Table 1 Immunohistochemically positive cells and apoptotic bodies counted in AA-exposed offspring

	AA in the drinking water (ppm)				AA-injection
	0	25	50	100	50 mg/kg/day, i.p.
Reelin (/mm^2)					
Males	22.0 ± 1.1[a] (3)	35.3 ± 12.3 (4)	44.9 ± 7.3 (8)**	37.8 ± 6.3 (5)*	37.9 ± 9.5 (4)
Females	24.4 ± 8.7 (7)	31.1 ± 6.8 (6)	37.2 ± 13.6 (6)	42.6 ± 8.9 (3)*	38.4 ± 9.0 (5)*
Males + Females	23.7 ± 8.9 (10)	32.8 ± 9.0 (10)*	41.6 ± 10.7 (14)**	39.6 ± 7.2 (8)**	38.2 ± 8.7 (9)**
GAD67 (/mm^2)					
Males	17.4 ± 8.9 (3)	33.1 ± 2.8 (4)	38.8 ± 8.6 (8)**	41.6 ± 6.7 (5)**	36.2 ± 7.4 (4)**
Females	28.8 ± 4.1 (7)†	31.3 ± 9.1 (6)	34.4 ± 7.0 (6)	30.9 ± 10.7 (3)	33.8 ± 9.9 (5)
Males + Females	25.4 ± 7.7 (10)	32.0 ± 7.0 (10)	36.9 ± 8.0 (14)**	37.6 ± 9.4 (8)**	34.9 ± 8.5 (9)*
Calb-D-28 K (/mm)					
Males	7.96 ± 1.30 (3)	12.47 ± 1.74 (4)*	9.87 ± 3.85 (8)	11.72 ± 4.27 (5)	9.28 ± 1.50 (4)
Females	8.08 ± 2.61 (7)	7.62 ± 3.58 (6)†	8.05 ± 2.71 (6)	11.10 ± 2.92 (3)	10.84 ± 3.64 (5)
Males + Females	8.04 ± 2.22 (10)	9.56 ± 3.80 (10)	9.09 ± 3.42 (14)	11.49 ± 3.60 (8)*	10.14 ± 2.85 (9)
Apoptotic body (/mm)					
Males	1.35 ± 0.94 (3)	0.56 ± 0.14 (4)	0.46 ± 0.35 (8)	0.44 ± 0.28 (5)	0.70 ± 0.63 (4)
Females	1.22 ± 1.02 (7)	3.52 ± 7.38 (6)	0.72 ± 0.34 (6)	0.21 ± 0.19 (3)*	0.19 ± 0.17 (5)*
Males + Females	1.25 ± 0.94 (10)	2.34 ± 5.71 (10)	0.57 ± 0.36 (14)	0.36 ± 0.26 (8)*	0.42 ± 0.49 (9)*
PCNA (/mm)					
Males	5.46 ± 1.34 (3)	2.54 ± 0.97 (4)*	3.18 ± 1.40 (8)*	2.88 ± 1.35 (5)*	2.86 ± 1.37 (4)
Females	3.07 ± 1.65 (7)	3.56 ± 1.16 (6)	3.26 ± 0.66 (6)	2.17 ± 1.16 (3)	2.25 ± 0.63 (5)
Males + Females	3.79 ± 1.88 (10)	3.16 ± 1.16 (10)	3.21 ± 1.11 (14)	2.62 ± 1.25 (8)	2.52 ± 1.00 (9)

AA, acrylamide; GAD67, glutamic acid decarboxylase 67; Calb-D-28 K, Calbindin-D-28 K; i.p., intraperitoneal injection; PCNA, proliferating cell nuclear antigen

* $P < 0.05$, ** $P < 0.01$ versus 0 ppm group

† $P < 0.05$ versus males of the same group

[a] Mean ± SD

() No. of animals examined

was a trend toward decrease in females and combined values at 100 ppm. Similarly, intraperitoneal AA exposure also resulted in appreciable but non-significant decreases in the number of PCNA-positive cells, particularly in males. There were no statistically significant differences between males and females in any group.

Discussion

In our previous study, we found no apparent AA-induced neurotoxicity in the axon terminals of rat pups following exposure of the mother to toxic levels through her drinking water (Takahashi et al. 2009). In the present study, we used identical animals and detected a clear increase in the number of Reelin-positive cells in the dentate hilus of offspring from 25 ppm, comparable to pups treated with intraperitoneal AA. A concomitant increase in GAD67-positive cells as well as an increasing tendency toward expression of Calb-D-28 K after AA exposure through both maternal and intraperitoneal transfer suggests an increase in the number of Reelin-producing GABAergic interneurons (Saegusa et al. 2010). In addition, we found a sex difference in the number of GAD67-positive cells in untreated controls. Although a number of studies exist demonstrating sex differences in GAD67-expression in mammals (Searles et al. 2000; Hays et al. 2002; Catalano et al. 2010), these reports all document differences in the GABAergic neurons of the hypothalamic sexually dimorphic nucleus. There have been no previous studies reporting differences in hilar GABAergic interneurons. We did not see any sex difference in Reelin- or Calb-D-28 K-positive cells in the hilar area of untreated controls. While further studies may be necessary regarding sexual dimorphism in the hilar GABAergic interneurons, we are confident that sexual dimorphism does not exist in the numbers of Reelin-, Calb-D-28 K- or PCNA-positive cells, nor apoptotic bodies.

We have recently shown aberrant increases of Reelin-expressing interneurons in the dentate hilus following developmental exposure to anti-thyroid agents during the gestation and lactation period (Saegusa et al. 2010). Considering the role of Reelin in neuronal development,

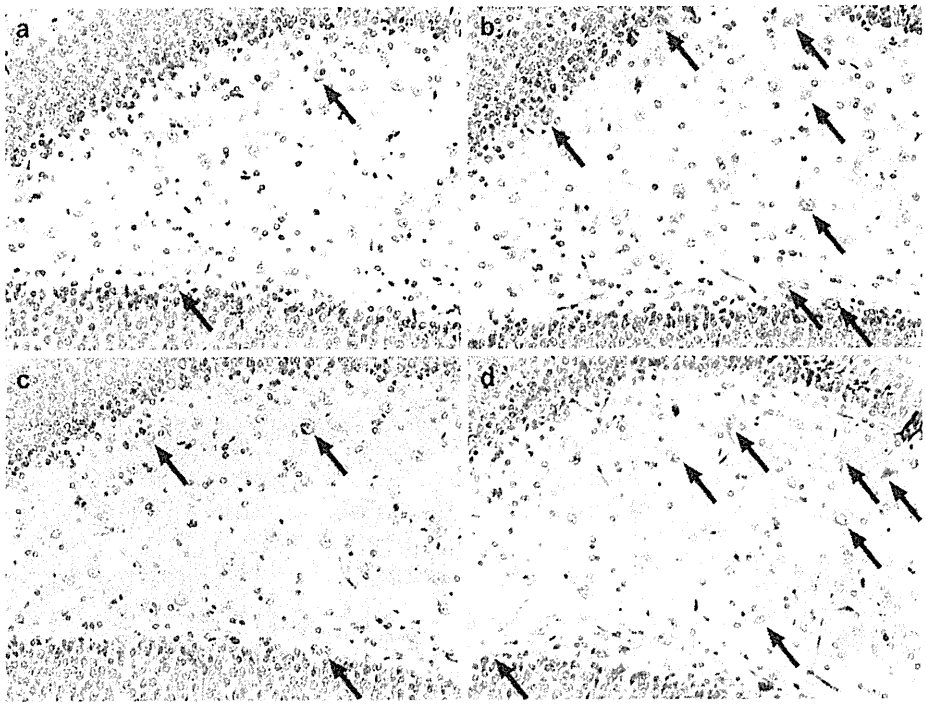

Fig. 2 Distribution of immunoreactive cells for Reelin and GAD67 in the hippocampal formation in rats at PND 21 after maternal exposure to AA. **a**, **b** Reelin-immunoreactive cells in the hilus of the dentate gyrus of offspring at 0 ppm (**a**) and 100 ppm (**b**). Reelin-positive cells with abundant cytoplasm (*indicated with arrows*) show scattered distribution within the hilar region. Note the higher number of Reelin-positive cells in (**b**) when compared with (**a**). Reelin immunohistochemistry, avidin–biotin complex method counterstained with hematoxylin. Magnification, × 200. **c**, **d** GAD67-immunoreactive cells in the hilus of the dentate gyrus of offspring at 0 ppm (**c**) and 100 ppm (**d**). Note the higher number of GAD67-positive cells indicated with arrows in (**d**) when compared with (**c**). GAD67-immunohistochemistry, avidin–biotin complex method counterstained with hematoxylin. Magnification, × 200

AA-driven increases in Reelin-expressing interneuron numbers may suggest abnormalities in neuronal migration and positioning in the dentate granular cell layer. Importantly, this aberrant increase continues until adult stages in cases of developmental hypothyroidism (Saegusa et al. 2010). Therefore, it is absolutely necessary to examine the reversibility of the interneuron changes induced by developmental AA exposure.

