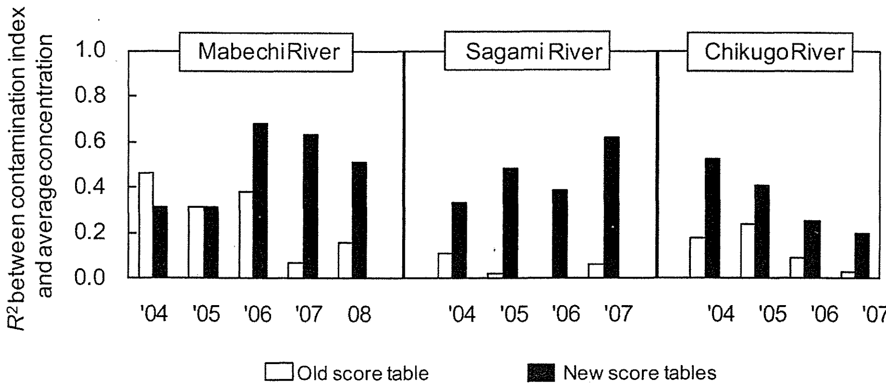

Fig. 2 – Comparison of the coefficients of determination (R^2) for plots of average pesticide concentrations versus contamination index values calculated with the new score tables and the old score table.

the new score tables (Score X was calculated from Eq. (2), and Scores Y and Z were obtained from Table 1) and the old score table (Scores A–C, Table 1S in the supplementary information). Fig. 1S in the supplementary information shows an example of the correlation between the contamination index values and average measured concentrations (sum of the measured concentrations divided by the number of water samples) of pesticides in the Chikugo River in 2004. Higher correlations were obtained with the new score tables than with the old score table. The coefficient of determination R^2 increased from 0.18 to 0.52 when the new score tables were applied (compare views A and B in Fig. 1S). However, the concentrations for any given contamination index value were scattered in a range around the logarithm of 2. The data scattering may have been due to the uncertainty for the application dates and application methods (including drift and adjuvant) for the pesticides; the acquisition of accurate application dates and the accurate evaluation for the effects of application methods were hard (Matsui et al., 2006a) and therefore these factors were not considered in the calculation of the contamination index. It may also be due to the accuracy of Score X values. In evaluating Score X value it is assumed that the quantity of pesticide applied was equal to the amount of the sales in that year. This assumption may, however, not be very accurate because all the purchased pesticides may not necessarily be applied in the agricultural field in a year after the purchase.

Fig. 2 summarizes the R^2 values for the plots of the average concentrations of pesticides against the contamination index values for the three rivers. Overall, the contamination index values calculated with the new score tables were better correlated with pesticide concentrations than the values calculated with the old score table, indicating that the new score tables are effective tools for preliminary ranking or prioritizing pesticides to be monitored.

3.2. *Use of the tables to select pesticides to be monitored*

3.2.1. *Estimating maximum pesticide concentration with the new score tables*

Fig. 3 shows the relationship between the highest observed pesticide concentrations and contamination index values. Since score is defined such that a score increase of 1 corresponds to a 10-fold increase in the pesticide concentration in river water, the relationship between highest observed concentration and contamination index, which is given by a sum of scores, is described, in the ideal situation, by the following equation:

$$\log C_H = \text{Contamination Index} - A \quad (6)$$

where C_H is the highest observed pesticide concentration (μg/L) and A is a constant. The mean A value was 11.6. The A value for the one-sided 95% upper confidence limit was 10.7. Therefore, C_U, the one-sided 95% upper confidence limit of the maximum pesticide concentration (μg/L), is predicted by the following equation:

$$\log C_U = \text{Contamination Index} - 10.7 \quad (7)$$

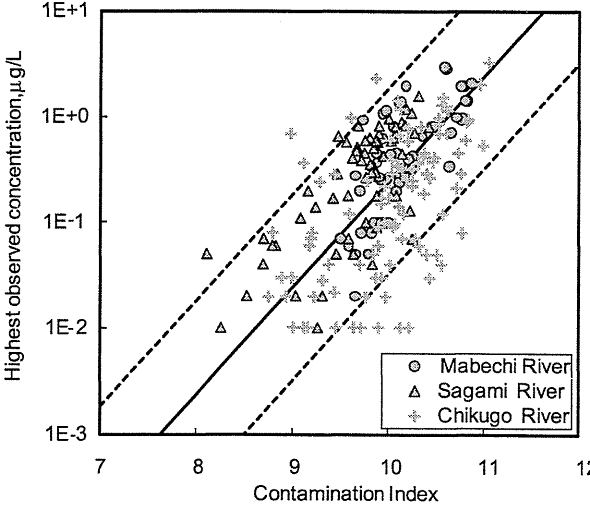

Fig. 3 – Relationship between the highest observed concentrations of pesticides and the contamination index values calculated with the new score tables. The solid line is a plot of the equation log C_H = Contamination index - 11.4. The dashed lines indicate one-sided 95% upper and lower confidence limits.

3.2.2. Use of possible maximum concentration to select pesticides to be monitored

JDWQG specifies that *DI* value, defined by Eq. (1), should not exceed 1. However, the calculation of *DI* value still has a potential problem that the value is heavily dependent on the number of monitored pesticides. In the current study, we predicted the maximum possible concentration of pesticide i, PV_i, in river water by using Eq. (7), and we then calculated PV_i/GV_i values. The PV_i/GV_i values were compared with the DV_i/GV_i values for the highest observed concentration.

Fig. 4 shows the DV_i/GV_i and PV_i/GV_i quotients of pesticides applied in the Sagami River basin in 2004. Because the quantification of pesticide concentrations above 1/100 of each GV_i value is recommended in evaluating *DI* value in JDWQG (MHLWJ, 2003b), a PV_i/GV_i quotient of 0.01 can be regarded as a threshold limit in selecting pesticides to be monitored and used for *DI* value calculation. That is, if the PV_i/GV_i value of pesticide *i* is less than 0.01, nonnecessity for monitoring that pesticide is suggested. By applying this threshold limit, we selected 16 pesticides from the 34 pesticides applied in the Sagami River basin for monitoring in the year 2004, as shown in Fig. 4. Among the selected pesticides, seven compounds DV_i/GV_i values exceeded the 0.01 threshold (iprobenfos, molinate, mefenacet, benthiocarb, esprocarb, simetryn, and bromobutide). However, other seven pesticides had DV_i/GV_i values of <0.01 although their PV_i/GV_i values were ≥0.01. This is understandable because PV_i is the maximum possible pesticide concentration predicted by the one-sided 95% upper confidence limit, and PV_i can be regarded as a conservative estimate. Two pesticides (diquat monohydrate and cafenstrole) were selected as pesticide candidates, but their concentrations were not actually measured.

