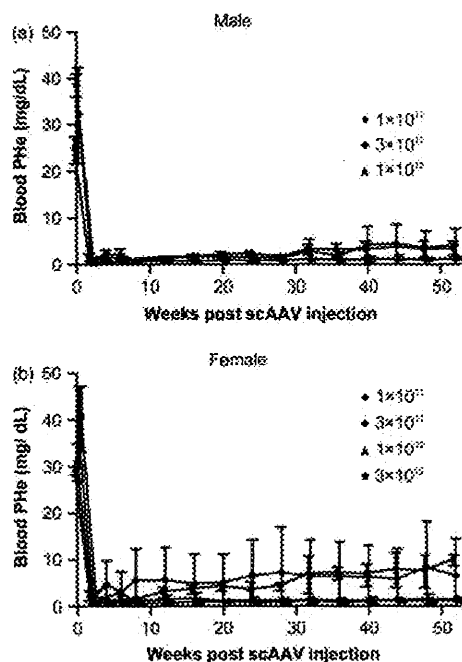

Figure 4. Long-term efficacy of i.p.-injected scAAV8/LP1-mPAH in *Pah*^{enu2} mice. (a) Male *Pah*^{enu2} mice were administered either 1×10^{11} vg (circles, $n = 4$), 3×10^{11} vg (diamonds, $n = 3$) or 1×10^{12} vg (triangles, $n = 3$) of scAAV8/LP1-mPAH, and the data are depicted as the mean ± SD blood Phe (mg/dl) versus weeks post-injection of scAAV8. (b) Female *Pah*^{enu2} mice were given either 1×10^{11} vg (circles, $n = 3$), 3×10^{11} vg (diamonds, $n = 3$), 1×10^{12} vg (triangles, $n = 4$) or 3×10^{12} vg (squares, $n = 4$) of scAAV8/LP1-mPAH, and the data are shown as the mean ± SD blood Phe (mg/dl)

Long-term correction of hyperphenylalaninemia in both genders

Encouraged by the shot-term efficacy of scAAV8/LP1-mPAH vector in female *Pah*^{enu2}, we treated several cohorts of PKU mice with the same vector for longer observation ($n = 3$–4 each). Male and female *Pah*^{enu2} mice (6–8 weeks of age) were given either 1×10^{11}, 3×10^{11} or 1×10^{12} vg of scAAV8/LP1-mPAH by i.p. injection. An additional female cohort was given a higher dose (3×10^{12} vg), in consideration of the possibility of temporary transgene expression in this gender. The treated mice were phlebotomized for blood Phe analysis every 2–4 weeks, and Figure 4 shows the kinetics of blood Phe concentration for 1 year following vector administration. In males, Phe levels decreased to normal with all doses (1×10^{11} vg, from 25.1 ± 2.3 to 1.1 ± 0.3 mg/dl; 3×10^{11} vg, from 38.3 ± 2.7 to 0.8 ± 0.6 mg/dl; 1×10^{12} vg, from 32.5 ± 9.7 to 0.4 ± 0.1 mg/dl) at week 2 (Figure 4a). All males with 1×10^{12} vg ($n = 3$) and two out of three males with 3×10^{11} vg maintained normal blood Phe (<1.7 mg/dl) throughout 1-year observation, whereas one with 3×10^{11} vg showed a mild elevation (8–9 mg/dl) from 32 weeks post-i.p. One male with 1×10^{11} vg upheld normal blood Phe, whereas three out of four in this cohort had slightly increased blood Phe from 32 weeks that was within the therapeutic range (2–6 mg/dl). A similar, although slightly limited long-term efficacy was observed in females (Figure 4b). In the female *Pah*^{enu2} mice with 3×10^{12} vg ($n = 4$) or 1×10^{12} vg ($n = 4$) of vector, blood Phe decreased to normal in 2 weeks (from 40.2 ± 7.0 to 0.7 ± 0.4 mg/dl, and 38.5 ± 8.7 to 0.6 ± 0.2 mg/dl, respectively) and maintained that level for 1 year. In females with 3×10^{11} vg, two out of three animals sustained normal blood Phe, whereas the other mouse lost control after 4 weeks with moderate hyperphenylalaninemia (10–15 mg/dl). In females with 1×10^{11} vg ($n = 3$), blood Phe was corrected initially (from 29.1 ± 1.0 to 1.5 ± 0.5 mg/dl at week 4), although the level was gradually increased to near-normal (2–6 mg/dl at weeks 12–28) and then mild hyperphenylalaninemic range (6–10 mg/dl at weeks 32–52). Despite such variability being observed in a limited number of animals, the reduction of blood Phe was significant in all the cohorts throughout the observation period. This result clearly demonstrated the superb efficacy of scAAV8/LP1-mPAH vector in treating murine PKU. We assumed that the vector threshold dose

Table 2. Remote phase blood Phe, *in vivo* Phe oxidation and vector DNA in the liver

Gender	Dose (vg)	Phe (mg/dl)	$\Delta^{13}CO_2$ (‰)	Vector DNA (c/dg)
Male	1×10^{11}	3.9	18.31	0.88
	1×10^{11}	3.0	32.54	0.57
	3×10^{11}	1.7	52.18	1.34
	3×10^{11}	2.1	19.54	1.16
	1×10^{12}	3.3	45.34	9.95
	1×10^{12}	1.2	ND	7.85
Female	1×10^{11}	12.4	3.96	0.17
	1×10^{11}	8.9	1.57	0.22
	3×10^{11}	4.2	5.62	0.48
	1×10^{12}	1.6	60.44	3.13
	3×10^{12}	1.3	23.31	6.38
	3×10^{12}	2.4	62.95	6.47
	3×10^{12}	1.2	55.60	9.26

ND, not determined.

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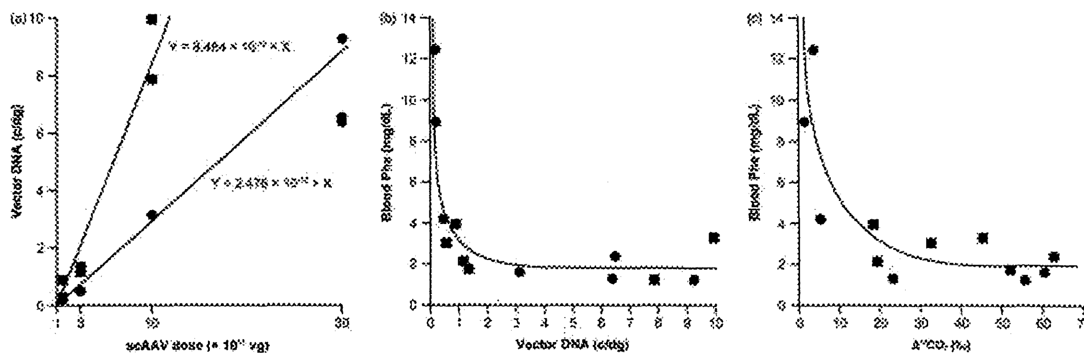

