)

During the premating period, AS produced no significant deviations in the estrous cycle of F0 and F1 females although a few control and AS-treated rats had persistent diestrus. The incidence of females with a normal estrous cycle also did not change significantly in either generation (data not shown).

The reproductive performance of F0 and F1 parental animals are summarized in Table 1. During the mating period, copulation was not observed in two males each in the control, 120 ppm and 3000 ppm groups and in one female of the control group in the F0 generation. In the F1 generation, one male in the control group, two males and one female in the 120 ppm group, one male in the 600 ppm group, and three males and one female in the 3000 ppm group did not copulate. Among females with successful copulation, one female each in the control and 3000 ppm group and two females at 120 ppm in the F0 generation and two females each in the control, 600 ppm and 3000 ppm groups, and four females at 120 ppm in the F1 generation were not impregnated. In addition, one pregnant F0 female each at 120, 600 and 3000 ppm and one pregnant F1 female at 120 ppm did not deliver live pups; however, there were no significant differences in the copulation, fertility or gestation index, and the precoital interval or gestation length between the control and AS-treated groups in F0 and F1 generation. No significant changes were observed in the number of implantations or pups delivered, and delivery index in either generation.

As for the sperm parameters examined for scheduled-sacrificed adults, in F0 generation, the absolute number of cauda epididymal sperm was significantly decreased at 3000 ppm ($253.8 \pm 61.3 \times 10^6$/cauda versus $286.3 \pm 40.3 \times 10^6$/cauda in the control); however, no significant changes were found in the number per gram of tissue. No such change was observed in F1 adults. There were no significant differences in the number of testis sperm, the percentage of motile sperm and progressively motile sperm, the swimming speed and pattern, and the percentage of morphologically abnormal sperm between control and AS-treated groups in either F0 or F1 adults (data not shown).

3.3. Developmental effects (F1 and F2)

Gross examination of delivered pups revealed one F1 pup with trauma in the perianal region and tail in the control group and one F1 pup with hemimelia and oligodactyly in the 120 ppm group, but no significant difference was found in the incidence between the control and AS-treated groups. No malformed F2 pups were found in any groups.

Table 2 shows sex ratio of delivered pups, and the viability and body weight during the preweaning period. No significant changes were found in the sex ratio of pups and the viability index in either generation. In the 3000 ppm group, the body weight of male and female F1 pups was significantly lower than the control on PND 21. Body weights of F2 female pups were also significantly lower than controls on PND 21 at 3000 ppm. There were no significant differences in the body weight of male F2 pups between the control and AS-treated groups during the preweaning period.

For the physical development of male and female F1 pups and male F2 pups, there was no significant difference in the completion rate of pinna unfolding, and the age at completion of incisor eruption and eye opening between the control and AS-treated groups. In female F2 pups, the completion rate of pinna unfolding on PND 2 was significantly lower in the 600 ppm group ($17.0 \pm 35.4\%$, compared with 45.8 ± 46.9 in controls), but no dose dependency was observed in this change. No significant changes were found in the completion rate of pinna unfolding on PND 1, 3 or 4 and in other physical developmental landmarks in female F2 pups. The AGD and AGD per cube root of the body weight ratio were not significantly different between control and AS-treated groups in male and female F1 and F2 pups (data not shown).

All male and female F1 pups in all groups achieved the surface righting reflex on PND 5, negative geotaxis reflex on PND 8 and mid-air righting reflex on PND 18. No significant changes were observed in the response time of surface righting and negative geotaxis reflex. In F2 pups, one female of the 600 ppm group failed in one of three trials of the mid-air righting reflex on PND 18; however, there was no significant difference in the mean success rate between the