The numbers of pesticide candidates for monitoring selected by this method are summarized in Table 2: 16–21 pesticide candidates among the 30–34 in the Mabechi River, 16–18 among the 31–34 in the Sagami River, and 22–25 among the 32–33 in the Chikugo River. The efficiency of the selection method was evaluated by comparison with pesticide monitoring data (columns E–H in Table 2). For all the data in the table, the groups of selected pesticides for which the PV_i/GV_i values were >0.01 included all the pesticide with DV_i/GV_i values of ≥0.01. There was no pesticide with a PV_i/GV_i value of <0.01 and a DV_i/GV_i value of ≥0.01. As shown in Column H of Table 2, 26–75% of the selected pesticides with PV_i/GV_i values of ≥0.01 had DV_i/GV_i values of ≥0.01 (the success rate in selecting pesticide candidates for monitoring was 26–75%). We feel these percentages constitute successful first efforts. It is notable that the pesticides with PV_i/GV_i values of <0.01 were all (100% in Column H of Table 2) with DV_i/GV_i values of <0.01 (the success rate in removing pesticides unnecessary for monitoring was 100%).

The three rivers we evaluated in the current study are currently monitored for pesticides selected on the basis of previous experience by the local water supply authorities rather than by means of our proposed method. However, for rivers where pesticides are not monitored or have no basis for determining the necessity of monitoring, our proposed method could save time and expense in identifying monitoring needs. The use of this methodology would also help determining the necessity of monitoring metabolites and degrades of pesticides if reaction pathway and kinetics of pesticide degradation are known and incorporated into the diffuse pollution hydrologic model.

A considerable number of water supply authorities in Japan monitor all or most of the 102 pesticides that are listed in Pesticide Group 1 of JDWQG without conducting any risk assessment to properly select the mentoring pesticides. Under such circumstances, our proposed new score tables and method could assist in the selection of pesticides to be monitored and the determination of which pesticides require no monitoring. No such decision could be reached by means of any other

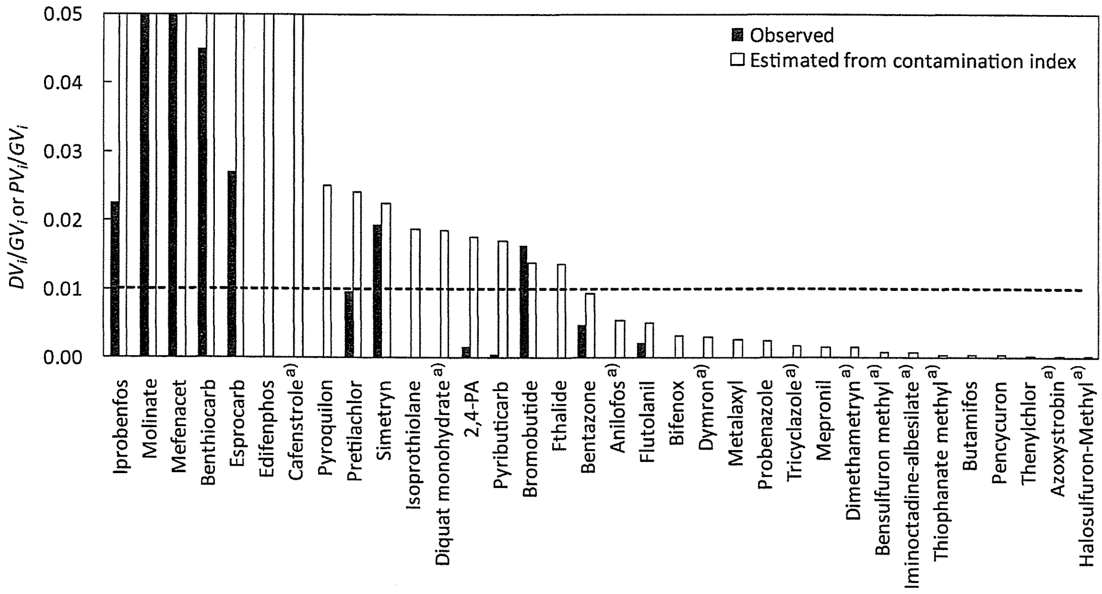

Fig. 4 – PV_i/GV_i (open bars) and DV_i/GV_i (solid bars) values for pesticides in the Sagami river basin in 2004. a) the concentrations were not measured (PV_i is the possible maximum predicted concentrations of pesticide i, GV_i is the reference concentration of pesticide i in JDWQG, and DV_i is the observed concentration of pesticide i).

Table 2 – Performance of new score tables for selecting pesticides (PV_i is the possible maximum predicted concentrations of pesticide i, GV_i is the reference concentration of pesticide i in JDWQG, and DV_i is the observed concentration of pesticide i).

Column A	Column B	Column C	Column D	Column E	Column F	Column G	Column H
	Year		Applied in rice-paddy	With measured concentration	With DVi/GVi < 0.01	With DVi/GVi ≥ 0.01	Success ratio (%)
Mabechi River	2004	Number of pesticides	34	18	13	5	
		With $PV_i/GV_i \geq 0.01$	21	14	9	5	36[a]
		With $PV_i/GV_i < 0.01$	13	4	4	0	100[b]
	2005	Number of pesticides	34	22	17	5	
		With $PV_i/GV_i \geq 0.01$	20	16	11	5	31[a]
		With $PV_i/GV_i < 0.01$	14	6	6	0	100[b]
	2006	Number of pesticides	33	18	12	6	
		With $PV_i/GV_i \geq 0.01$	19	14	8	6	43[a]
		With $PV_i/GV_i < 0.01$	14	4	4	0	100[b]
	2007	Number of pesticides	30	18	12	6	
		With $PV_i/GV_i \geq 0.01$	18	13	7	6	46[a]
		With $PV_i/GV_i < 0.01$	12	5	5	0	100[b]
	2008	Number of pesticides	30	18	9	9	
		With $PV_i/GV_i \geq 0.01$	16	12	3	9	75[a]
		With $PV_i/GV_i < 0.01$	14	6	6	0	100[b]
Sagami River	2004	Number of pesticides	34	23	16	7	
		With $PV_i/GV_i \geq 0.01$	16	14	7	7	50[a]
		With $PV_i/GV_i < 0.01$	18	9	9	0	100[b]
	2005	Number of pesticides	31	26	20	6	
		With $PV_i/GV_i \geq 0.01$	17	15	9	6	40[a]
		With $PV_i/GV_i < 0.01$	14	11	11	0	100[b]
	2006	Number of pesticides	32	30	23	7	
		With $PV_i/GV_i \geq 0.01$	18	17	10	7	41[a]
		With $PV_i/GV_i < 0.01$	14	13	13	0	100[b]
	2007	Number of pesticides	31	28	22	6	
		With $PV_i/GV_i \geq 0.01$	17	16	10	6	38[a]
		With $PV_i/GV_i < 0.01$	14	12	12	0	100[b]
Chikugo River	2004	Number of pesticides	32	23	16	7	
		With $PV_i/GV_i \geq 0.01$	23	20	13	7	35[a]
		With $PV_i/GV_i < 0.01$	9	3	3	0	100[b]
	2005	Number of pesticides	32	29	23	6	
		With $PV_i/GV_i \geq 0.01$	24	23	17	6	26[a]
		With $PV_i/GV_i < 0.01$	8	6	6	0	100[b]
	2006	Number of pesticides	33	29	23	6	
		With $PV_i/GV_i \geq 0.01$	22	21	15	6	29[a]
		With $PV_i/GV_i < 0.01$	11	8	8	0	100[b]
	2007	Number of pesticides	32	28	21	7	
		With $PV_i/GV_i \geq 0.01$	25	22	15	7	32[a]
		With $PV_i/GV_i < 0.01$	7	6	6	0	100[b]

a Number of pesticides with $DV_i/GV_i \geq 0.01$/number of pesticides with $PV_i/GV_i \geq 0.01$.
b Number of pesticides with $DV_i/GV_i < 0.01$/number of pesticides with $PV_i/GV_i < 0.01$.