In contrast to the increase in apoptosis in the subgranular zone induced by developmental exposure to anti-thyroid agents (Saegusa et al. 2010), maternal transfer of AA from drinking water to offspring causes decreased apoptosis in this zone at 100 ppm, as does intraperitoneal injection of AA. Although the reason for the decrease in apoptosis is unclear, the anti-apoptotic cellular signaling machinery may be activated as a neuroprotective mechanism to compensate for an impairment of neurogenesis caused by AA. Further studies may be necessary to delineate the exact mechanisms underlying this regulation of apoptosis. Recently, impairment of adult neurogenesis, as revealed by a decrease in the number of proliferating cells in the subgranular zone, has been reported in mice treated with intraperitoneal injections of AA at 50 mg/kg with a total of 12 doses in 2 weeks (Park et al. 2010). Although the dose-relationship was not clear, we also detected a decrease in PCNA-positive (i.e. proliferating) cells in the subgranular zone in all groups of maternally exposed male offspring, as well as a similar but non-significant decrease in male pups receiving intraperitoneal injections with AA. In contrast, we did not see any apparent decrease in PCNA-positive cells in females following either maternal exposure or intraperitoneal injections. The use of a bromodeoxyuridine-based consecutive in vivo labeling method for S-phase cells such as that used by Park et al. (2010) may detect more subtle changes in proliferation activity than the method of detecting proliferating cells at the time of killing as used in the present study.

Although neurogenesis in the dentate gyrus can be affected by AA exposure during the postnatal period (Park et al. 2010), high doses of AA administered by intraperitoneal injections are necessary to induce increases in Reelin-producing interneurons during the lactation period in the present study. Importantly, we here found that much lower doses are able to stimulate changes in Reelin expression if administered maternally from the gestation period onwards. In humans, the internal level of AA in

neonates by transplacental exposure was estimated to be at least equal to that of the mother (Schettgen et al. 2004). Considering that only about a tenth of maternal AA is transferred to offspring by lactation (Takahashi et al. 2009), transplacental rather than lactational transfer of AA may be responsible for disruption of neuronal migration and neurogenesis.

Previous study has shown that short-term oral treatment with AA in rats resulted in moderate dose-dependent effects on plasma thyroxine (increased) and thyroid stimulating hormone (decreased), as well as some follicular cell hypertrophy, suggesting an acute effect of AA exposure on the thyroid (Khan et al. 1999). Conversely, an extensive recent study has shown no effect of sub-chronic AA exposure on gene expression, neurochemistry, hormone levels, or histopathology in the hypothalamus–pituitary–thyroid axis of rats (Bowyer et al. 2008). These results may suggest that AA at least does not cause developmental hypothyroidism that can trigger impaired brain development in rats.

Systemic growth retardation in offspring caused by maternal and/or offspring toxicity may cause a delay in brain development. In previous studies of developmental hypothyroidism (Shibutani et al. 2009; Saegusa et al. 2010), we observed a suppression of systemic growth in the offspring after exposure to anti-thyroid agents that caused apparent hypothyroidism in both dams and offspring. Thus, there is a possibility that the increase in the number of Reelin-expressing interneurons occurs in concert with the delayed brain growth accompanying systemic growth retardation rather than the hypothyroidism-related impairment of thyroid hormone signaling in the brain. With regard to the effects of developmental exposure to AA on offspring growth, maternal administration of AA at dose levels inducing maternal neurotoxicity has been shown to lead to reductions in offspring body size (Friedman et al. 1999; Takahashi et al. 2009). However, we have recently revealed that the systemic growth retardation in offspring resulting from maternal protein restriction affects brain growth but does not change the cellular distribution of immunoreactivity for Reelin, Calb-D-28 K, or GAD67 or of NeuN-positive post-mitotic neurons in the dentate hilus, either at weaning or in adulthood (Ohishi et al. in press). This result may suggest that the growth retardation arising from AA toxicity in offspring does not affect the population of GABAergic interneurons involved in Reelin synthesis.

In summary, using study animals known to suffer no apparent axon terminal neurotoxicity following exposure to maternally toxic levels of AA through drinking water, we here found a clear increase in the number of Reelin-producing interneurons in the dentate hilus of offspring following maternal exposure to AA at and above 25 ppm. We also observed decreased apoptosis in the neuroblast-producing subgranular zone at 100 ppm, comparable to pups given high doses of AA directly via intraperitoneal injections during the lactation period. These results suggest a compensatory mechanism that regulates and protects against the impaired neurogenesis and mismigration arising following maternal exposure to AA from low doses during neuronal development. The lowest-observed-adverse-effect level of AA was determined to be 25 ppm (3.7 mg/kg body weight/day).

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ORGAN TOXICITY AND MECHANISMS

Life stage-related differences in susceptibility to acrylamide-induced neural and testicular toxicity

Miwa Takahashi · Kaoru Inoue · Naoki Koyama · Midori Yoshida · Kaoru Irie · Tomomi Morikawa · Makoto Shibutani · Masamitsu Honma · Akiyoshi Nishikawa

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Abstract In order to assess age-dependence of susceptibility to acrylamide (ACR)-induced neural and testicular toxicity, 3- and 7-week-old male SD rats were given ACR at 0, 50, 100, or 200 ppm in the drinking water for 4 weeks, and the nervous and male reproductive systems were examined histopathologically. Testicular genotoxicity was evaluated with the comet assay and the micronucleus (MN) test. Glutathione *S*-transferase (GST) activity and glutathione (GSH) content in the liver and testis were also measured. In both young and adult animals, neurotoxicity was evident from 100 ppm and increased in proportion to ACR intake per body weight. In the testis, marked degeneration and exfoliation, mainly of spermatids, were observed from 100 ppm limited to young animals. The comet assay revealed ACR to significantly induce DNA damage from 100 ppm in both life stages, while MNs were found only in young rats from 100 ppm. The level of GST activity in the testis of young rats at the end of experiment was significantly lower than that of adult animals, regardless of the ACR treatment. There were no life stage-related differences in GSH contents in the liver and testis. These results suggest that susceptibility to neurotoxicity might not differ between young and adult rats when exposure levels are adjusted for body weight. Regarding testicular toxicity, young animals around puberty proved more susceptible than adult animals, possibly due to their lower level of testicular GST activity than that in adult animals.

Keywords Acrylamide · Age · Susceptibility · Neurotoxicity · Testicular toxicity · Rat

M. Takahashi · K. Inoue · M. Yoshida · K. Irie · T. Morikawa · M. Shibutani · A. Nishikawa
Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

N. Koyama · M. Honma
Division of Genetics and Mutagenesis, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

M. Shibutani (✉)
Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan
e-mail: mshibuta@cc.tuat.ac.jp

Introduction

Acrylamide (ACR), a proven carcinogen in animals known to be a neuro- and reproductive toxicant, has found many industrial and laboratory uses and therefore is a potential hazard to man. Recently, it was found to be formed on heating from carbohydrate and amino acid components of food, so that public exposure has become a worldwide concern (Exon 2006; Parzefall 2008). Mean daily intake of ACR for adults is estimated as 1 μg/kg body weight/day, but values for infants and children are estimated to be two- to threefold higher when expressed on a body weight basis (WHO/IPCS 2006). So far, since toxicity studies of ACR have mainly been performed using adult animals, to evaluate toxicity targeting children is important for risk assessment of ACR exposure in man.

Even the limited studies on susceptibility to neurotoxicity of ACR in relation with the life stage have not generated consistent results controversial. For example, whereas a rotarod test revealed slightly higher sensitivity with adult when compared to immature rats regarding disruption of locomotor activity (Kaplan and Murphy 1972), earlier onset of neurological symptoms as well as faster induction of severe myelin disruption was found with

suckling rats given ACR at 50 mg/kg by intraperitoneal injection 3 times weekly when compared with adult rats (Suzuki and Pfaff 1973). Similarly, mice given ACR at 400 ppm in the drinking water revealed earlier onset of neurological symptoms and terminal nerve swelling by administration starting from 3 weeks of age than from 8-weeks of age (Ko et al. 1999).