Figure 5. Relationships among vector dose, blood Phe, *in vivo* Phe oxidation and vector DNA content in remote phase. (a) Relationship between the given dose of scAAV8/LP1-mPAH (vg; *x*-axis) and vector DNA content in the liver (c/dg; *y*-axis). Plots with males (squares) fit a regression line of $y = 8.484 \times 10^{-12}x$, whereas plots with females (circles) fit a line of $y = 2.478 \times 10^{-12}x$. (b) Relationship between the vector DNA in the liver (c/dg; *x*-axis) and blood Phe (mg/dl; *y*-axis). Males (squares) and females (circles) fit a single hyperbolic-like curve. (c) Relationship between *in vivo* Phe oxidation ($\Delta^{13}CO_2$‰; *x*-axis) and blood Phe (mg/dl; *y*-axis). Plots of males (squares) and females (circles) fit a single hyperbolic-like curve

to correct hyperphenylalaninemia was 1×10^{11} vg or less for male Pah^{enu2} mice, and slightly over 1×10^{11} vg for females that would be a half-log higher. A gender-specific difference appears to exist with scAAV8 as well, although the barrier in female animals may be smaller than that with ssAAV vectors.

Correlation between transduction rate and function in remote phase

After confirming the long-term efficacy of the scAAV8 vector in both genders, we examined *in vivo* Phe oxidation and tissue DNA in several *Pah*^{enu2} mice later than 1 year after scAAV8 injection. Table 2 summarizes the data obtained for six males and seven females evaluated at 60–80 weeks post-i.p., and Figure 5 shows the relationships among the parameters chosen. The amount of vector DNA in the liver showed a linear relationship with the given vector dose, and was not saturated within the dose range used in the present study (Figure 5a). Compared with the data for 8 weeks post-i.p. (Table 1), the vector DNA content in the treated females was approximately one-tenth in the remote phase, suggesting a slow but substantial vector loss. The lines best fit the data were $y = 8.484 \times 10^{-12}x$ ($r^2 = 0.962, p < 0.0001$) for males and $y = 2.478 \times 10^{-12}x$ ($r^2 = 0.967, p < 0.0001$) for females, respectively (x, vector dose in vg; y, vector DNA in the liver in c/dg). Thus, male livers were more efficiently transduced than female livers by 3.4-fold, in agreement with the above assumption of vector doses for correcting hyperphenylalaninemia in these genders.

Once the liver was transduced, the vector appeared to impact on Phe metabolism in the same, or nearly identical mode in both genders. Figure 5b shows the relationship between liver vector copy number and blood Phe. Plots for male (squares) and female (circles) livers appear to fit into quite analogous, if not identical, hyperbolic-like curves. Blood Phe levels were steeply reduced to normal as the vector copy number increased from 0 to 1 c/dg, and no further reduction was observed with more vector DNA in the liver. Therefore, Phe was normalized by the presence of 1 or more vector c/dg, regardless of gender. The result suggests that the larger dose requirement of scAAV8 to correct female hyperphenylalaninemia resulted not from poorer transgene expression but from a lower transduction efficiency. Figure 5c shows the relationship between *in vivo* Phe oxidation and blood Phe. Again, plots of both males (squares) and females (circles) appear to fit an almost identical hyperbolic-like curve. Earlier preclinical studies showed that recovery of 10–20% of normal PAH activity was sufficient to normalize blood Phe in *Pah*^{enu2} mice, in agreement with accumulated clinical data [1,9,31]. Because we observed a significant baseline deviation and fluctuation of $\Delta^{13}CO_2$ in WT animals (Table 1) partly because of the relative BH_4 insufficiency *in vivo* [27], it was difficult to calculate the actual recovery of Phe oxidation capacity of the treated *Pah*^{enu2} mice. Nevertheless, it is noteworthy that some animals showed physiological or even higher levels of Phe catabolism, even for extended periods (60–80 weeks) after scAAV8 injection.

Discussion

In the present study, we showed a long-term efficacy of a scAAV8 vector for murine PKU in both genders. To our knowledge, the vector allowed the longest duration (up to 80 weeks) of correcting hyperphenylalaninemia, particularly in female *Pah*^{enu2}, by gene transfer. We previously showed successful correction of hyperphenylalaninemia in *Pah*^{enu2} mice with a ssAAV5 vector, although the effect was partial and transient in females [7]. Type 8-pseudotyping of that vector increased the initial efficacy by more than ten-fold, although the therapeutic benefit waned over time in female *Pah*^{enu2}. Other studies also recognized a similar female disadvantage with ssAAV8, although it was

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milder than that with ssAAV2 and ssAAV5 [14,32]. On the other hand, Ding *et al.* [10] normalized blood Phe in both male and female Pah^{enu2} mice with a ssAAV8 vector up to 42 weeks. Following an injection of relatively large dose (5×10^{12} vg) of ssAAV8, they found very high copy number (>1000 c/dg) of vector DNA in the liver of their animals, which was 20–50-fold greater than than that in other studies including our own. If this was indeed the case, such an excess amount of vector, at least in part, may account for the adequate PAH expression that would overcome the female disadvantage.

In our experimental setting, the scAAV genome structure finally allowed a robust and stable liver transduction in female mice. In accordance with our results, recent studies have demonstrated the advantage of scAAV vectors in liver-directed gene transfer, particularly in combination with the serotype 8 capsid [32–35]. Pañeda *et al.* [32] extensively studied the efficiency of pseudotyped ssAAV and scAAV vectors and showed a significant advantage of scAAV8 in female mice. Furthermore, using the same vector platform (scAAV8/LP1) as in the present study, Vaessen *et al.* [33] showed a near-physiological transgene expression in a murine apolipoprotein A-I deficiency model, whereas Wu *et al.* [34] suggested that further fine-tuning of the vector should increase the efficacy. Because the therapeutic end point for PKU gene transfer is relatively high (>10% of normal PAH activity), these investigations are quite encouraging. Interestingly, Nathwani *et al.* [35] observed that preadministration of bortezomib, a proteasome inhibitor, augmented liver transduction with a scAAV8 vector in female mice by two-fold, whereas the enhancement in male animals was more modest. A detailed understanding of the molecular events in AAV transduction should allow such pharmacological support for better gene transfer in actual clinical settings.