ranking tool, such as the old score table, which prioritizes pesticides but does not have an absolute criterion for selection. Pesticide compounds with a similar property can be analyzed with the same multi-residue method. The monitoring costs thus do not simply depend on the mere number of compounds to analyze, but on the number of analytical methods to run and the individual costs of these methods. However, the analysis requires the standard solution and the accuracy control of analysis for each compound, and therefore the proper selection of monitoring pesticides has a strong merit.

4. Conclusions

1) New, improved score tables designed for selecting pesticides on the basis of their properties were prepared by restructuring and refinement based on sensitivity analyses conducted with a pesticide diffuse pollution model. The correlations between observed pesticide concentrations and contamination index values calculated with the new score tables were greatly improved over correlations obtained with the old score table.
2) Possible maximum concentrations of pesticides (PV_i) were estimated from the one-sided 95% upper confidence limit for the regression line for the contamination index. The number of pesticide candidates for monitoring selected on the basis of the threshold PV_i/GV_i quotient of 0.01 was roughly two-thirds of the number of pesticides applied. All the pesticides that actually detected in the river waters with DV_i/GV_i quotients larger than 0.01 were included in the list of selected pesticide candidates. The new score tables give contamination index values and then PV_i/GV_i values,

which can be expected to be useful criteria for determining whether or not a pesticide should be monitored on the basis of a threshold value of 0.01.

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Appendix. Supplementary data

Supplementary data related to this article can be found online at doi:10.1016/j.watres.2011.11.036.

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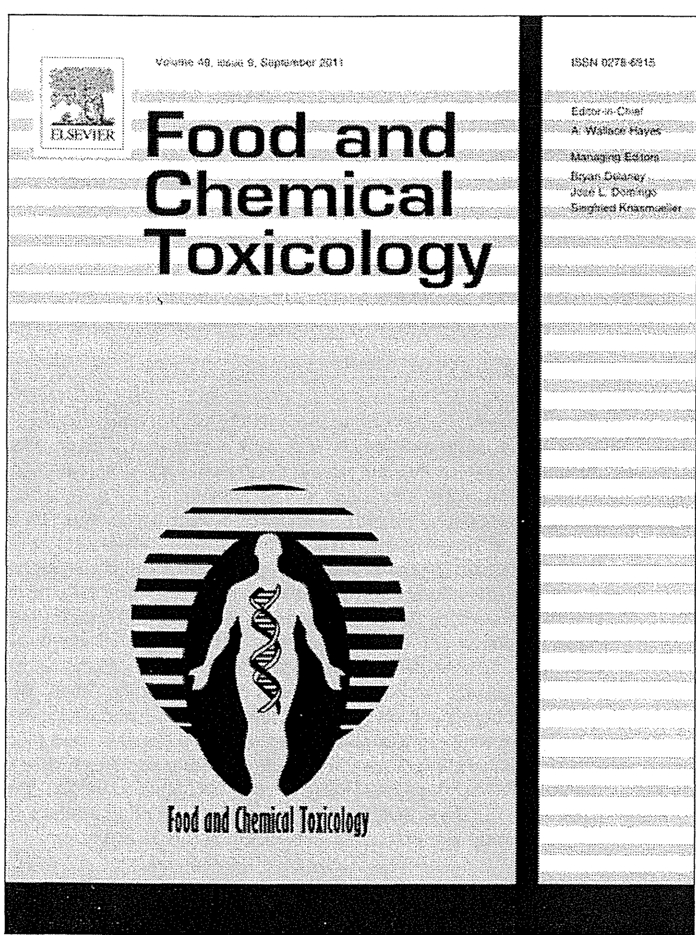

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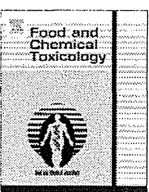

Evaluation of the reproductive and developmental toxicity of aluminium ammonium sulfate in a two-generation study in rats

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ABSTRACT

Aluminium ammonium sulfate (AAS) was tested for reproductive/developmental toxicity in a two-generation study. Male and female rats were continuously given AAS in drinking water at 0, 50, 500 or 5000 ppm. Water consumption was decreased in all AAS-treated groups, and the body weight of parental animals transiently decreased in the 5000 ppm group. In either generation, no compound-related changes were found in estrous cyclicity, sperm parameters, copulation, fertility and gestation index, number of implantations and live birth pups, sex ratios of pups or viability during the preweaning period. Male and female F1 pups in the 5000 ppm group showed a lower body weight on postnatal day 21, while there were no differences in the birth weight of F1 and F2 pups between the control and AAS-treated groups. Preweaning body weight gain in F2 males and females indicated a similar decreasing tendency at 5000 ppm. In F1 and F2 weanlings, the weight of the liver, spleen and thymus decreased at 5000 ppm, but no histopathological changes were found in these organs. In F1 females in the 5000 ppm group, vaginal opening was delayed slightly. There were no compound-related changes in male preputial separation or in other developmental landmarks. In behavioral tests conducted for F1 animals at 4–6 weeks of age, no compound-related changes were found in spontaneous locomotor activity and performance in a water-filled multiple T-maze. In conclusion, the NOAEL of AAS for two-generation reproductive/developmental toxicity was considered to be 500 ppm in rats. Considering the aluminium content in the basal diet, the total ingested dose of aluminium from drinking water and food in this 500 ppm group was calculated to be 5.35 mg Al/kg bw/day.

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1. Introduction

Aluminium compounds are widely used as food additives in various food products; for example, aluminium ammonium sulfate [AAS; CAS No.: 7784-25-0 (anhydrous), 7784-26-1 (dodecahydrate)] is added as a firming agent or stabilizer in egg products, processed fish and vegetables, candied fruit, etc. (Codex Alimentarius Commission, 2010). Other aluminium compounds used as food additives include acidic and basic sodium aluminium phosphate (SALP), sodium and calcium aluminium silicate, aluminium sodium sulfate, and aluminium lakes of various food dyes and colors (IPCS, 2007). While aluminium is ubiquitous in the environment (IPCS, 1997), the major route of aluminium exposure by the general public is considered to be dietary exposure, particularly through foods containing such aluminium compounds as food additives (WHO, 2008). Total dietary exposure to aluminium has been calculated to range from 14 to 280 mg Al/week in the adult population (IPCS, 2007).