As for testicular toxicity, no data on the susceptibility to ACR in relation with testicular development have been reported. In our previous study, although retardation of spermatogenesis was observed in preweaning rats exposed to ACR via maternal drinking water or direct intraperitoneal injection, no histopathological changes suggestive of testicular toxicity were noted (Takahashi et al. 2009). Therefore, testes prior to spermatogenesis could be less sensitive to ACR.

In the present study, to elucidate factors influencing susceptibility to ACR-induced neuro- or testicular toxicity, we compared histopathological changes of the nervous and male reproductive systems between rats exposed to ACR during the puberty growth period and the young adult stage. The comet assay and micronucleus (MN) test were also conducted to evaluate testicular genotoxicity induced by ACR. Additionally, to compare the capacity for ACR detoxification between the young and adult animals, glutathione *S*-transferase (GST) activity and glutathione (GSH) content were measured in the liver and testis.

Materials and methods

Experimental design

Thirteen pregnant Crj:CD (SD) rats were obtained from Charles River Japan Inc. (Yokohama, Japan) at gestational day 10. They were housed individually in polycarbonate cages with wood chip bedding and maintained in an air-conditioned animal room (temperature: 24 ± 1°C, relative humidity: 55 ± 5%, 12-h light/dark cycle) with basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) and tap water available ad libitum. After delivery, 40 male pups on weaning at 21 days of age were allocated to 4 groups, each consisting 10 animals from different dams, given ACR (Sigma, St. Louis, MO, USA; CAS #79-06-1) at 0, 50, 100, and 200 ppm in the drinking water for 4 weeks (**young group**). Similarly, forty male SD rats at 6 weeks of age were obtained from Charles River Japan Inc., and acclimatized with basal diet (CRF-1) and tap water ad libitum for 1 week. Then, they were randomly divided into 4 groups and given ACR at 0, 50, 100, and 200 ppm in the drinking water for 4 weeks (**adult group**). The highest dose was set as the dose that induces neurotoxicity and testicular toxicity within 4 weeks in adult male rats (Lee et al. 2005). Observations for clinical signs and mortality were conducted daily. Body weights and food consumption were recorded every week. In addition, animals were scored with respect to the appearance of gait abnormalities, as previously reported (Moser 1991; Shell et al. 1992; Lee et al. 2005), as follows: grade 1, normal gait; grade 2, slightly abnormal gait with slight degrees of ataxia, hopping gait, and foot splay; grade 3, moderately abnormal gait with moderate degrees of ataxia, foot splay, and limb abduction; grade 4, severely affected gait, including inability to support the body weight as well as foot splay.

At necropsy, all animals were killed under deep anesthesia by exsanguination, and the brain, testes, and epididymides were removed and weighed. The trigeminal nerve was also removed. The brains from 5 animals per each group were fixed in methacarn solution at 4°C overnight. The brains from another 5 rats per each group, along with the trigeminal nerves and epididymides were fixed in neutrally buffered 10% formalin. One testis was used for comet assays, and the other was fixed in Bouin's solution at room temperature overnight. All fixed tissues were routinely processed for paraffin embedding, sectioned at 3 μm, and stained with hematoxylin and eosin (HE). The sciatic nerves were exposed at autopsy and subjected to in situ fixation by immersion in ice-cold 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 min (Takahashi et al. 2009). The portion located at the ankle position was carefully dissected and further fixed with fresh fixative overnight, postfixed in 1% osmium tetroxide (TAAB Laboratories Equipment Ltd., Berkshire, UK) in the same buffer for 2 h at 4°C, and embedded in epoxy resin (TAAB Laboratories Equipment Ltd.). Semithin sections, 1 μm in thickness, were stained with toluidine blue for light microscopic assessment.

As a satellite study, 3- or 7-week-old male SD rats were similarly given ACR at 0 or 200 ppm in the drinking water for 4 weeks. The livers and unilateral testes were collected for measurement of GST activity and GSH content. Small portions of each testis were fixed in Bouin's solution to confirm ACR-induced lesions microscopically.

The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Morphometric assessment

To evaluate aberrant dot-like structures immunoreactive with synaptophysin (SYP) in the cerebellar molecular layer, methacarn-fixed cerebellum sections were subjected to immunohistochemistry for SYP, as described previously (Takahashi et al. 2008), with rabbit polyclonal antibody Ab-4 (1: 200, Lab Vision Corp., Fremont, CA, USA) as the primary antibody.

For morphometry of axonal degeneration in the sciatic nerves and SYP-immunoreactive aberrant dot-like structures in the cerebellar molecular layer, photomicrographs were taken with a digital camera attached to a microscope (DP71, Olympus Corp., Tokyo, Japan). Measurement was then performed using image analysis software (WinROOF, Version 5.7.1, Mitani Corp. Tokyo, Japan). The total number of axons/unit area and the numbers of degenerated axons and the diameters of axons were assessed in one cross-sectional area at 400× magnification of toluidine blue-stained specimens from each animal, and the density, percentage of degenerated axons, and percentage of myelinated axons less than 3 μm in diameter were calculated. For evaluation of SYP-immunoreactive aberrant dot-like structures, numbers of dots in the left cerebellar hemisphere were counted following measurement of the length of the cortex in one cross-sectional area at 12.5× magnification and the number of SYP-immunoreactive dots/unit length of the cortex was calculated.

For testicular toxicity, approximately 400–650 circularly sectioned seminiferous tubules for each rat were assessed microscopically, and then the percentages of tubules with histopathological changes were calculated.

Comet assay

The procedures for preparing and processing comet assays were performed according to the recommendation by an expert working group on the comet assay in the International Workshop on Genotoxicity Testing (IWGT) (Tice et al. 2000; Burlinson et al. 2007) and slightly modified for testes. Briefly, each testis was washed with cold mincing/homogenizing buffer containing Hanks' Balanced Salt (HBSS) Solutions (Invitrogen Corporation, Carlsbad, CA, USA), 20 mM EDTA·2Na, and 10% DMSO, minced with scissors, and placed on ice for 15–30 s to precipitate clumps of cells. The supernatant was suspended in 0.5% Nusieve GTG agarose (Lonza, Basel, Switzerland), quickly layered on a MAS-coated slide (Matsunami Glass Ind. Ltd., Osaka, Japan), immersed in lysing solution (pH10, 100 mM EDTA·2Na, 2.5 M NaCl, 10 mM Tris(hydroxymethyl)aminomethane containing 1% Triton-X and 10% DMSO) at 4°C overnight, and electrophoresed for 15 min in alkaline buffer after the unwinding treatment. Then, the cells were fixed with ethanol and stained with SYBER green (Molecular Probes, Eugene, OR, USA) according to the manufacturer's recommendation. We observed the cells under a fluorescence microscope (BX50, Olympus Co.). Round-shaped cells, considered as spermatocytes and early spermatids, were captured with a CCD camera. At least 100 cells were observed and the tail intensity of each comet image was measured using an image analysis software (Comet assay IV, Perceptive Instruments Ltd., Suffolk, UK).

Micronucleus (MN) test

The MN test for the testis was conducted according to the method of Tates et al. (1983) with a slight modification. Briefly, the testes excised from each animal were minced in HBSS Solution. The cell suspensions were incubated in 2 mg/ml collagenase solution (Wako Pure Chemicals Ind., Osaka, Japan) for 30 min at 37°C in a shaking water bath, filtered through a cell strainer, washed, and fixed in methanol. The cells were stained with DAPI (4′,6-diamidino-2-phenylindole dihydrochloride), and the slide specimens were prepared with acridine orange coating (TOYOBO Co., Ltd., Tokyo, Japan). We observed 1,000 early spermatids per animal under a fluorescence microscope (BX50, Olympus Co.).

Measurement of GST activity and GSH content

The livers and testes obtained from the satellite groups were rinsed with PBS solution to remove any red blood cells. Total GSH concentrations were determined with the Glutathione Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), and GST activity was measured using a Glutathione *S*-Transferase Assay Kit (Cayman Chemical). Sample preparation and measurement were conducted according to the instructions of the manufacturer.

Statistical analysis

Variance in data for body weights, food consumption, water consumption, values from morphometric assessment in the sciatic nerves, cerebellar molecular layer and testis, and data for the comet assay were checked for homogeneity by Bartlett's procedure. If the variance was homogeneous, the data were assessed by one-way analysis of variance. If not, the Kruskal–Wallis test was applied. When statistically significant differences were indicated, the Dunnett's multiple test was employed for comparisons between the 0 ppm and ACR-treated groups. Data for the MN tests, GST activity, and GSH content were analyzed by Student's or Welch's t test following a test for equal variance.