One of the issues addressed in the present study was how many AAV vector copies in the liver would be required for persistent phenotypic correction of PKU. In the analysis at 8 weeks post-i.p., we found 1.5 ± 0.3 c/dg following 1×10^{11} vg i.p. and 27.3 ± 16.0 c/dg following 1×10^{12} vg i.p. (Table 1), whereas other studies detected more AAV genomes in their mouse liver. For example, Harding *et al.* [9] detected 16–51 c/dg of vector DNA in the mouse liver 8–17 weeks after they injected 5×10^{11} vg of ssAAV8, and Nakai *et al.* [36] found 58.2 ± 10.9 c/dg of vector 6 weeks after 3×10^{11} vg of ssAAV8. Presumably, our method of AAV administration (i.p.) was relatively less efficient compared to the PV injection that these investigators carried out. However, the reason for the large discrepancy compared to another study [10] (>1000 c/dg) is unknown. After initial transduction of the liver, the recombinant AAV genomes may be gradually degraded or diluted because they are present as episomes [37]. Indeed, we observed that the vector DNA was decreased to one-tenth in the animal liver after 1 year. At this stage, we demonstrated that the threshold of the therapeutic vector DNA in the tissue was approximately 1 c/dg. To extend gene transfer efficacy, a new strategy may be required. Integrating vectors are maintained if they do not elicit immunological responses, although most of them have some genotoxicity at the expense of stability through mitosis. Site-specific integrases may offer safer and stable transduction [38], although the delivery vehicle for such a strategy is currently underdeveloped. Alternatively, targeting extrahepatic tissues for PKU gene therapy has been discussed. In particular, skeletal muscle is an attractive target because of its slow turnover and easy accessibility [3,39,40]. One of the problems with this approach is how to deliver cofactor BH_4 to the exogenously expressed PAH in a clinically feasible way.

In summary, a single injection of scAAV8 vector corrected hyperphenylalaninemia in male and female PKU mice for the longest period reported to date. Although it is difficult to translate murine experiments into human PKU directly, the accumulating safety and efficacy studies of AAV gene transfer to larger animals are very informative. Together with an encouraging result obtained in a haemophilia B clinical trial started recently [41], these investigations will help to develop a safe and long-lasting gene transfer strategy for PKU treatment.

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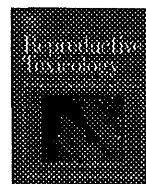

Two-generation reproductive toxicity study of aluminium sulfate in rats

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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.

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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.

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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

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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/F1 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 ± 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

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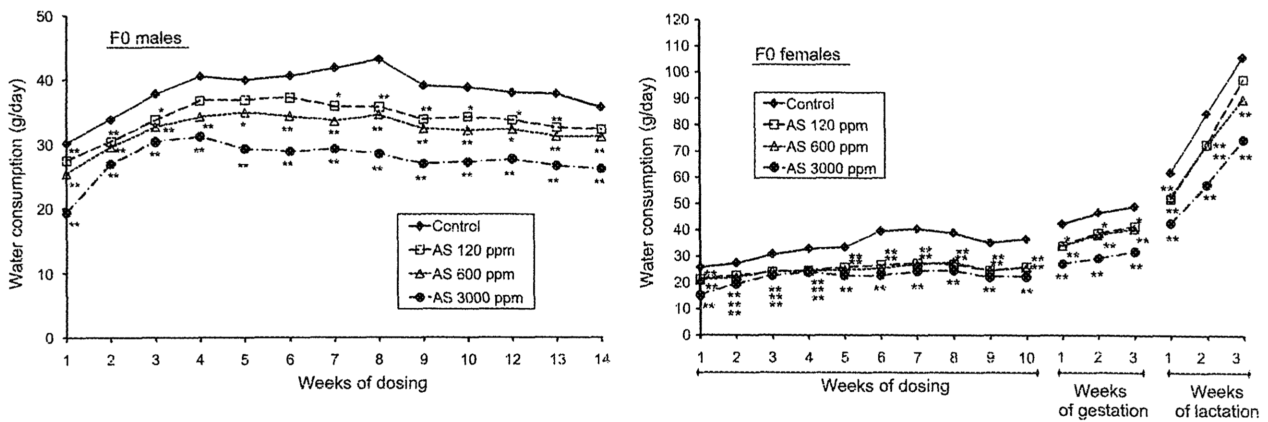

Fig. 1. Water consumption of F0 parental animals. *Significantly different from the control, $P < 0.05$, **significantly different from the control, $P < 0.01$.

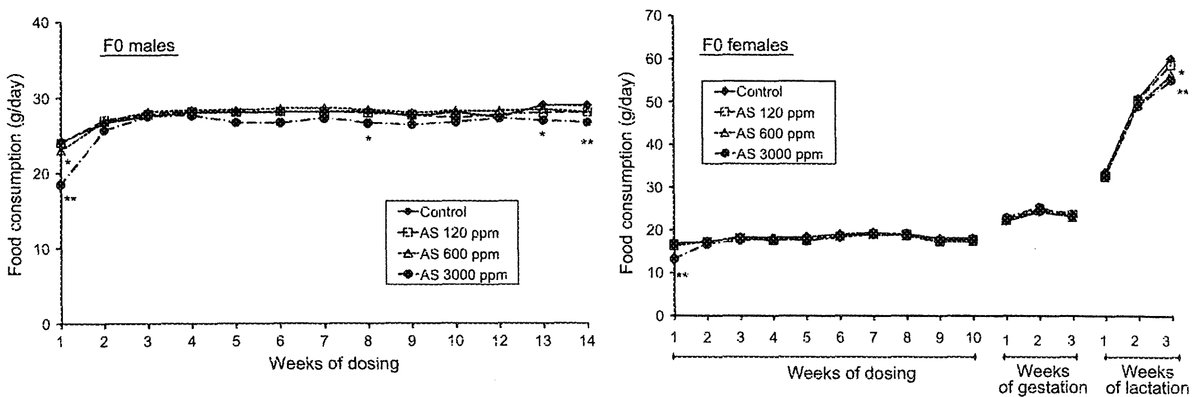

Fig. 2. Food consumption of F0 parental animals. *Significantly different from the control, $P < 0.05$, **significantly different from the control, $P < 0.01$.

control and 600 ppm groups (100 ± 0.0% versus 98.4 ± 7.3%). The surface righting reflex on PND 5 and negative geotaxis reflex on PND 8 were achieved in all male and female F2 pups in all groups, and no significant changes were found in the response time (data not shown).

As for the sexual development of F1 male and female animals, vaginal opening was significantly delayed at 3000 ppm (31.4 ± 1.7, compared to 29.5 ± 2.1 in control). At this dose, body weight at the time of vaginal opening was slightly heavier than the control (119.0 ± 13.3 g versus 109.6 ± 11.6 g) although not statistically significant. No significant differences between control and AS-treated groups were noted in the age at preputial separation or body weight at the time of completion in males.