Concerning the effects of aluminium on human health, many international and national organizations have conducted toxicological evaluations (ATSDR, 2008; EFSA, 2008; IPCS, 1997; WHO, 2007). Recently, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) re-evaluated aluminium from all sources, including food additives. While it established a provisional tolerable weekly intake (PTWI) of 1 mg Al/kg bw for all aluminium compounds in food, a review of the toxicology database disclosed that there is a need for an appropriate study of developmental toxicity and a multigeneration study incorporating neurobehavioral endpoints to be conducted on relevant aluminium compound(s) (WHO, 2007).

Aluminium has been reported to affect the male reproductive system, causing necrosis of spermatocytes/spermatids in the testes, decreases in testicular spermatid counts and epididymal spermatozoa counts, and reduction of fertility etc., in various laboratory animals (Guo et al., 2005; Kamboj and Kar, 1964; Krasovskii et al., 1979; Llobet et al., 1995; Roy et al., 1991; Yousef et al., 2005). Although the oral bioavailability of aluminium is extremely low

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(less than 1%) (Jouhanneau et al., 1997; Zafar et al., 1997), such effects were observed in oral gavage studies (Krasovskii et al., 1979; Roy et al., 1991; Yousef et al., 2005), the lowest effective dose of which was reported to be 2.5 mg Al/kg bw/day in a 6-month exposure study in rats. However, because the toxicokinetics after a bolus dose by gavage must differ significantly from those after actual continuous exposure via the diet in humans, the relevance of these oral gavage studies for human risk assessment is unclear. Further, the dietary intake of aluminium was not considered in oral gavage studies even though laboratory animal feed contains a significant portion of aluminium (ranging from 60 to 8300 ppm) (ATSDR, 2008). There is therefore a possibility that the toxic effects of aluminium on the male reproductive system might have been overestimated in these studies. In fact, Hicks et al. (1987) demonstrated that 28-day continuous exposure to diets containing basic SALP or aluminium hydroxide did not affect testicular histopathology up to 302 mg Al/kg bw/day in rats. In the 26-week feeding study of SALP basic in dogs, germinal epithelial cell degeneration and atrophy in the seminiferous tubules were observed at 75 mg Al/kg bw/day (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). These dietary exposure studies took into account the aluminium content in the basal diet, but they all used water-insoluble or sparingly-soluble forms of aluminium (ATSDR, 2008; IPCS, 2007), which are widely assumed to be less bioavailable than soluble compounds, such as AAS, aluminium chloride and aluminium lactate (IPCS, 2007; Taimei Chemicals Co. Ltd., year not specified a). Thus, it is necessary to investigate the effects on the male reproductive system after continuous exposure to water-soluble aluminium compounds.

As for developmental toxicity, most of the reported continuous exposure studies focused on specific neurobehavioral endpoints (Bernuzzi et al., 1986, 1989; Muller et al., 1990). In particular, a group of researchers from the University of California have provided many reliable results in this field (Donald et al., 1989; Golub and Germann, 2001; Golub et al., 1992, 1995, 2000). In these studies, Swiss Webster mice were given a diet containing aluminium lactate throughout the gestation and/or lactation period, and neurobehavioral effects, such as greater or lower grip strength, decreased sensitivity to heat and impaired learning in a maze, were found in the offspring from dams given ⩾500 mg Al/kg diet (100–210 mg Al/kg bw/day). In contrast, insufficient information is available regarding the effects of continuous aluminium exposure on the physical and sexual development of offspring.

In order to fill these data gaps, we previously conducted a two-generation reproductive toxicity study of aluminium sulfate with continuous administration in drinking water, according to OECD test guidelines under GLP (Hirata-Koizumi et al., 2011). Aluminium sulfate is a water-soluble salt of aluminium, and is primarily used as a flocculant for water purification, paper-sizing agent, fire extinguisher material, etc. (Donaldson, year not specified; Taimei Chemicals Co. Ltd., year not specified b). The results of a two-generation study showed possible effects on postnatal growth, but no definitive effects were found in reproductive or developmental toxicity, including neurobehavioral parameters up to 3000 ppm (31.2–87.0 mg Al/kg bw/day). In the present study, a two-generation reproductive toxicity study was conducted of another water-soluble aluminium compound, AAS, which is used as a food additive.

2. Materials and methods

This study was conducted in accordance with OECD guideline 416 "Two-generation Reproduction Toxicity Study" (OECD, 2001) and the Japanese guidelines for the designation of food additives and revision of the standards for the use of food additives (MHW, 1996). All procedures involving the use and care of animals complied with the principles for Good Laboratory Practice (ME, METI and MHLW, 2003, 2008) and applicable animal welfare regulations ["Act on Welfare and Management of Animals"(Japanese Animal Welfare Law, 2005), "Standards Relating to the Care, Management of Laboratory Animals and Relief of Pain"(ME, 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"(MHLW, 2006)].

2.1. Animals and housing conditions

Crl:CD(SD) rats (4 weeks old) were purchased from Atsugi Breeding Center, Charles River Laboratories Japan, Inc. (Yokohama, Japan). They were acclimated to the laboratory for 8 days, and rats found to be in good health were divided into 4 groups of 24 males and 24 females each by stratified random sampling based on body weight. Ear tattoos were used for parent animal identification, and limb tattoos for live pup identification (after postnatal day (PND) 4).

Throughout the study, animals were maintained in an air-conditioned room with controlled temperature (22 ± 3 °C) and humidity (50 ± 20%). Light was provided on a 12-h light/dark cycle (light: 8:00–20:00). The animals were housed individually, except for the acclimation, mating and nursing periods, in metal bracket-type cages with wire-mesh floors. From day 17 of gestation to day 21 after delivery, individual dams and litters were reared using wood chips as bedding (White Flake; Charles River Laboratories Japan, Inc.). All animals were fed *ad libitum* with a standard rat diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan), and were supplied with drinking water containing different concentration of AAS, as mentioned below, through two generations. Aluminium concentration in the standard diet, analyzed by flame atomic absorption spectrometry for each lot of diet, ranged from 22 to 29 ppm.

2.2. Chemical and dosing

AAS (99.5% pure anhydrous; Lot No. A81009) was obtained from Taimei Chemicals Co., Ltd. (Nagano, Japan). The bulk of the sample was stored with a desiccating agent at room temperature (20–25 °C). The test article was dissolved in ion-exchanged water, and served as drinking water for the animals, which were 5 weeks old when the treatment started. Control rats were given ion-exchanged water alone as drinking water, in which AAS was contained at less than the quantitation limit (5 μg/mL). Before the start of the study, the stability of AAS in ion-exchanged water at concentrations of 0.05 and 10 mg/mL was confirmed after 5-day storage at room temperature following 7-day refrigerated storage; therefore, dosing solutions were prepared at least once every 7 days and kept in a cool place until serving, and the drinking water was replaced at least once every 5 days. During the study, the concentrations of AAS in drinking water were analyzed at the first and last preparation and once every 3 months, and were confirmed to be 99.4–104.4% of the target by high performance liquid chromatography.