Results

In-life parameters and intake of ACR

Suppression of body weight gain was observed in the young groups at 100 and 200 ppm from week 2 (Fig. 1a).

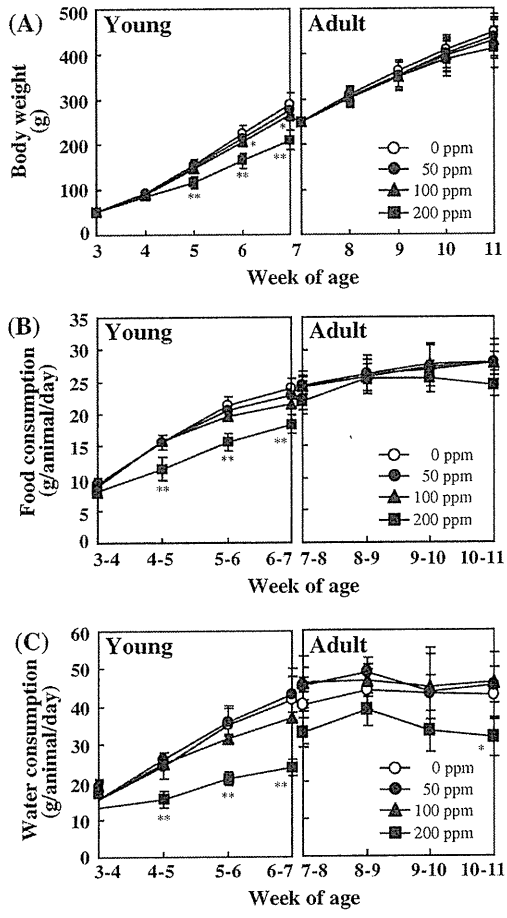

Fig. 1 Time course of change in body weights (**a**), food consumption (**b**), and water consumption (**c**) in young and adult rats given ACR in the drinking water for 4 weeks. Data are mean ± SD. *, **$P < 0.05$ and $P < 0.01$ vs. 0 ppm

In the adult groups, there were no intergroup differences in the body weight curves. Also, food consumption was suppressed only in the young group at 200 ppm (Fig. 1b). Water consumption was lowered at 200 ppm both in young and adult groups (Fig. 1c). Mean daily intakes of ACR are summarized in Table 1. Compared to adult groups, mean daily intake of ACR per kg body weight was higher in young groups at each dose.

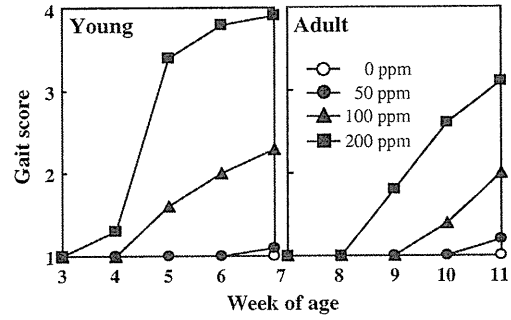

Fig. 2 Scores for gait abnormalities of young and adult rats given ACR in the drinking water for 4 weeks

Both in young and adult groups, apparent gait abnormalities were found at 100 and 200 ppm, and their severity advanced during the exposure in a dose-dependent manner (Fig. 2). Young groups showed earlier occurrence of gait abnormalities and faster progression of the symptoms than adult groups. At 200 ppm, slightly abnormal gait appeared in the young animals from week 1, and symptoms rapidly progressed so that the gait score reached 3.4 at week 2. Adult animals at 200 ppm exhibited mild gait abnormality from week 2, which progressed to score 3.1 at week 4.

Final body and organ weights are summarized in Table 2. In young groups, body weights were significantly depressed at 100 and 200 ppm. Alteration of the brain weight in young rats appeared to reflect body weight decrease. Decreases in absolute weights of the testis and epididymides observed in young and adult rats could have been linked with the histopathological changes described below.

Morphometric analysis

Data for histopathology and morphometry of lesions developing in the nervous system are shown in Table 3. Representative histopathological illustrations of the nervous systems of young and adult groups are summarized in Fig. 3 and Fig. 4, respectively. In both young and adult groups, central chromatolysis of ganglion cells in the trigeminal nerves was apparent from 100 ppm. The density of

Table 1 Mean daily intake of ACR in young and adult rats

Group	Acrylamide in the drinking water (ppm)				
		0	50	100	200
	No. of animals examined	10	10	10	10
Young	(mg/kg/day)	0 ± 0[a]	8.27 ± 0.32	15.73 ± 1.51	26.37 ± 3.51
Adult	(mg/kg/day)	0 ± 0	6.26 ± 1.10	12.63 ± 1.97	19.07 ± 3.46

[a] Mean ± SD

Table 2 Body and organ weights of young and adult rats given ACR in the drinking water for 4 weeks

		Acrylamide in the drinking water (ppm)			
		0	50	100	200
Young					
Body weight	(g)	287.4 ± 24.6[a]	273.5 ± 15.7	263.7 ± 14.4*	210.4 ± 24.3**
Brain	(g)	1.97 ± 0.05	1.94 ± 0.09	1.83 ± 0.09*	1.66 ± 0.04**
	(g%)	0.69 ± 0.06	0.71 ± 0.05	0.69 ± 0.03	0.80 ± 0.08**
Testes	(g)	2.57 ± 0.15	2.44 ± 0.22	2.39 ± 0.19	1.87 ± 0.36**
	(g%)	0.90 ± 0.07	0.90 ± 0.09	0.91 ± 0.07	0.89 ± 0.12
Epididymides	(g)	0.40 ± 0.04	0.35 ± 0.02**	0.37 ± 0.04	0.30 ± 0.02**
	(g%)	0.14 ± 0.02	0.13 ± 0.01	0.14 ± 0.02	0.15 ± 0.02
Adult					
Body weight	(g)	444.3 ± 38.0	433.0 ± 42.0	426.7 ± 42.1	409.2 ± 45.5
Brain	(g)	2.07 ± 0.06	2.08 ± 0.11	2.02 ± 0.09	1.99 ± 0.07
	(g%)	0.47 ± 0.03	0.48 ± 0.04	0.48 ± 0.04	0.49 ± 0.06
Testes	(g)	3.30 ± 0.26	3.39 ± 0.39	3.25 ± 0.20	3.19 ± 0.24
	(g%)	0.74 ± 0.07	0.78 ± 0.08	0.77 ± 0.08	0.79 ± 0.09
Epididymides	(g)	0.97 ± 0.05	1.04 ± 0.09	0.97 ± 0.07	0.84 ± 0.06**
	(g%)	0.22 ± 0.02	0.24 ± 0.02	0.23 ± 0.03	0.21 ± 0.02

10 animals per each group were examined

[a] Mean ± SD

*, ** $P < 0.05$, $P < 0.01$ vs. 0 ppm group

Table 3 Histopathology and morphometry of lesions developing in the nervous system

		Acrylamide in the drinking water (ppm)			
		0	50	100	200
Young					
Trigeminal nerve					
No. of animals examined		10	10	10	10
Central chromatolysis (+/++/+++)[a]		0	3 (3/0/0)	10 (0/5/5)[##]	10(0/0/10)[##]
Sciatic nerve (distal portion)					
No. of animals examined		10	10	10	10
Density	(/100 μm^2)	2.56 ± 0.32[b]	2.73 ± 0.17	2.92 ± 0.25**	2.42 ± 0.25
Degenerated axons	(%)	0.28 ± 0.15	0.39 ± 0.14	0.82 ± 0.19**	7.51 ± 3.25**
Myelinated axons, <3 μm in diameter	(%)	18.01 ± 3.45	16.74 ± 2.79	18.80 ± 2.73	21.57 ± 4.07
Cerebellar cortex					
No. of animals examined		5	5	5	5
SYP-immunoreactive aberrant dots	(/mm cortex)	0.50 ± 0.20	0.41 ± 0.18	1.49 ± 0.59	6.09 ± 1.62*
Adult					
Trigeminal nerve					
No. of animals examined		10	10	10	10
Central chromatolysis (+/++/+++)[a]		0	3 (3/0/0)	10 (3/7/0)[##]	10 (0/3/7)[##]
Sciatic nerve (distal portion)					
No. of animals examined		10	10	10	10
Density	(/100 μm^2)	2.10 ± 0.23	2.03 ± 0.15	2.10 ± 0.24	2.15 ± 0.24
Degenerated axons	(%)	0.39 ± 0.16	0.65 ± 0.27	0.96 ± 0.37*	1.74 ± 0.77**
Myelinated axons, <3 μm in diameter	(%)	13.96 ± 2.75	12.30 ± 2.39	13.45 ± 2.68	14.16 ± 2.82
Cerebellar cortex					
No. of animals examined		5	5	5	5
SYP-immunoreactive aberrant dots	(/mm cortex)	0.54 ± 0.12	0.47 ± 0.09	1.71 ± 0.81	5.88 ± 2.61*