3.4. Behavioral effects (F1)

Spontaneous locomotor activity at 10-min intervals and for 60 min was not significantly different between control and AS-treated groups in male and female F1 rats. In the water-filled *T*-maze test, pre-test swimming trials in the straight channel

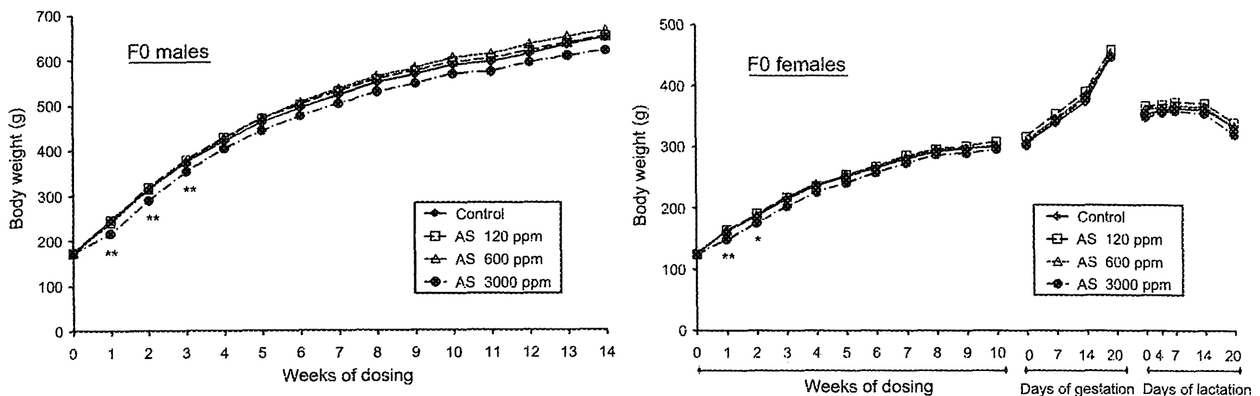

Fig. 3. Body weight of F0 parental animals. *Significantly different from the control, $P < 0.05$, **significantly different from the control, $P < 0.01$.

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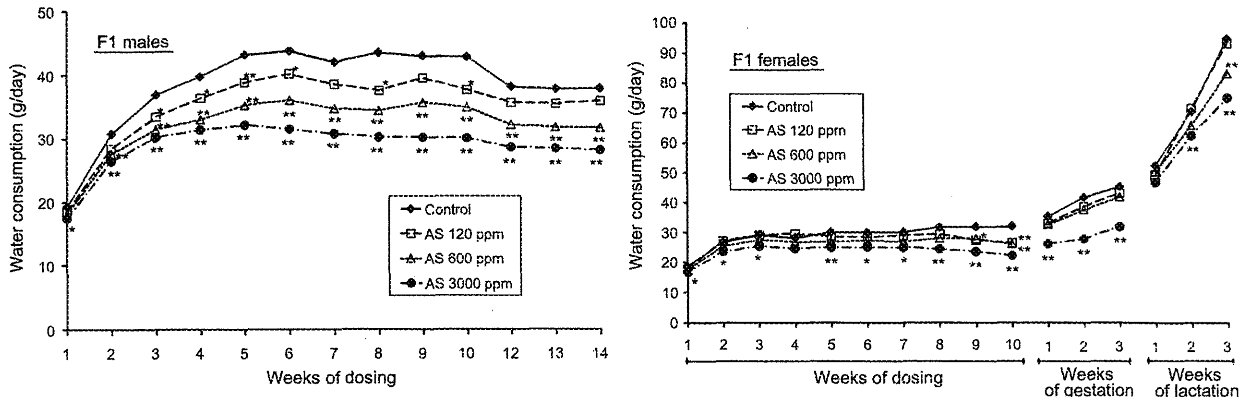

Fig. 4. Water consumption of F1 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

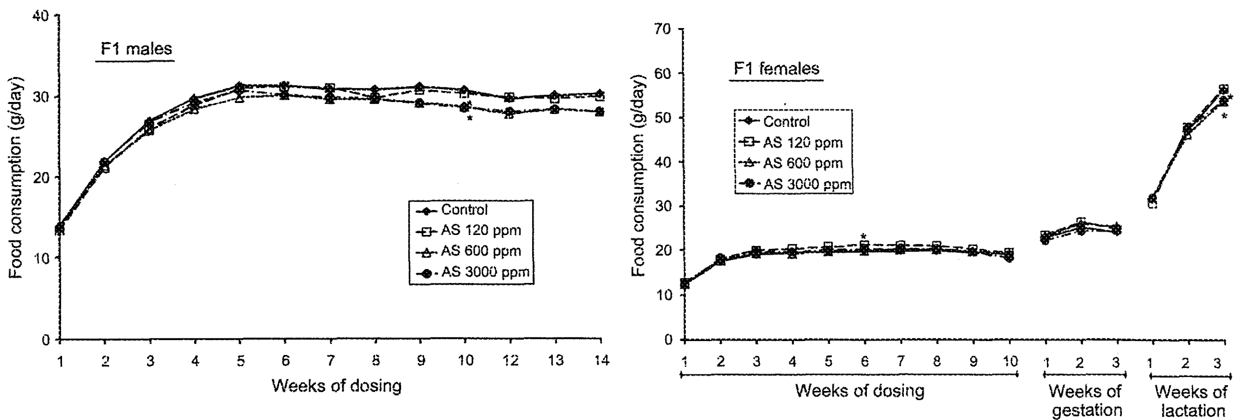

Fig. 5. Food consumption of F1 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

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 the straight channel. On days 2–4 of the *T*-maze test, no significant changes were observed in the elapsed time and number of errors in males. In females, the elapsed time and the number of errors on day 2 of the *T*-maze was significantly lowered at 600 ppm, but there were no significant differences in the elapsed time or number of errors on days 3 and 4 of the *T*-maze test between control and AS-treated groups (data not shown).

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

In F0 males, absolute and relative liver weights were significantly decreased at 3000 ppm. Absolute spleen weight was also

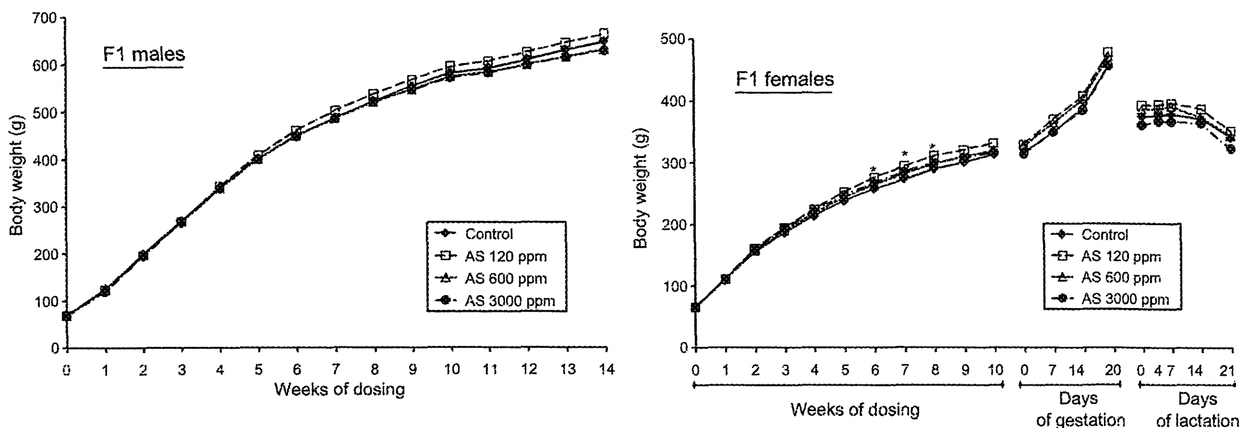

Fig. 6. Body weight of F1 parental animals. *Significantly different from the control, $P<0.05$, **significantly different from the control, $P<0.01$.