Prior to the present two-generation reproductive toxicity study, a dose-finding study was performed in male and female rats (6/sex/dose) given drinking water containing AAS at 0, 300, 1000, 3000 or 10,000 ppm. In that study, males were dosed for 7 weeks, beginning 14 days before mating, and females were dosed from 14 days before mating to day 4 of lactation throughout the mating and gestation periods. AAS reduced water consumption in all treatment groups, and there were decreases in body weight at 3000 ppm and above. At necropsy, thickening of the limiting ridge in the stomach was detected at 10,000 ppm, although no animals died at any doses. There were no changes in any other reproductive/developmental parameters. Taking into account the results of this dose-finding study, the dose levels of AAS in the present study were set as 50, 500 or 5000 ppm.

2.3. Experimental design

A graphic representation of the experimental design is presented in Fig. 1. The study began with 24 rats/sex/group (F0 generation), and they were exposed to AAS in drinking water at 0, 50, 500 or 5000 ppm. After 10-week administration of AAS, each female was mated with a male from the same dosage group, and pregnant females were allowed to deliver and nurse their pups. F0 parental male rats were necropsied after the parturition of paired females, and F0 females were necropsied after weaning of their pups. Administration of AAS was continued throughout the mating, gestation and lactation periods until necropsy.

For the second generation, 24 male and 24 female weanlings (1 or 2 weanlings/sex in each litter) in each group were selected as F1 parents on PNDs 21–25 to equalize the mean body weights among groups as much as possible. 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 mated after 10-week administration. They were 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. Parental data (F0 and F1)

Throughout the study, all F0 and F1 parental rats were observed twice daily for general appearance and behavior, as well as for any signs of AAS intake. Food and water consumption was determined once and twice a week, respectively, throughout the exposure period, except during cohabitation. The body weights of males

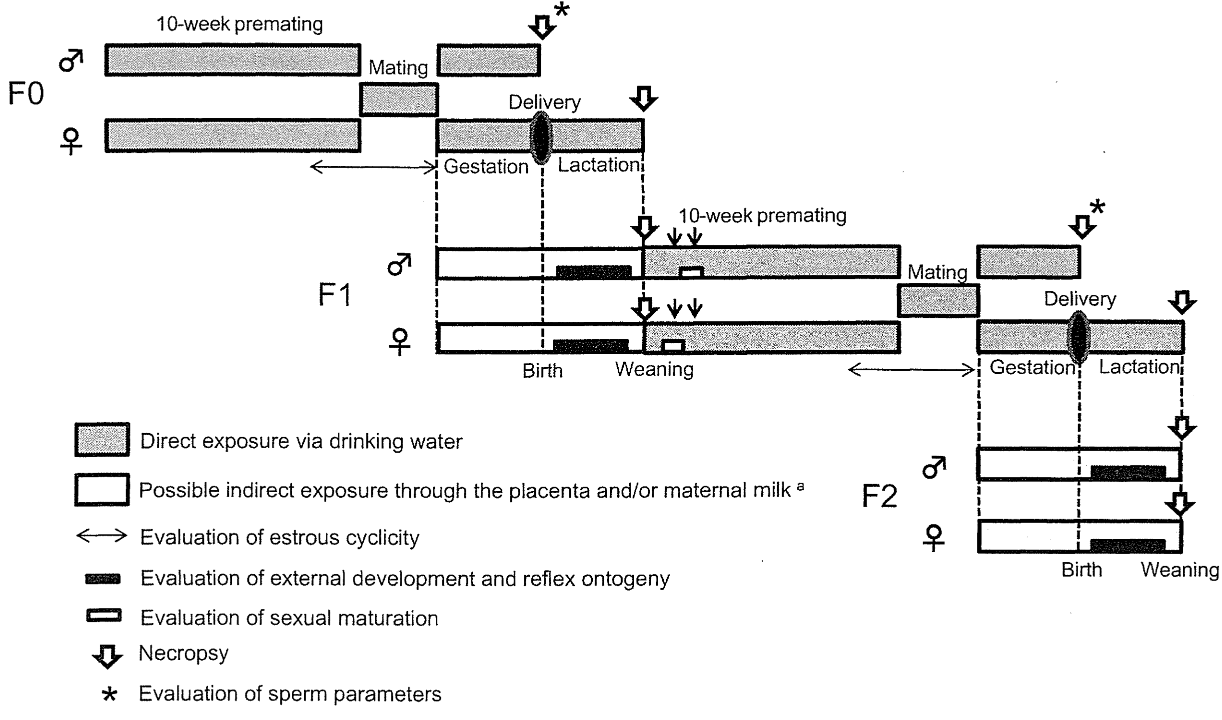

Fig. 1. Presentation of study design. [a]Pups could be also exposed to AAS directly via drinking water at the end of weaning period.

were measured weekly throughout the study. Body weight measurement for females was also performed weekly until evidence of copulation was detected, and thereafter on days 0, 7, 14 and 20 of gestation and on days 0, 4, 7, 14 and 21 of lactation.

2.4.1. Assessment of reproductive performance

After the 10-week premating period, each female was transferred to the home cage of a randomly chosen male from the same exposure group, and cohabited on a 1:1 basis until successful copulation occurred or the mating period of 2 weeks had elapsed. Beginning 2 weeks before the cohabitation period, vaginal smears were prepared daily to determine the stage of estrus for each F0 and F1 female until evidence of copulation was detected. The presence of sperm in the vaginal smear and/ or a vaginal plug was considered as evidence of successful mating, and 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 within not less than 7 days. Following confirmation of mating, females were returned to their home cages, and allowed to deliver spontaneously and nurse their pups until PND 21 (day of weaning). They were checked at least three times daily on days 21–25 of gestation, and 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.

2.4.2. Necropsy and histopathology

F0 and F1 parental males were euthanized by exsanguination under ether anesthesia after the parturition of paired females. The females were evaluated for estrous cycle stage by examination of the vaginal smear after weaning of pups, and euthanized in the proestrus stage by exsanguination under ether anesthesia. After external examination of these parental animals, the abdomen and thoracic cavity were opened and gross internal examination was performed. The number of uterine implantation sites was recorded for each female. The brain, pituitary, thyroid, thymus, liver, kidney, spleen, adrenal, testis, epididymis, seminal vesicle, ventral prostate, uterus and ovary were weighed (weights of the thyroid and seminal vesicle were measured after fixation). 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.