[a] *Grade of change* + mild, ++ moderate, +++ severe

[b] Mean ± SD

*, ** $P < 0.05$, $P < 0.01$ vs. 0 ppm group

[##] $P < 0.01$ vs. 0 ppm group (Fisher's exact test)

SYP synaptophysin

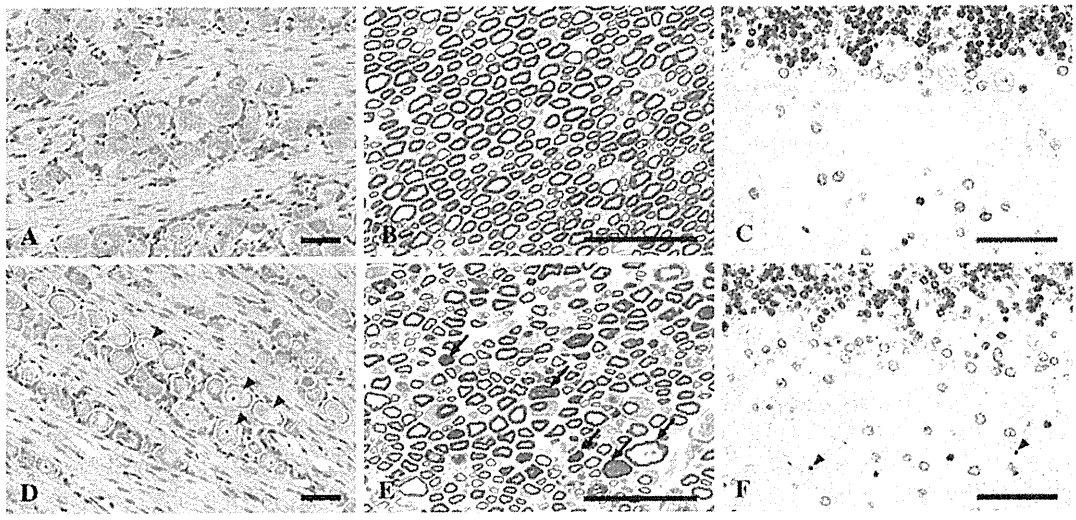

Fig. 3 Histopathology of the trigeminal nerve (**a**, **d**), sciatic nerve (**b**, **e**), and cerebellar molecular layer (**c**, **f**) in young rats given ACR at 0 or 200 ppm for 4 weeks. (**a–c**) Normal tissues of a young rat from the 0 ppm group. (**d–f**) At 200 ppm, central chromatolysis of ganglion cells (**d** *arrowheads*) in the trigeminal nerve was apparent. Increases in degenerated axons (**e** *arrows*) in the sciatic nerve and dot-like SYP-immunoreactive structures (**f** *arrowheads*) in the cerebellar molecular layer were also found. **a**, **d** hematoxylin and eosin. **b**, **e** resin-embedded semithin sections stained with toluidine blue. **c**, **f** immunohistochemical staining for SYP. *Bar* = 50 μm

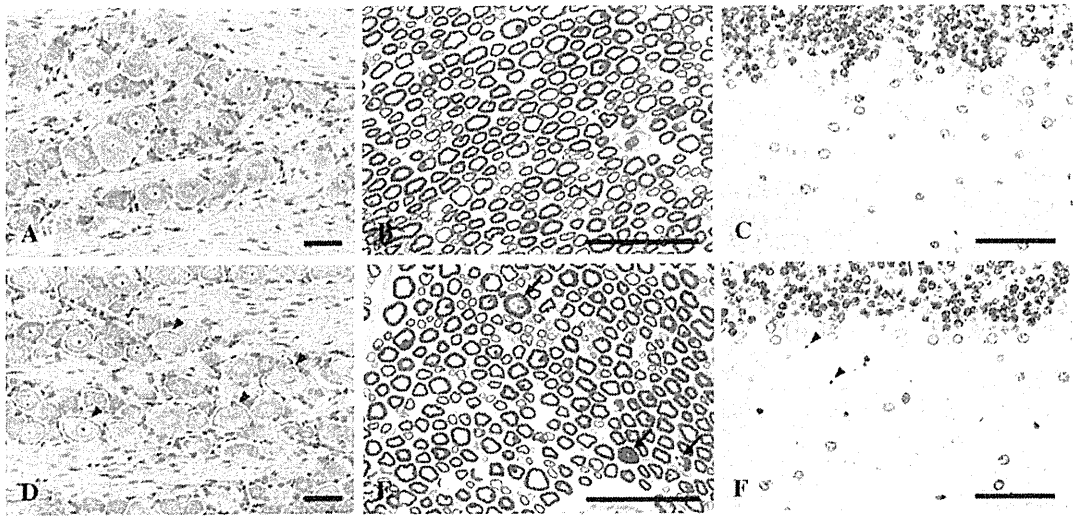

Fig. 4 Histopathology of the trigeminal nerve (**a**, **d**), sciatic nerve (**b**, **e**), and cerebellar molecular layer (**c**, **f**) of adult rats given ACR at 0 or 200 ppm for 4 weeks. (**a–c**) Normal tissues of an adult rat from 0 ppm group. (**d–f**) Similar to the young group, central chromatolysis of ganglion cells (**d** *arrowheads*) in the trigeminal nerve, increases in degenerated axons (**e** *arrows*) in the sciatic nerve and dot-like SYP-immunoreactive structures (**f** *arrowheads*) in the cerebellar molecular layer were observed at 200 ppm **a**, **d** hematoxylin and eosin. **b**, **e** resin-embedded semithin sections stained with toluidine blue. **c**, **f** immunohistochemical staining for SYP. *Bar* = 50 μm

axons in the sciatic nerve was increased only at 100 ppm in the young group, but without dose dependence. In both young and adult groups, significant increase in degenerated axons in the sciatic nerve was observed from 100 ppm, and increase in dot-like SYP-immunoreactive structures in the cerebellar molecular layer was also found at 200 ppm. Although not statistically significant, myelinated nerves <3 μm in diameter showed a tendency for increase at 200 ppm in both young and adult groups. At 200 ppm, most parameters were higher in young groups compared to adult counterparts.

In the testis, marked degeneration and loss of or decrease in spermatids was observed from 100 ppm in young animals (Fig. 5 and Table 4). Elongate spermatids