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Table 1
Reproductive performance of F0 and F1 parental animals.

AS (ppm)		0 (control)	120	600	3000
F0 generation					
No. of rats (male/female)		24/24	24/24	24/24	24/24
Copulation index (%)[a]	Males	91.7	91.7	100	91.7
	Females	95.8	100	100	100
Precoital interval (days)[b]		3.2 ± 1.1	3.2 ± 1.8	2.9 ± 1.3	2.8 ± 1.6
Fertility index (%)[c]	Males	95.5	90.9	100	95.5
	Females	95.7	91.7	100	95.8
Gestation index (%)[d]		100	95.5	95.7	95.7
Gestation length (days)[b]		22.4 ± 0.5	22.5 ± 0.6	22.1 ± 0.4	22.3 ± 0.5
Delivery index (%)[b,e]		94.3 ± 5.6	88.6 ± 21.0	90.7 ± 20.8	92.0 ± 20.5
F1 generation					
No. of rats (male/female)		24/24	23/24	24/24	24/24
Copulation index (%)[a]	Males	95.8	91.3	95.8	87.5
	Females	100	95.8	100	95.8
Precoital interval (days)[b]		3.3 ± 3.2	3.0 ± 2.0	2.7 ± 1.5	2.3 ± 1.1
Fertility index (%)[c]	Males	91.3	81.0	91.3	95.2
	Females	91.7	82.6	91.7	91.3
Gestation index (%)[d]		100	94.7	100	100
Gestation length (days)[b]		22.4 ± 0.5	22.3 ± 0.5	22.2 ± 0.4	22.2 ± 0.4
Delivery index (%)[b,e]		94.0 ± 9.9	87.5 ± 22.6	91.4 ± 10.7	94.6 ± 6.8

[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.
[e] Delivery index (%) = (no. of pups delivered/no. of implantations) × 100.

decreased significantly in this group, but no significant change was found in the relative weight. In F1 males, the absolute weights of the adrenals at 3000 ppm and the testes at 600 ppm were significantly decreased without significant changes in the relative weight. There were no significant changes in the absolute and relative weights of any organ in F0 and F1 female adults (data not shown).

No dose-related gross lesions were found in F0 or F1 adults. Histopathological examination of the reproductive organs revealed no compound-related alterations. There was no significant difference in the number of primordial follicles in the ovary of F1 females between control and 3000 ppm groups (data not shown).

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

Absolute and relative organ weights of male and female F1 weanlings are shown in Table 3. The 3000 ppm treated males and females had a significantly lower body weight at scheduled sacrifice than the controls. In this group, absolute and relative liver weights were significantly lower than the controls. Absolute spleen weight was also decreased significantly in both sexes of the 3000 ppm group, accompanied by a significant decrease in the relative weight in males. In addition, significant decreases in the absolute weight were found for the thymus in both sexes and for the kidneys, testes and epididymides in males at 3000 ppm, and for the uterus in females at 600 and 3000 ppm. Relative brain weight was significantly increased in both sexes of the 3000 ppm group.

Table 4 presents absolute and relative organ weights of male and female F2 weanlings. The mean body weight at scheduled sacrifice was significantly lowered in both sexes of the 3000 ppm group. In males, the absolute and relative weights of the thymus and spleen were significantly decreased in the 3000 ppm group. Significant decreases were also found in the absolute weight of the liver and epididymides at 3000 ppm. The relative brain weight was significantly increased at this dose. At 120 ppm, the only significant change was a non-dose-related decrease in the relative thymus weight. In F2 females, there were significant decreases in the absolute and relative weights of the liver, and the absolute weight of the spleen, ovary and uterus, and a significant increase in the relative brain weight at 3000 ppm. In addition, a significant decrease in the absolute brain weight was observed only in the 600 ppm group.

External and internal gross observations revealed no compound-related alterations either in F1 and F2 weanlings or in pups found dead during the preweaning period. There were no dose-related histopathological changes in the liver and spleen of male and female F1 and F2 weanlings.

4. Discussion

AS administered via the drinking water to male and female rats resulted in decreased water consumption for both sexes in all treatment groups. Since the dosing solution containing AS was pH 3.57–4.20, the acidity would decrease the palatability of drinking water in AS-treated groups. Decreased water consumption was associated with decreased food consumption by F0 and F1 males and females in the 600 and 3000 ppm groups and decreased body weight in F0 male and females in the 3000 ppm group. Since water-deprived animals typically reduce their levels of feed consumption and consequently lower their body weight [46], decreased food consumption and body weight observed in the present study could be considered secondary to the decreased water consumption. In the present study, food consumption and body weight fell notably during the early dosing period in F0 males and females. Food consumption also decreased in F0 and F1 females at the end of the lactation period, when F1 or F2 pups would commence eating and drinking for themselves [37]. Campbell et al. [46] reported that animals have a certain amount of "buffering" capacity in the form of physiological mechanisms acting to reduce fluid loss. This might explain notable changes around the time when rats start drinking AS-containing water.

Continuous drinking of AS-contained water for two generations did not result in changes in copulation, fertility or gestation indices, pre-coital or gestation length, the number of implantations or pups delivered, or the incidence of pups with malformations or variations. In addition, adverse effects were not found in estrous cyclicity or sperm parameters, and the histopathology of reproductive tissues in male and female parental animals. Previous studies have demonstrated that parenterally administered aluminium affected

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Table 2
Sex ratio, viability and body weight for F1 and F2 pups.