Histopathological evaluations were performed for the testes, epididymides, seminal vesicles, ventral prostate, coagulating gland, ovaries, uterus and vagina of all F0 and F1 animals in the control and highest dose groups. These organs were embedded in paraffin by a routine procedure. They were then sectioned, stained with hematoxylin–eosin and examined histopathologically under a light microscope.

The number of primordial follicles was counted for 10 F1 females, each randomly selected from the control and highest dose groups. The right ovary was dehydrated and embedded in paraffin in longitudinal orientation by routine procedures, and sectioned serially at 5 μm. Every 20th section was mounted on a slide and stained with hematoxylin–eosin. About 40 sections per ovary were used to determine the primordial follicles.

2.4.3. Sperm parameters

At the time of F0 and F1 parental male sacrifice, the right testis was immediately removed, weighed and homogenized in a physiological solution, and the number of homogenization-resistant spermatid heads was counted in a hemacytometer. Right epididymal cauda was also extracted and weighed, and an epididymal sperm suspension was prepared to assess the sperm number, motility, and morphology. Caudal sperm numbers were enumerated with a hemacytometer under a light microscope. Sperm motility (percentage of motile sperm and progressively motile sperm, swimming speed and pattern) was determined using a computer-assisted cell motion analyzer (TOX IVOS; Hamilton Thorne Bioscience, Beverly, MA, USA). Sperm morphology was evaluated for 200 sperm per male under a light microscope, using sperm slides stained with eosin.

2.5. Offspring evaluation (F1 and F2)

All pups derived from F0 and F1 parents (F1 and F2 litters, respectively) were examined as soon as possible on the day of birth to determine the number and sex of pups, the number of liveborn and stillborn members of each litter, and gross abnormalities. To reduce variability among litter size, each litter was randomly adjusted to eight pups of four males and four females on PND 4. The pups 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.

2.5.1. Developmental landmarks

All F1 and F2 live pups were evaluated for pinna unfolding daily for 4 days after birth. The anogenital distance (AGD) of these animals was measured using calipers on PND 4, and the AGD/cube root of the body weight ratio was calculated to correct the influence of body size on the AGD value (Gallavan et al., 1999). One male and one female F1 and F2 pup selected from each dam were observed for incisor eruption beginning on PND 8 and eye opening beginning on PND 12 until each pup achieved the criterion. For the same F1 and F2 pups, the surface righting reflex, negative geotaxis and mid-air righting reflex were assessed on PND 5, 8 and 18, respectively. 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 the criteria were fulfilled.

2.5.2. Necropsy and histopathology

F1 weanlings not selected to become parents and all F2 weanlings were euthanized under ether anesthesia on PND 26. For one male and one female F1 and F2 weanling selected from each dam, major organs were removed, fixed and preserved, as described for the adults. Weights of the brain, thymus, liver, kidney, spleen, adrenal, testis, epididymis, ventral prostate, uterus and ovary were recorded before fixation.

Since test substance-related change was found in the thymus, liver and spleen weight of males and in the liver and spleen weight of females in the highest dose group, histopathological evaluations of these organs were performed for 10 male and 10 female F1 and F2 weanlings in the control and highest groups. The examined animals were selected randomly from the animals whose organs were stored. Paraffin sections were routinely prepared, stained with hematoxylin–eosin and examined histopathologically under a light microscope.

2.6. Neurobehavioral evaluation

Spontaneous locomotor activity was evaluated at 4 weeks of age for 10 male and 10 female F1 rats randomly selected from each group. The animals were placed individually in transparent polycarbonate cages [285(W) × 450(D) × 210(H) mm, CL-0108-1; CLEA Japan Inc., Tokyo, Japan], and spontaneous motor activity was measured with SUPERMEX (Muromachi Kikai Co., Ltd., Tokyo, Japan), which was mounted above the cage to detect changes in heat across multiple zones of the cage with an infra-red sensor. Spontaneous motor activity was determined for 10 min periods and for a total of 60 min.

Spatial learning ability was assessed using a water-filled multiple T-maze (Biel's type) for 10 male and 10 female F1 rats selected from each group at 6 weeks of age. The water temperature of the maze was maintained at 21.0–22.0 °C. On the first day, the animals were given triplicate swimming trials in a straight channel, and the time required to reach the goal was recorded. On the following three days, they were subjected to three maze trials per day, and the time required to reach the goal and the number of errors were recorded. None of the rats were allowed to remain in the water for more than 3 min in any trial to prevent exhaustion.

2.7. Statistical analysis

The body weight of parental animals, food and water consumption, length of estrous cycle, gestational length, precoital interval, 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 evaluated by Bartlett's test for homogeneity of variances ($P < 0.05$). The body weight of preweaning pups, 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 applied ($P < 0.10$). If a significant difference was found, Dunnett's test was used for pairwise comparisons between control and individual treatment groups ($P < 0.01$ or 0.05). Data without homogeneity were subjected to the Kruskal–Wallis rank sum test ($P < 0.10$), and if significant differences were detected, the Mann–Whitney U test was used to compare AAS-treated groups with the control group ($P < 0.01$ or 0.05). The incidence of parental animals with clinical signs and necropsy and histopathological findings, 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 control and each dosage group using Fisher's exact test ($P < 0.01$ or 0.05). Wilcoxon's rank sum test was performed for the incidence of pups with clinical signs or necropsy findings per litter, completion rate of pinna unfolding in each litter, and the success rate of surface and mid-air righting reflex ($P < 0.01$ or 0.05). The number of primordial follicles was compared between the control and highest dose groups using Student's t-test ($P < 0.01$ or 0.05).

3. Results

3.1. Clinical observations, water and food consumption and body weight of parental animals (F0 and F1)

Throughout the study, there were no treatment-related parental deaths or clinical signs of toxicity at any of the three exposure levels evaluated. Water consumption was dose-dependently reduced in males and females of both generations, as shown in Fig. 2. Significant changes were observed throughout or almost throughout the dosing period in F0 males of all AAS-treated groups, and in F0 females and F1 males and females in the 500 and 5000 ppm groups. Significant decreases in water consumption were also found during weeks 1, 9 and 10 of dosing, week 1 of gestation and week 1 of lactation in 50 ppm-treated F0 females and during weeks 4 and 8–10 of dosing in 50 ppm-treated F1 females. Food consumption was significantly lower during week 1 of dosing in F0 males of the 5000 ppm group and in F0 females of the 500 and 5000 ppm groups (data not shown). In 5000 ppm-treated F0 and F1 females, there were also significant decreases in food consumption in the 2nd and 3rd weeks of lactation. Body weight was significantly lower in the 2nd week of dosing in both sexes of F0 rats and on day 21 of lactation in F0 females at 5000 ppm (Fig. 3). In the 5000 ppm group, the body weight of F1 males and females was significantly lower in the first 2 and 3 weeks of dosing, respectively (Fig. 3).