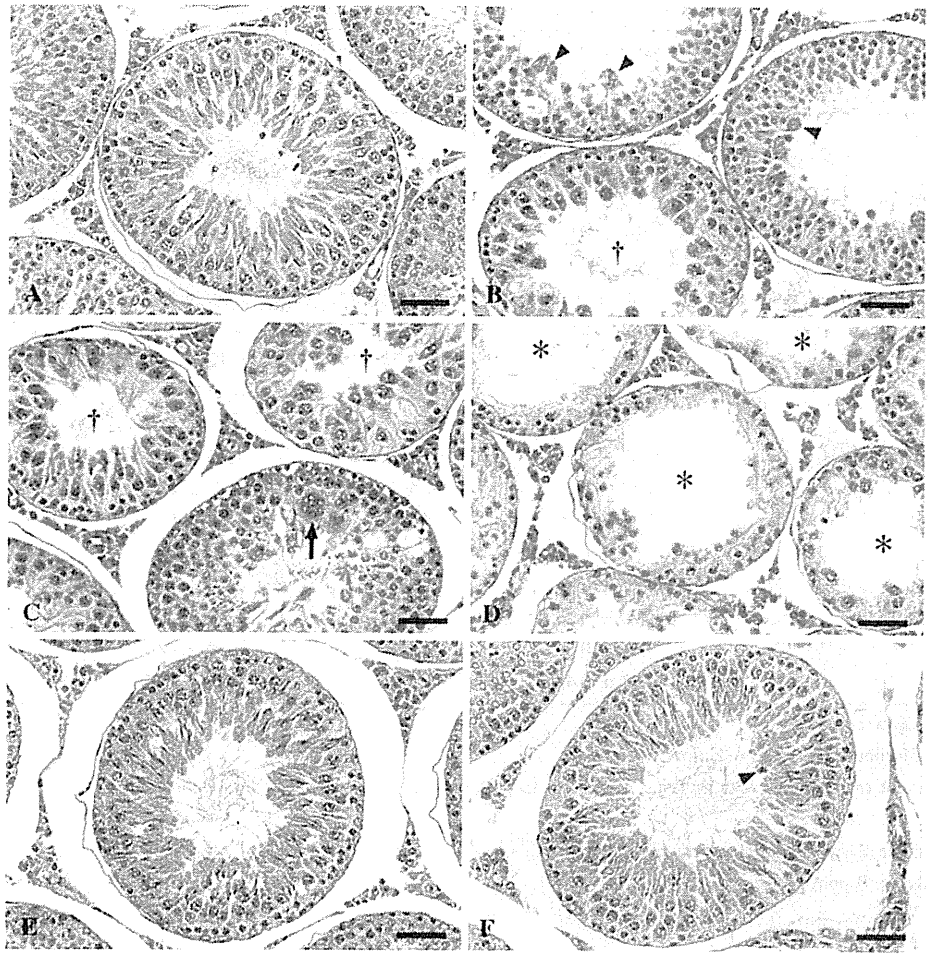

Fig. 5 Histopathology of the testis of young and adult rats given ACR at 0 or 200 ppm for 4 weeks. **a** Normal seminiferous tubules of a young rat from the 0 ppm group. (**b–d**) Degeneration of spermatids (*arrowheads*), loss of or decreased in elongated spermatids (†), and multinucleated giant cells (*arrow*) are apparent in a young rat at 200 ppm. In severely affected cases, many seminiferous tubules showed marked germ cell depletion (*). **e** Normal seminiferous tubules of an adult rat from the 0 ppm group. **f** Only a small number of exfoliated germ cells (*arrowhead*) was found in the lumina of tubules in a case of the adult group at 200 ppm. HE stain. All *bars* = 50 μm

appeared to be most vulnerable to ACR, and in severely affected cases, many seminiferous tubules showed marked germ cell depletion. In addition, exfoliation of germ cells and appearance of multinucleated giant cells were also found. Many exfoliated germ cells were observed in the epididymal duct. In the adult groups, only small numbers of exfoliated germ cells was found in lumina of seminiferous tubules. Sertoli cells were morphologically unaffected in both young and adult animals. Similar histopathological changes were also observed in the testis of each young and adult animal at 200 ppm in the satellite groups used for measuring GST activity and GSH contents.

Data for relationships between ACR intake per kg body weight and neurotoxicity parameters, including the gait score at week 4, the number of degenerated axons in the sciatic nerves, and the number of SYP-immunoreactive structures in the cerebellar molecular layer are shown in Fig. 6a–c. All these parameters increased in proportion to ACR intake. For testicular toxicity, the relationship between ACR intake per kg body weight and the percentage of affected seminiferous tubules is shown in Fig. 6d. With increase in ACR dose, affected tubules profoundly increased in the young group, while the magnitude of increase was very small in the adult group.

Comet assays and MN tests

The comet assay revealed that ACR significantly induced DNA damage in a dose-dependent manner from 100 ppm

Table 4 Histopathological data for the testes of young and adult rats given ACR in the drinking water for 4 weeks

Findings (%)[a]	Acrylamide in the drinking water (ppm)			
	0	50	100	200
Young				
Affected tubules[b]	3.51 ± 1.68[c]	9.03 ± 18.81	16.93 ± 12.23*	66.59 ± 26.96**
Exfoliation of germ cells	3.39 ± 1.61	3.93 ± 3.07	9.80 ± 6.22*	10.44 ± 9.87
Multinucleated giant cells	0.02 ± 0.06	0.07 ± 0.14	0.57 ± 0.71	1.67 ± 3.06**
Degeneration of spermatids	0.10 ± 0.18	0.84 ± 2.54	3.95 ± 6.47	20.90 ± 13.37**
Loss of or decrease in elongated spermatids	0 ± 0	4.99 ± 15.74*	5.62 ± 8.87**	20.43 ± 14.61**
Loss of or decrease in round spermatids	0.02 ± 0.06	0 ± 0	1.51 ± 3.19	12.68 ± 10.97**
Atrophic tubules[d]	0 ± 0	0.17 ± 0.54**	0.12 ± 0.38**	24.03 ± 30.83**
Sertoli cell vacuolation	0.60 ± 0.57	1.07 ± 0.55	0.99 ± 0.62	1.06 ± 0.92
Adult				
Affected tubules[b]	0.47 ± 0.30	0.58 ± 0.23	1.17 ± 0.60*	1.53 ± 0.67**
Exfoliation of germ cells	0.45 ± 0.30	0.56 ± 0.23	1.17 ± 0.60*	1.46 ± 0.71**
Multinucleated giant cells	0 ± 0	0 ± 0	0 ± 0	0.07 ± 0.17
Degeneration of spermatids	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Loss of or decrease in elongated spermatids	0.02 ± 0.06	0.02 ± 0.07	0 ± 0	0 ± 0
Loss of or decrease in round spermatids	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Atrophic tubules[d]	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Sertoli cell vacuolation	0.62 ± 0.45	0.53 ± 0.50	0.72 ± 0.43	0.81 ± 0.53

10 animals per each group were examined

[a] Approximately 400–650 tubules/rat were examined

[b] Affected tubules represent total tubules with findings, except for tubules showing only Sertoli cell vacuolation

[c] Mean ± SD

[d] Atrophic tubules are those showing marked germ cell depletion

*, ** $P < 0.05$, $P < 0.01$ vs. 0 ppm group

in young and adult groups (Fig. 7a). Although the values did not greatly differ between the groups, the values in the young group were higher than those in the adult group at 200 ppm. On the other hand, MN was clearly induced only in young group in a dose-dependent manner (Fig. 7b) with statistical significance at both 100 and 200 ppm ($P < 0.05$). ACR slightly induced MN in the adult group at 200 ppm.

GST activity and GSH contents in the liver and testis

In the liver, compared to the 0 ppm group, GST activity was significantly increased at 200 ppm in the young group (Fig. 8a). Although not statistically significant, GST activity in the adult animals at 200 ppm also showed a tendency for increase. GSH contents were unchanged in both groups. There were no differences in the level of GST activity and GSH contents in the liver between young and adult groups. In the testis, although GST activity and GSH contents in both groups were not changed by ACR treatment, the levels of GST activity in the young group were significantly lower than those in the adult group (Fig. 8b).

Discussion

In the present study, ACR dose-related suppression of body weight, and food and water consumption was observed only in young rats. When gait abnormalities progressed, animals became unable to support their body weights, and it was difficult to take food and water from containers set in the upper part of the cage. Therefore, in the housing conditions designed for adult animals, the suppressions might be due to immature body size of young animals causing difficulty in access to food and water associated with the development of neurotoxicity.

On clinical observation, although both young and adult animals exhibited similar symptoms from 100 ppm, earlier occurrence and faster progression of the symptoms were

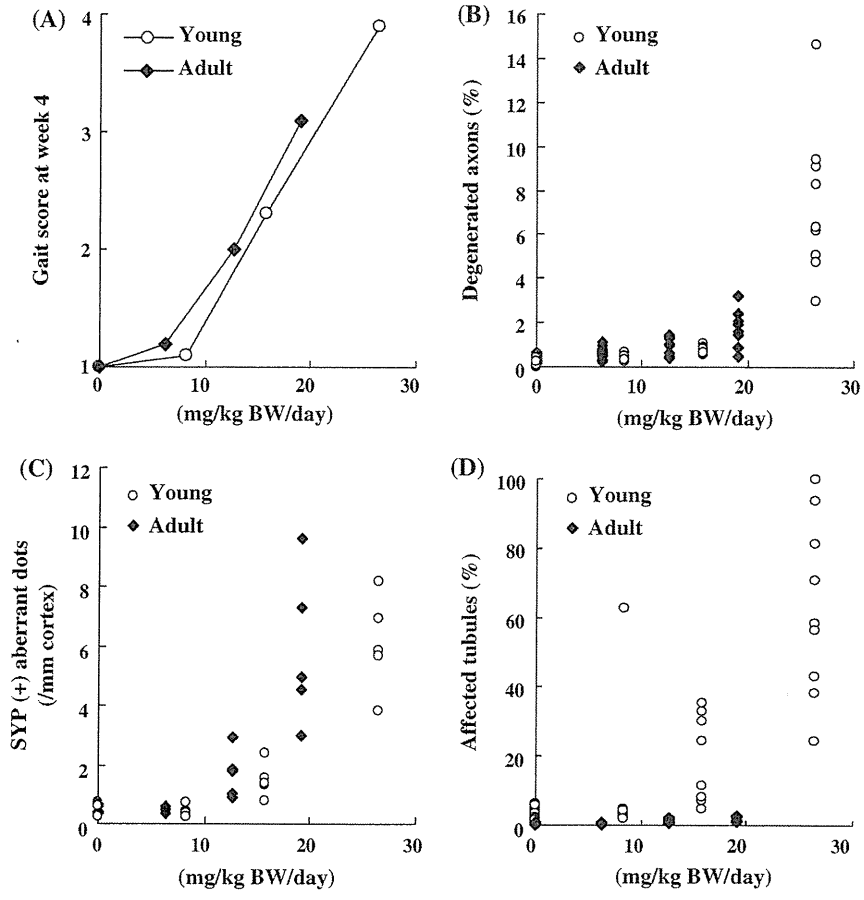

Fig. 6 Relationship between ACR intake per body weight and changes in neurotoxicity and testicular toxicity parameters in young and adult rats