AS (ppm)	0 (control)	120	600	3000
F1 offspring				
No. of litters	22	21	22	22
No. of pups delivered[a]	13.9 ± 1.7	12.4 ± 4.7	13.1 ± 4.1	13.1 ± 3.4
Sex ratio of pups[b]	0.503	0.462	0.513	0.536
Viability index of pups (%)[a]				
On PND 0[c]	100.0 ± 0.0	99.3 ± 2.3	99.7 ± 1.6	99.5 ± 2.4
On PND 4[d]	98.7 ± 2.9	95.2 ± 21.8	98.8 ± 2.6	98.0 ± 5.4
On PND 21[e]	99.4 ± 2.7	100.0 ± 0.0	100.0 ± 0.0	99.4 ± 2.7
Male pup weight during lactation (g)[a]				
On PND 0	7.05 ± 0.61	7.25 ± 0.99	6.74 ± 0.69	6.96 ± 0.76
On PND 4	11.04 ± 0.85	11.41 ± 1.99	10.86 ± 1.37	11.00 ± 1.06
On PND 7	18.91 ± 1.29	19.36 ± 2.77	18.59 ± 1.71	18.47 ± 1.35
On PND 14	37.70 ± 2.63	37.97 ± 3.08	37.39 ± 2.59	36.34 ± 2.41
On PND 21	62.48 ± 4.50	62.63 ± 6.14	60.77 ± 4.01	57.34 ± 4.86**
Female pup weight during lactation (g)[a]				
On PND 0	6.61 ± 0.55	6.89 ± 0.83	6.35 ± 0.57	6.60 ± 0.64
On PND 4	10.46 ± 0.89	11.06 ± 1.71	10.27 ± 1.33	10.43 ± 0.83
On PND 7	18.03 ± 1.27	18.56 ± 2.31	17.69 ± 1.61	17.61 ± 1.21
On PND 14	36.29 ± 2.71	36.94 ± 3.03	35.67 ± 2.60	35.31 ± 2.24
On PND 21	60.17 ± 4.16	60.87 ± 5.68	57.68 ± 4.33	55.60 ± 4.34**
F2 offspring				
No. of litters	22	18	22	21
No. of pups delivered[a]	13.1 ± 3.6	13.2 ± 3.8	12.6 ± 3.9	14.0 ± 1.9
Sex ratio of pups[b]	0.528	0.502	0.536	0.457
Viability index of pups (%)[a]				
On PND 0[c]	99.68 ± 1.51	99.49 ± 2.14	98.42 ± 3.57	98.69 ± 3.60
On PND 4[d]	94.72 ± 14.54	98.07 ± 5.45	99.07 ± 3.15	99.01 ± 2.49
On PND 21[e]	100.00 ± 0.00	98.61 ± 4.04	100.00 ± 0.00	100.00 ± 0.00
Male pup weight during lactation (g)[a]				
On PND 0	6.97 ± 0.68	6.92 ± 0.81	6.87 ± 0.74	6.89 ± 0.60
On PND 4	10.73 ± 1.62	10.53 ± 1.27	11.27 ± 1.81	10.52 ± 1.15
On PND 7	17.96 ± 2.05	17.51 ± 2.12	18.83 ± 2.39	17.72 ± 1.60
On PND 14	35.79 ± 3.52	36.18 ± 3.63	37.32 ± 4.15	35.44 ± 2.73
On PND 21	59.61 ± 5.45	59.44 ± 5.67	60.12 ± 7.12	56.36 ± 4.47
Female pup weight during lactation (g)[a]				
On PND 0	6.66 ± 0.69	6.38 ± 0.78	6.41 ± 0.65	6.50 ± 0.49
On PND 4	10.22 ± 1.63	9.70 ± 1.23	10.36 ± 1.54	9.98 ± 0.91
On PND 7	17.03 ± 1.99	16.36 ± 2.35	17.40 ± 2.18	16.89 ± 1.23
On PND 14	34.82 ± 3.52	34.17 ± 3.58	34.96 ± 4.24	34.01 ± 2.09
On PND 21	57.33 ± 4.90	56.11 ± 5.54	56.41 ± 6.04	54.16 ± 2.82*

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

male reproductive systems, causing decreases in testicular and epididymal sperm counts, necrosis of spermatocytes/spermatids in the testes, and reduction of fertility, etc., in rats and mice [19,20,47]. Although aluminium has extremely low oral bioavailability (less than 1%) [48,49], male reproductive toxicity was also observed in oral gavage studies [22,23,50]. In the 6-month oral gavage study in rats, changes in the number of spermatozoa and motility, and the substantial proliferation of interstitial cells in the testes were observed at doses as low as 2.5 mg Al/kg/day (as aluminium chloride) [50]. The primary reason why such effects were not detected in the present study might be a difference in the administration method because the toxicokinetic behavior of chemicals given as a bolus dose by gavage must differ significantly from those after continuous administration via drinking water. Another possible factor is aluminium content in the laboratory animal feed, for which substantial brand-to-brand and lot-to-lot variations, ranging from 60 to 8300 ppm, have been reported [51]. Since the dietary intake of aluminium was not considered in these oral gavage studies, toxic effects of aluminium could be overestimated. As for continuous exposure studies, taking into account the aluminium content in the basal diet, Hicks et al. [52] demonstrated that 28-day continuous dietary exposure to basic sodium aluminium phosphate or aluminium hydroxide did not affect the testicular histopathology up to 302 mg Al/kg/day in Sprague–Dawley rats. In the 26-week feeding study of basic sodium aluminium phosphate in beagle dogs, germinal epithelial cell degeneration and atrophy in the seminiferous tubules were observed at 75 mg Al/kg bw/day [21], but such effects on male reproductive organs were not detected up to 88 mg Al/kg/day in the similar subchronic dietary study for acidic sodium aluminium phosphate in beagle dogs [53]. These dietary studies used water-insoluble or sparingly soluble forms of aluminium [8,51]. Since it is widely assumed that insoluble aluminium compounds are less bioavailable than soluble compounds, such as AS, aluminium chloride and aluminium lactate [8], there is a possibility that the male reproductive toxicity of aluminium was underestimated in these previous dietary exposure studies. The present two-generation study provided useful information that the male reproductive system is not affected even after continuous exposure to a water-soluble aluminium compound, at least up to around 50 mg Al/kg bw/day.

In the present study, some developmental effects were observed. Male and female F1 pups and female F2 pups in the

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

AS (ppm)		0 (control)	120	600	3000
Males					
No. of animals		22	20	22	22
Body weight	(g)	90.8±6.9	93.4±10.5	89.7±6.1	79.4±7.5**
Brain	(g)	1.69±0.06	1.73±0.08	1.72±0.07	1.68±0.05
	(g/100 g bw)	1.88±0.13	1.87±0.19	1.92±0.09	2.14±0.17**
Thymus	(mg)	375±55	384±86	357±58	305±51**
	(mg/100 g bw)	414±56	409±64	398±59	383±36
Liver	(g)	4.33±0.43	4.40±0.60	4.22±0.45	3.49±0.53**
	(g/100 g bw)	4.77±0.30	4.71±0.33	4.70±0.27	4.37±0.30**
Kidney[a]	(g)	1.06±0.09	1.09±0.14	1.03±0.11	0.95±0.13**
	(g/100 g bw)	1.17±0.06	1.16±0.07	1.15±0.08	1.20±0.07
Spleen	(mg)	394±49	410±68	388±74	301±43**
	(mg/100 g bw)	436±63	437±40	432±73	379±37**
Testis[a]	(mg)	596±65	583±67	569±65	539±51*
	(mg/100 g bw)	657±64	626±49	635±64	682±58
Epididymis[a]	(mg)	81.8±8.6	76.8±10.9	76.5±8.4	72.0±9.9**
	(mg/100 g bw)	90.4±10.3	82.0±6.1	85.4±8.4	91.5±14.6
Females					
No. of animals		22	20	22	21
Body weight	(g)	84.3±6.3	85.9±9.2	80.5±7.0	75.8±6.4**
Brain	(g)	1.64±0.06	1.66±0.06	1.63±0.05	1.63±0.07
	(g/100 g bw)	1.96±0.12	1.95±0.18	2.04±0.17	2.16±0.14**
Thymus	(mg)	383±66	373±74	345±46	313±33**
	(mg/100 g bw)	453±63	433±64	429±57	415±41
Liver	(g)	3.83±0.47	3.92±0.48	3.61±0.35	3.24±0.34**
	(g/100 g bw)	4.53±0.30	4.57±0.31	4.48±0.30	4.27±0.25*
Kidney[a]	(g)	0.99±0.11	0.99±0.09	0.93±0.10	0.93±0.10
	(g/100 g bw)	1.17±0.08	1.15±0.07	1.15±0.09	1.23±0.09
Spleen	(mg)	337±62	356±55	341±64	292±43*
	(mg/100 g bw)	400±67	415±44	422±53	386±47
Ovary[a]	(mg)	25.3±4.8	25.3±3.8	22.5±4.6	24.7±3.2
	(mg/100 g bw)	30.1±5.1	29.7±5.0	27.9±5.0	32.5±4.2
Uterus	(mg)	70.6±16.6	74.2±32.0	59.2±11.9*	55.4±13.4**
	(mg/100 g bw)	83.8±19.2	85.5±32.4	73.3±11.9	73.3±18.0