3.2. Daily intake of AAS and aluminium in parental animals (F0 and F1)

For each of the AAS-treated groups, daily AAS intake was estimated based on water consumption and body weight during the premating and postmating periods in males and during the premating, gestation and lactation periods in females. Calculated average intake of AAS during the whole period was 3.78, 33.5 and 305 mg/kg bw in F0 males, 6.52, 58.6 and 500 mg/kg bw in F0 females, 4.59, 41.8 and 372 mg/kg bw in F1 males, and 6.65, 61.9 and 517 mg/kg bw in F1 females for the 50, 500 and 5000 ppm groups, respectively. Considering aluminium content in the basal diet, dietary aluminium exposure of F0 and F1 animals was estimated from the food consumption and body weight in the control and AAS-treated groups. Average aluminium intake from drinking water and food combined was calculated to be 1.56, 1.98, 5.35 and 36.3 mg Al/kg bw/day in F0 males, 2.20, 2.89, 8.81 and 59.0 mg Al/kg bw/day in F0 females, 1.83, 2.35, 6.57 and 44.2 mg Al/kg bw/day in F1 males, and 2.39, 3.10, 9.36 and 61.1 mg Al/kg bw/day in F1 females for control through high-dose groups.

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

During the premating period, a few AAS-treated F0 and F1 female rats had persistent diestrus; however, the incidence of females with normal estrous cycles (4–5 days) was not changed significantly compared with the control. There were no significant differences in the estrous cycle between control and AAS-treated groups (data not shown).

Reproductive performance of F0 and F1 parental animals is summarized in Table 1. Although some animals failed to copulate, impregnate or deliver live pups, no significant changes were found in the copulation, fertility or gestation index between the control and AAS-treated groups in F0 and F1 generations. There were also no significant differences in the precoital interval and gestation length in either generation.

Sperm analysis of schedule-sacrificed F0 and F1 adults revealed no significant differences in the number of testis sperm and cauda epididymal 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 AAS-treated groups (data not shown).

3.4. Developmental effects (F1 and F2)

No significant changes were found in the number of implantations or pups delivered, delivery index, sex ratio of pups and the viability index during the preweaning period in either generation (Table 2). During the preweaning period, external gross examination revealed microphthalmia, a rudimentary tail, trauma and scab

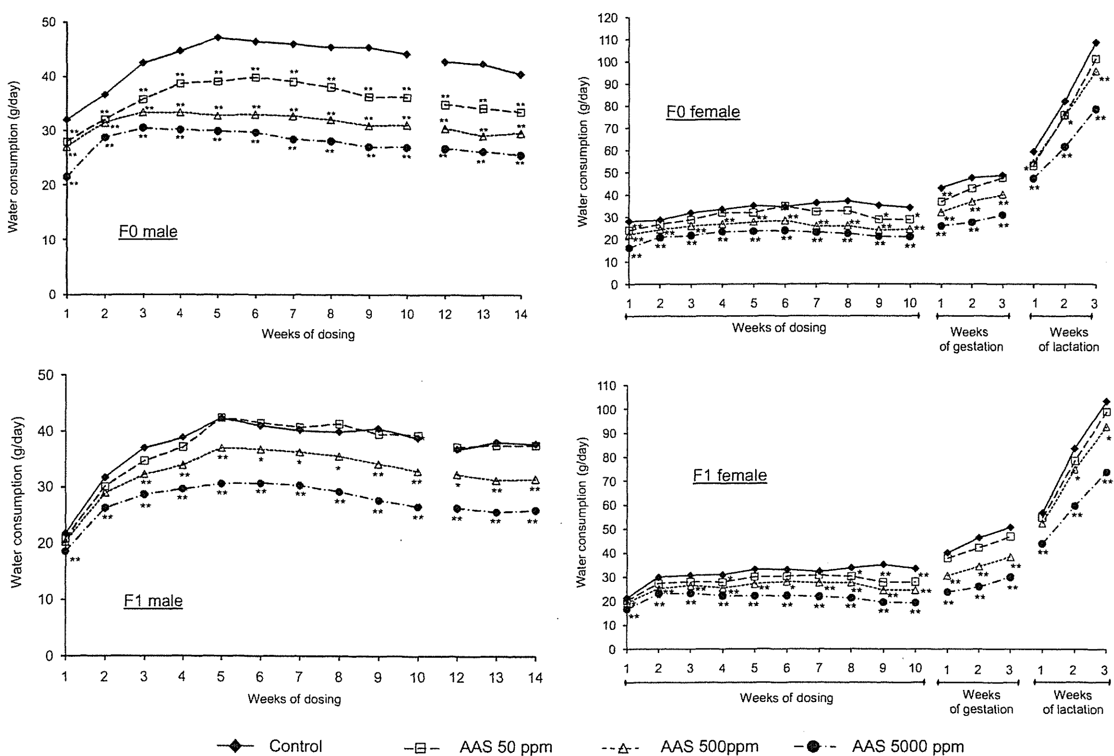

Fig. 2. Water consumption of F0 and F1 parental animals. *Significantly different from the control. $P < 0.05$, **Significantly different from the control, $P < 0.01$.

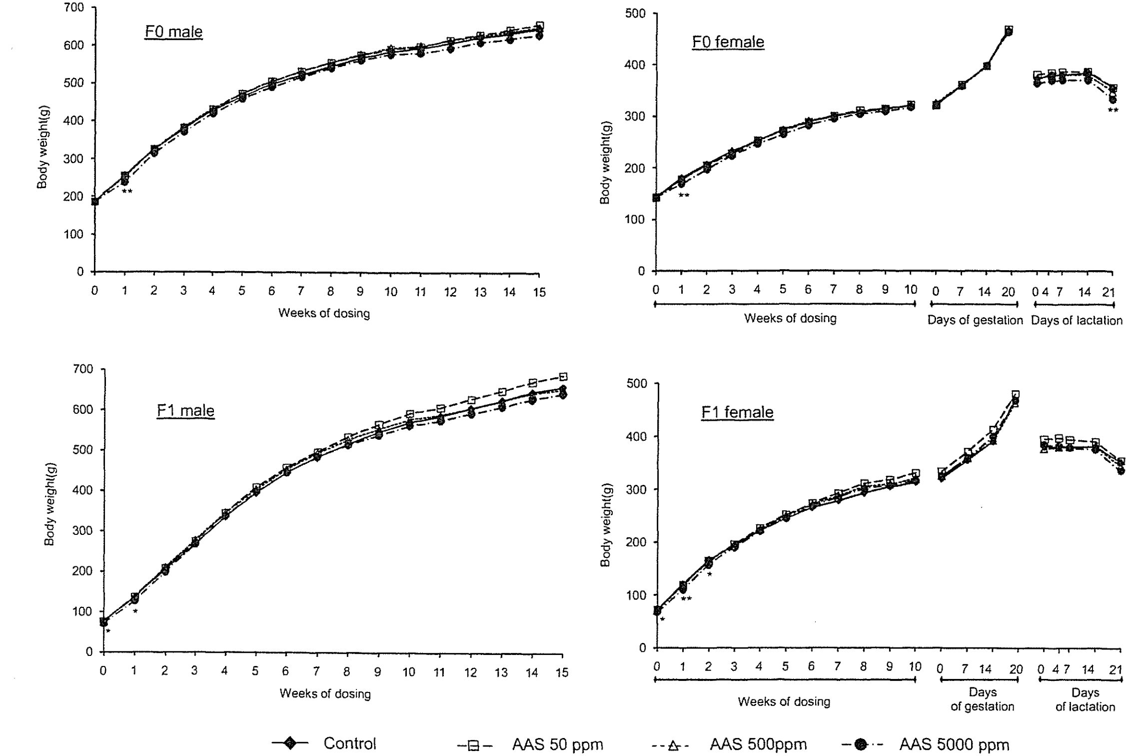

Fig. 3. Body weight change of F0 and F1 parental animals. *Significantly different from the control, $P < 0.05$. **Significantly different from the control, $P < 0.01$.