here observed in the young group. Also, neurotoxic lesions such as central chromatolysis of ganglion cells in the trigeminal nerves, degenerated axons in the sciatic nerve and dot-like SYP-immunoreactive structures in the cerebellar molecular layer, were evident from 100 ppm in both young and adult groups. The magnitude of changes in these parameters was higher in the young group than in the adult group, especially at the highest dose, and neurotoxicity appeared stronger in young animals, though the types of lesions observed were similar between the young and adult groups. Compared to adult animals, intake of ACR per kg body weight was higher in young animals at each dose and the parameters indicating the neurotoxicity increased in proportion to ACR intake. Accordingly, the stronger neurotoxicity in the young animals can be considered to be a reflection of larger amount of ACR intake per body weight. These results suggest that the susceptibility to ACR-induced neurotoxicity in young and adult rats is qualitatively similar under the given experimental conditions. As mentioned in the Introduction section, a few studies have demonstrated life stage-related differences in susceptibility to ACR neurotoxicity, though the experimental conditions, such as age of animals, dosing methods, and parameters examined, were different. While Suzuki and Pfaff concluded that suckling rats were more susceptible (Suzuki and Pfaff 1973), it seems that there was not much difference in number of injections to cause apparent symptoms and myelin degeneration between suckling and adult rats. In the report by Ko et al., earlier occurrence and faster progression of neurological abnormalities in young animals were similar to those observed in our study (Ko et al. 1999). Although the authors stated that the daily intake was not significantly different between the young and adult groups, intake of ACR per body weight at the beginning of the experiment might have been higher in the young group, because younger animals usually take more water than older ones. Taken together, clear evidence of the susceptibility difference in neurotoxicity between young and adults animals is considered to be undetermined.

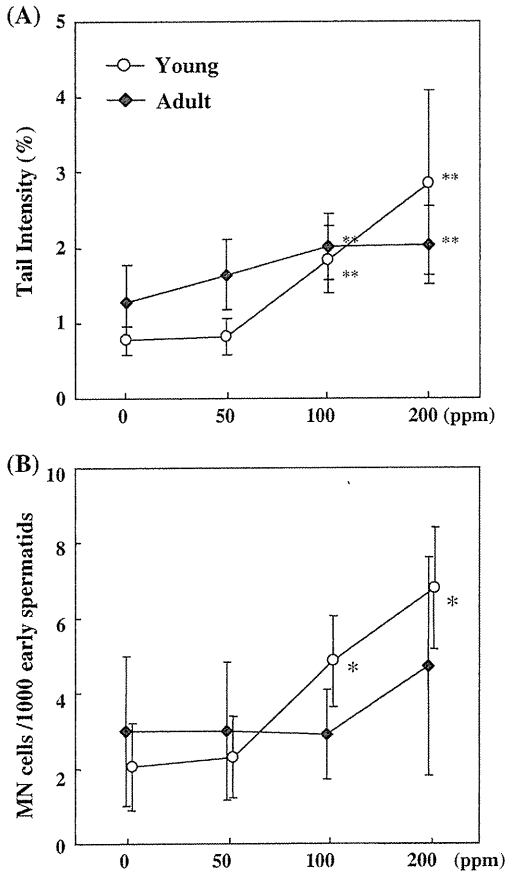

Fig. 7 Tail intensity of the comet image (**a**) and micronuclei frequency (**b**) obtained from young and adult rats given ACR in the drinking water for 4 weeks. Data are mean ± SD. $^{**}P < 0.01$ vs. 0 ppm

Regarding the susceptibility to ACR testicular toxicity in the present study, young animals showed apparently diverse and more profound lesions exceeding the dose–effect relationship observed in adult animals. ACR is known to interfere with motor proteins such as kinesin found in the sperm flagellum and alkylate protein sulfhydryl groups in the sperm tail (Sickles et al. 2007; Friedman et al. 2008). Therefore, it is considered that elongate spermatids are highly susceptible to ACR. In the comet assay, although DNA damage in the young group was higher than that in the adult group at 200 ppm, the values were not greatly different. However, the MN test revealed that ACR clearly induced MN in the young group, but not in the adult group. These results well correspond with the observations on histopathological examination. Because the comet assay and MN test in the testis target spermatocytes and early spermatids, the late stage of spermatogenesis may be more susceptible to ACR-induced genotoxicity in young than in adult animals.

As reported by others (Yousef and El-Demerdash 2006), the basal level of testicular GST activity in our cases was much lower than that in the liver. Although there were no life stage differences in the liver levels of GST activity, testicular GST activity in the present study was significantly lower in the young groups, irrespective of the ACR treatment. The activity of GST is low at birth and then increases gradually, but it has been known that the developmental profiles of antioxidant enzymes including GST in the testis differ greatly from those in the liver (Peltola et al. 1992). A study of the immunolocalization of GST-Yo, a member of the mu class expressed at high levels in the testis and epididymides, revealed that this enzyme was not detectable until 39 days of age and then appeared mainly in the elongate spermatids, with expression reaching maturity by day 49 (Papp et al. 1994). Therefore, the detoxification capacity of the testis in young animals was considered to be much lower than that in the adult animals during the experimental period in the present study, and such a difference might reasonably account for the high susceptibility to ACR-induced testicular toxicity observed in our young animals. In the liver, although GST activity was increased at 200 ppm, there were no apparent life-stage differences. Considering that the liver is the main organ involved in detoxification of ACR, similar level of GST activity may have contributed to the lack of differences in susceptibility to neurotoxicity between young and adult rats. Increase in GST activity in ACR-treated rats has been reported and considered to be due to increased formation of S-conjugates between ACR and GSH (Yousef and El-Demerdash 2006). ACR is known to cause GSH depletion (Zhang et al. 2009); however, decrease in GSH contents was not found in the present study. Because recovery or rather increase in liver GSH contents after depletion by treatment animals with acetaminophen has been reported (Ishii et al. 2009), the level of GSH in the present study might possibly have recovered after repeated treatment with ACR during the experimental period.

In summary, our results suggest that susceptibility to ACR neurotoxicity in young animals might not be different from that in adult ones when exposure levels are adjusted for the body weight. Regarding testicular toxicity, young animals proved more vulnerable than adults, and this might be due to a low level of testicular GST activity.