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

3000 ppm group had a lower body weight on PND 21 while no difference was found in the birth weight. Such inhibition of the preweaning body weight gain may be simply attributable to the decreased palatability of the drinking water, which would decrease the water intake of pups themselves or might decrease the amount of maternal breast milk; however, similar developmental effects of aluminium were reported in the previous three dietary exposure studies, in which aluminium lactate was mixed in the diet and fed to mice from day 0 of gestation throughout the lactation period [28,30,32], and the lowest effect level was 500 ppm, which was estimated to be equal to 94–273 mg Al/kg bw/day based on the body weight and food consumption during the lactation period [28]. In these dietary exposure studies, food consumption was decreased, but Golub et al. [28] indicated that the mean body weight of pups on PNDs 15 and 20 in the 1000 ppm group was lower than that of the pair-fed control. In contrast, the effects on body weight of preweaning mice were not found in the other two dietary exposure studies [29,31], which were conducted using a similar study design by the same research group. Donald et al. [29] discussed differences in the constituents of the diet as a possible cause of the inconsistent results, but this speculation appears to be contradicted by a subsequent study [30]. Although it is still unclear why different results were obtained in these dietary exposure studies, these results suggest that the fall in body weight around weaning in the present study might not be explained only by decreased water intake. Aluminium ingested by pups themselves and/or taken via maternal milk may affect preweaning growth, or impairment of maternal nursing behavior or the lactation status could be considered possible factors.

In F1 and F2 weanlings, various organ weight changes were found in the 3000 ppm group. Among them, an increase in the relative brain weight is considered to be a secondary change that occurs with the fall in body weight because the absolute weight did not change. Similarly, decreased absolute weights of the kidneys, testes, epididymides, uterus, etc., without changes in the relative weight, were thought to be associated with decreased body weight. On the other hand, the effects on the liver and spleen, the absolute and relative weights of which were decreased in both generations, could not be explained only by the fall of body weight. Since similar effects were observed in the above-mentioned dietary exposure study of aluminium lactate [28], the effects on the liver and spleen weight might be a direct effect of aluminium ingested by pups themselves and/or taken via maternal milk; however, in the present study, no histopathological changes were detected in the liver and spleen. Furthermore, the changes in the liver or spleen weight were not detected in adults, except for F0 males in the 3000 ppm group; therefore, organ weight changes observed in F1 and F2 weanlings in the 3000 ppm group were not deemed to be adverse effects. Organ weight changes in the 120 and 600 ppm groups were not considered to have toxicological significance because these changes were not dose-dependent or were inconsistent across generations.

As for effects on the developmental landmarks, vaginal opening was slightly delayed in F1 females in the 3000 ppm group while no compound-related changes were found in the other developmental landmarks, including male preputial separation. Vaginal opening generally occurs around the time of first ovulation in response to an increase in serum estradiol levels as females enter puberty [54], and therefore, it is widely used as a marker of female puberty.

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

AS (ppm)		0 (control)	120	600	3000
Males					
No. of animals		21	18	22	21
Body weight	(g)	87.7 ± 5.8	89.0 ± 8.7	87.0 ± 9.6	79.2 ± 6.8**
Brain	(g)	1.66 ± 0.05	1.69 ± 0.06	1.70 ± 0.06	1.67 ± 0.06
	(g/100 g bw)	1.90 ± 0.13	1.91 ± 0.17	1.97 ± 0.16	2.13 ± 0.17**
Thymus	(mg)	382 ± 50	348 ± 49	357 ± 66	305 ± 36**
	(mg/100 g bw)	439 ± 70	392 ± 52*	411 ± 57	386 ± 40**
Liver	(g)	3.93 ± 0.37	4.04 ± 0.64	3.91 ± 0.39	3.45 ± 0.41**
	(g/100 g bw)	4.49 ± 0.34	4.52 ± 0.44	4.50 ± 0.24	4.36 ± 0.23
Kidney[a]	(g)	1.02 ± 0.09	1.01 ± 0.13	0.99 ± 0.13	0.94 ± 0.10
	(g/100 g bw)	1.16 ± 0.08	1.14 ± 0.06	1.14 ± 0.07	1.19 ± 0.06
Spleen	(mg)	368 ± 54	381 ± 62	361 ± 49	296 ± 48**
	(mg/100 g bw)	421 ± 64	427 ± 50	416 ± 48	372 ± 42**
Testis[a]	(mg)	559 ± 67	549 ± 98	543 ± 77	534 ± 54
	(mg/100 g bw)	637 ± 60	615 ± 81	624 ± 47	680 ± 92
Epididymis[a]	(mg)	75.3 ± 6.9	78.3 ± 8.8	75.1 ± 10.7	70.5 ± 5.7*
	(mg/100 g bw)	86.1 ± 8.3	88.4 ± 9.0	86.5 ± 9.0	89.4 ± 8.2
Females					
No. of animals		22	18	21	21
Body weight	(g)	80.8 ± 6.0	80.0 ± 7.2	80.8 ± 9.1	73.8 ± 4.4**
Brain	(g)	1.60 ± 0.06	1.61 ± 0.05	1.64 ± 0.05*	1.61 ± 0.04
	(g/100 g bw)	1.99 ± 0.14	2.03 ± 0.16	2.05 ± 0.20	2.19 ± 0.15**
Thymus	(mg)	337 ± 45	364 ± 36	347 ± 49	312 ± 37
	(mg/100 g bw)	419 ± 61	457 ± 50	431 ± 47	424 ± 54
Liver	(g)	3.56 ± 0.35	3.61 ± 0.39	3.61 ± 0.48	3.07 ± 0.26**
	(g/100 g bw)	4.41 ± 0.21	4.51 ± 0.26	4.47 ± 0.26	4.17 ± 0.29**
Kidney[a]	(g)	0.95 ± 0.07	0.93 ± 0.10	0.92 ± 0.10	0.88 ± 0.08
	(g/100 g bw)	1.18 ± 0.08	1.16 ± 0.09	1.14 ± 0.06	1.20 ± 0.07
Spleen	(mg)	320.9 ± 46.7	331.8 ± 59.3	331.3 ± 57.1	269.9 ± 55.2**
	(mg/100 g bw)	398.4 ± 59.0	414.8 ± 64.3	409.0 ± 42.2	365.0 ± 67.4
Ovary[a]	(mg)	23.9 ± 3.7	22.8 ± 3.6	23.2 ± 3.5	20.2 ± 2.3**
	(mg/100 g bw)	29.7 ± 4.9	28.8 ± 5.6	29.0 ± 4.7	27.5 ± 3.5
Uterus	(mg)	60.5 ± 17.0	63.8 ± 18.4	65.0 ± 41.7	49.3 ± 11.6*
	(mg/100 g bw)	74.6 ± 19.2	79.3 ± 19.3	78.7 ± 40.4	67.0 ± 16.2