on right hindlimb and crushing of incisor/malocclusion in a few F1 pups in control and AAS-treated groups; however, there were no significant differences in incidence between the control and AAS-treated groups (data not shown). No gross abnormalities were found in any F2 pups. While there were no significant differences in birth weight between the control and AAS-treated groups, the

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.

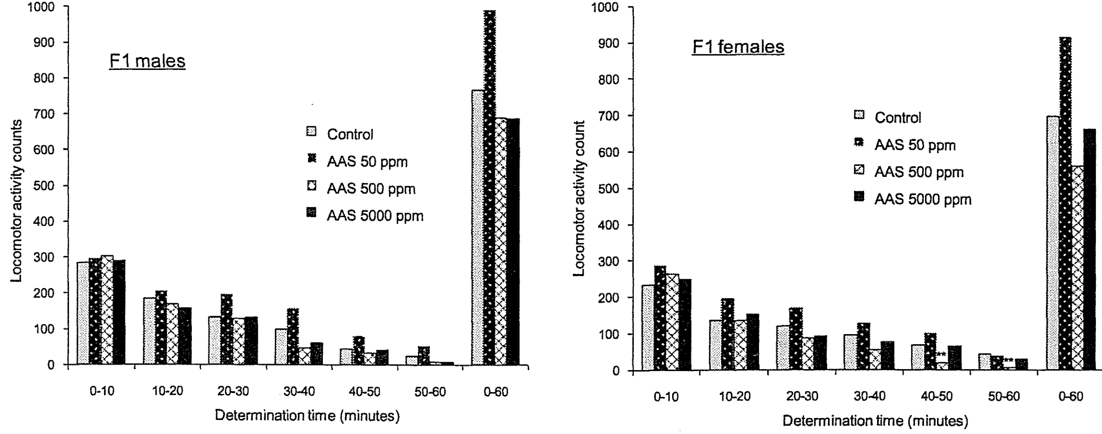

Fig. 4. Locomotor activity count in F1 parental rats. Data are presented as the mean of 10 animals/sex/group. **Significantly different from the control, $P < 0.01$.

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.

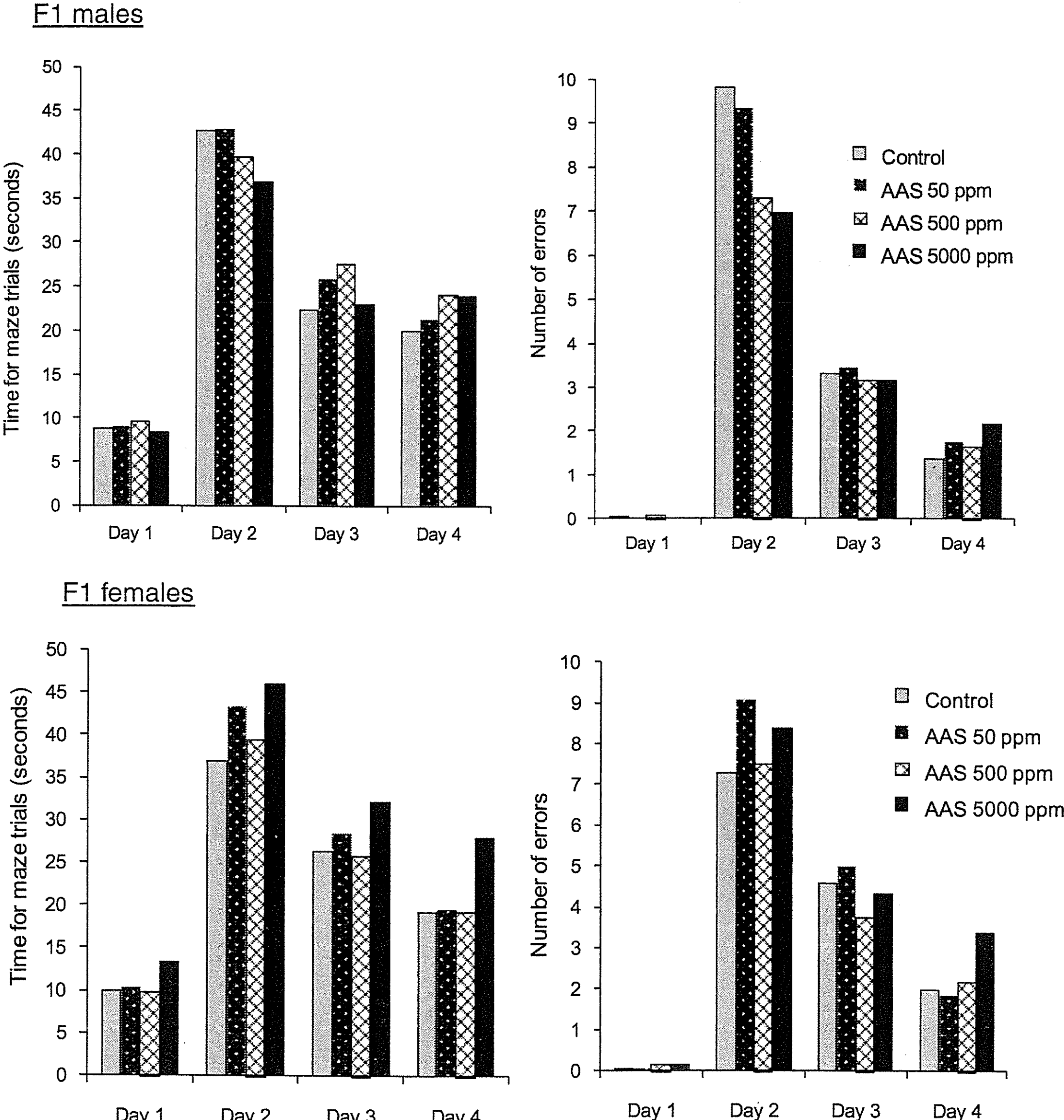

Fig. 5. Performance in a water-filled multiple T-maze in F1 parental rats. Data are presented as the mean of 10 animals/sex/group. There were no statistically significant differences between the control and AAS-treated groups.

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*