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

On the other hand, vaginal opening is closely related to body weight, and growth retardation can delay the day of acquisition [55]. In the present study, body weight at the time of vaginal opening was slightly heavier in F1 females of the highest dose group, compared with the control. However, other hormone-dependent events, including estrous cyclicity and AGD, were not changed in AS-treated groups. It seems unlikely that aluminium have a clear impact on the hormonal event. Further studies are required to draw a definitive conclusion on the effects of aluminium on the sexual maturation.

Aluminium has been reported to affect the developing nervous system [56,57]. In continuous exposure studies using rodents, the neurobehavioral effects were reported as follows: dietary exposure of pregnant rats to aluminium chloride or lactate at more than 96 mg Al/kg bw/day impaired the righting reflex, gasping reflex, negative geotaxis and/or locomotor coordination of preweaning pups [25,26]. Impaired performance of operant conditioning tasks, accompanied by a decrease in locomotor activity, was observed on PND 65 in the offspring of rats receiving a diet containing aluminium lactate at 400 mg Al/kg bw/day during gestation [58], suggesting that the effects continue after maturation. Unfortunately, aluminium concentration in the basal diet was not reported in these feeding studies using rats. Similar neurobehavioral effects were found in the preweaning, juvenile and adult periods of the offspring after dietary exposure of mice to aluminium lactate throughout the gestation and lactation periods [28–32]. These mouse studies took the basal diet content of the aluminium diet into consideration, and the lowest observed effect level was 500 ppm, corresponding to 100 mg Al/kg bw/day at the beginning of pregnancy and 210 mg Al/kg/day near the end of lactation [29]. In the present study, no changes were found in the reflex ontogeny of F1 and F2 pups and in spontaneous locomotor activity tested at 4 weeks of age for F1 animals. As for the performance in a water-filled multiple *T*-maze, a transient decrease in the elapsed time and the number of errors were found in F1 females in the 600 ppm group, but this change was not considered to be treatment-related because of the lack of dose-dependency. These findings indicate that previous findings of developmental neurotoxic effects were possibly related to the toxic effects of aluminium given at higher doses than those given in this study.

In summary, AS administered via drinking water at 120, 600 or 3000 ppm resulted in decreased water consumption. This change was associated with decreased food consumption in the 600 and 3000 ppm groups and decreased body weight in the 3000 ppm group. In the 3000 ppm group, male and female pups had a lower body weight on PND 21. At this dose, vaginal opening was slightly delayed. No definitive effects were found in the other reproductive/developmental parameters, including developmental neurobehavioral toxicity. Although there is a possibility that observed developmental effects are results of decreased water consumption, more definitive conclusions could not be reached since paired-comparison data are not available to assess the effects of decreased water intake in the absence of AS exposure. Based on these considerations, a conservative evaluation of the data led to the conclusion that the no observed adverse effect level of AS in this two-generation study is 600 ppm (41.0 mg/kg bw/day) for parental systemic toxicity and reproductive/developmental toxicity. The total ingested dose of aluminium from food and drinking

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water combined in this 600 ppm group was calculated to be 8.06 mg Al/kg bw/day.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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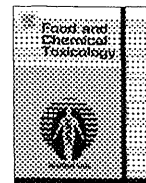

Evaluation of the reproductive and developmental toxicity of aluminium ammonium sulfate in a two-generation study in rats

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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 $\geqslant$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

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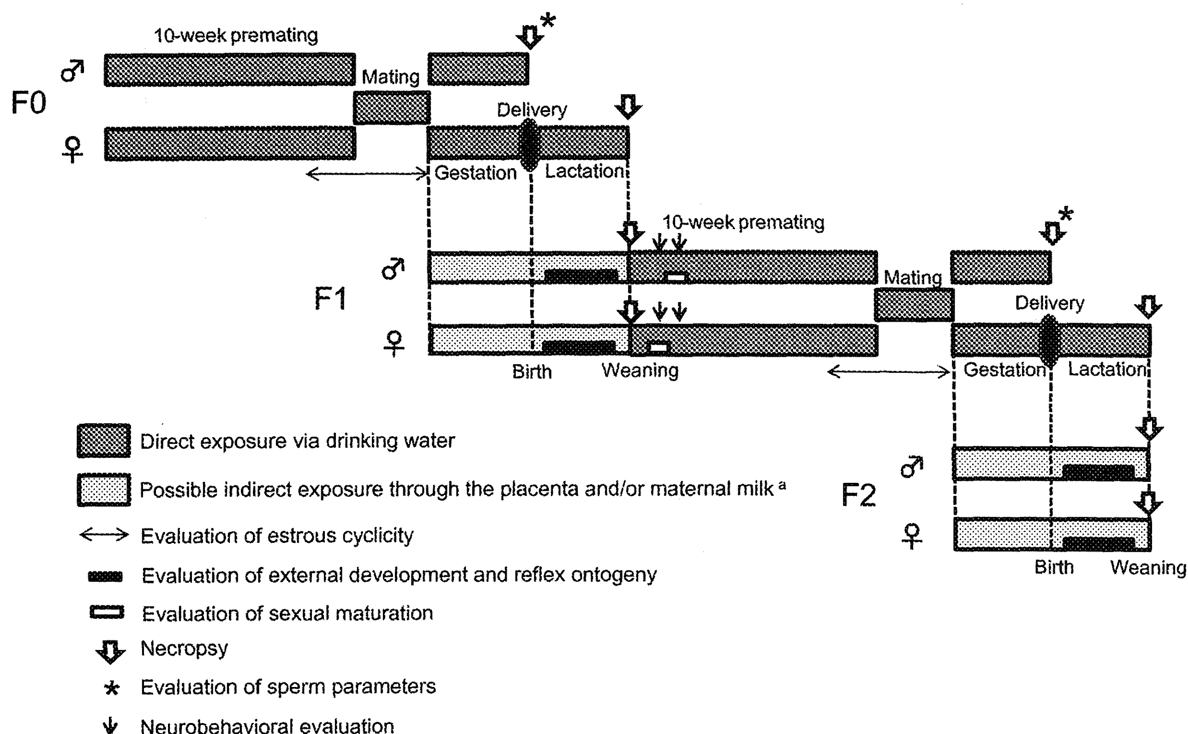

Fig. 1. Presentation of study design. [a]Pups could be also exposed to AAS directly via drinking water at the end of weaning period.

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

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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