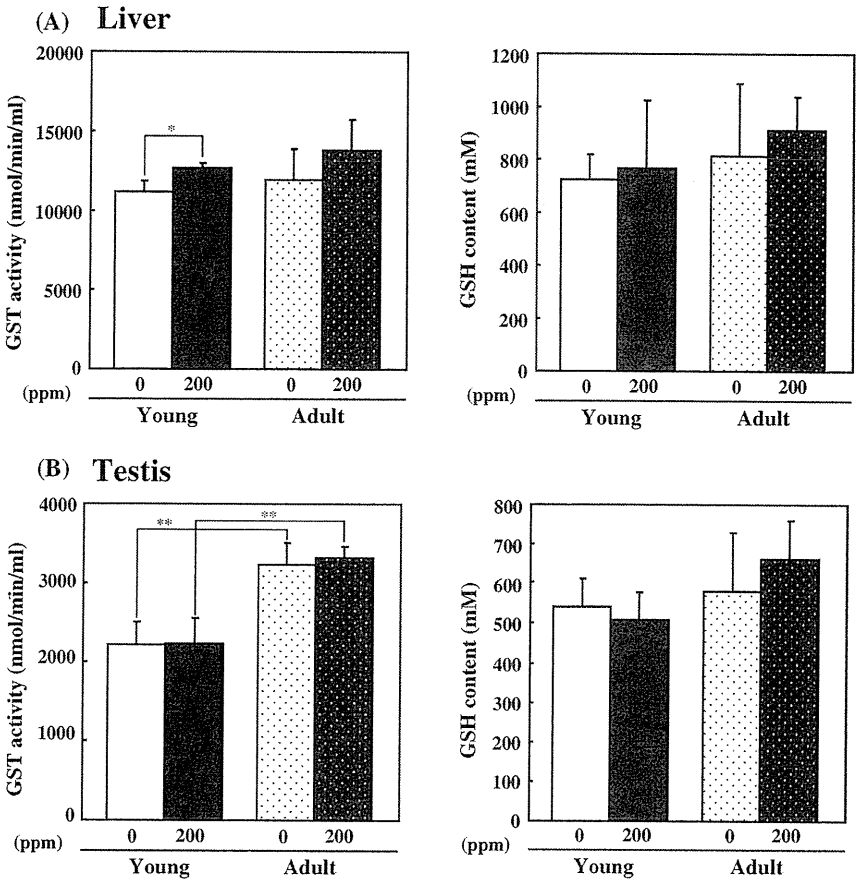

Fig. 8 GST activity and GSH contents in the liver (**a**) and testis (**b**) of young and adult rats given ACR at 0 or 200 ppm for 4 weeks. Data are mean ± SD. *, **$P < 0.05$ and $P < 0.01$

Acknowledgments This work was supported by Health and Labour Sciences Research Grants (Research on Food Safety) from the Ministry of Health, Labour and Welfare of Japan. We thank Miss Ayako Kaneko for technical assistance in conducting the animal study.

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Reply to Letter to the Editor

Reply to Comment on "Impaired oligodendroglial development by decabromodiphenyl ether in rat offspring after maternal exposure from mid-gestation through lactation" [Reprod. Toxicol. 31(1) (2011) 86–94]

Keywords:
Decabromodiphenyl ether (DBDE)
Brominated flame retardants (BFRs)
Impaired brain development
Developmental neurotoxicity (DNT)
Maternal exposure
Hypothyroidism
White matter hypoplasia
Rat

We thank Biesemeier et al. for their interests in our recent report regarding the developmental toxicity study of decabromodiphenyl ether (DBDE), one of the representative brominated flame retardants (BFRs), indicating impaired oligodendroglial development in rat offspring after maternal exposure from mid-gestation through lactation [1]. Biesemeier and his colleague have also recently reported an oral developmental neurotoxicity (DNT) study on DBDE using rats as an industry study, and found no apparent DNT at doses up to 1000 mg/kg-day [2]. They addressed four points on our study in their comment: (1) failure to control for potential litter effects; (2) citation of study reports that failed to control for litter effect; (3) effect of data variability between studies conducted in our laboratory in relation with small sample size; and (4) lack of further studies regarding the brain changes in relation with functional abnormalities. We persevere in our main conclusion and give the following explanations and clarification.

First of all, we have to state that our DBDE study by Fujimoto et al. [1] has been performed in a series of our studies to establish a rapid screening system of DNT. As we have mentioned in the Introduction section, regular DNT studies require large numbers of animals for detection of subtle dose–response changes. For screening purposes of many new chemicals, smaller scale studies, preferably with short-term experiments, employing suitable and sensitive neurodevelopmental endpoints focusing on histopathological parameters need to be established. For histopathological analysis including immunohistochemistry, we, in our DBDE study, designed to use 10 pups or more/group/time point [1]. Similar number of animals (10 pups/sex/group/time point) was recommended to use to the neuropathology endpoint in a recent DNT testing guideline developed by Organisation for Economic Co-operation and Development (OECD) [3].

OECD testing guideline for DNT includes morphometric analysis on brain tissues as well as conventional neuropathological analysis; however, any efficient histopathological parameters based on the mechanism of DNT have remained optional [3]. Thus, our studies were focused on establishing a rapid and sensitive screening system of DNT based on the detection of functional abnormalities of the brain cellular components in a small scale animal study. As an experimental model to build up an evaluation system, we utilized a model of developmental hypothyroidism because of its unique influence on neuronal migration and oligodendroglial development, both of which are essential for brain development [4–6]. The outcome of this type of impairment is permanent and is accompanied by apparent structural and functional abnormalities. Also, some environmental chemicals, such as BFRs, are thought to potentiate a thyroid hormone-disrupting effect that may lead to abnormal brain development [7]. Therefore, there is a growing concern regarding the DNT of these chemicals [8,9].

With regard to the failure to control for potential litter effects, we understand the viewpoint by Biesemeier et al. that the DNT should be evaluated using sufficient number of animals avoiding possible litter effect (1 pup from 1 dam/group). In the study by Fujimoto et al. [1], 8 dams were used per group, and 10 male and 10–11 female offspring were examined at both PND 20 and PND 77 in every group. Therefore, 2 pups each were provided from 2 or 3 dams (mostly 2 pups each from 2 dams) in addition to 1 pup each from 6 dams in each group for examination. With regard to the data that have shown statistically significant difference between the untreated controls and treatment groups by analysis using the individual animal as the experimental unit, we recalculated them using the litter as the experimental unit. As a result, we could not find any difference in the items showing significant difference between the use of individual animal and the litter as the experimental unit (Supplementary data: Tables 1–3). In addition, inconsistency in the study design between the reports by Teshima et al. [10] and Fujimoto et al. [1] was due to the mistakes in the former study. Description in the latter study was correct, and therefore, speculation of the potential litter effects with $n=6$ dams/group by Biesemeier et al. is not the case.

With regard to the citation of study reports that have been judged to fail to control for litter effect by others, a series of studies by Eriksson and/or Viberg [11–17] as pointed out by Biesemeier et al. have employed direct oral administration of test compound to delivered pups, and therefore, potential litter effect due to the difference in the prenatal environment among litters was considered to be rather low as compared with the exposure cases from prenatal periods. In experimental rodent studies using animals with identical (or mostly identical) genetic background, potential litter effect due to genetics as pointed out by Biesemeier et al. may not be the case. On the other hand, as pointed out by Johnson et al. [18], the study by Xing et al. have failed to control for litter effect [19]. We have cited the report by Viberg et al. [15] to discuss the possibility of the direct effect of DBDE on the brain to cause white matter hypoplasia in the study by Fujimoto et al. [1]. Viberg et al. [15] have

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shown increases of the amount of radioactivity after neonatal exposure to [U-^{14}C]DBDE in the brain during the first week after single administration; however, questions have been raised by others on the analytical methodology applied and data calculation [20,21]. As we have discussed [1], the biological relevance to our study results are unclear regarding the neurobehavioral effects in both mice and rats by Viberg et al. after single neonatal exposure to DBDE [15,16]. Although further study should be addressed regarding the role on developmental neurotoxic effects. Zhang et al. [22] recently reported tissue distribution including the brain of DBDE and its debrominated metabolites in sucking rat pups after prenatal and/or postnatal exposure, suggesting the possibility of direct effect of DBDE on the brain. In another recent study, Ibhazehiebo et al. [23] reported that DBDE disrupted thyroid hormone-mediated transcription in a reporter gene assay in fibroblast derived CV-1 cells, at concentrations as low as 0.01 nM. Such effect appeared to be due to interference with the thyroid receptor–DNA binding domain, rather than to competitive inhibition of T3-binding to the thyroid receptor or to alterations of thyroid receptor–cofactor binding. At similar low concentrations, they also reported that DBDE was found to inhibit thyroid hormone-induced dendrite arborization of Purkinje cells in vitro. However, as we have mentioned in the conclusion, mild hypoplastic changes of the white matter due to decreased number of oligodendrocytes observed in our DBDE study might be the hypothyroidism-related effects of DBDE because of sustained mild hypothyroidism at least at the high dose group [1].

The study conducted under the compliance of Good Laboratory Practice provides reliability in the data with regard to the parameters examined. It is said that DNT testing has been proven to be effective in identifying compounds with DNT potential [24]. However, it does not mean that current DNT testing guidelines [3,25] cannot be improved. Indeed, there is still controversy regarding the sensitivity; one has pointed out that they may be overly sensitive and produce a high rate of false positives [26], but another pointed out that they may be too insensitive and not enough comprehensive [27]. From neuropathology point of view, positive results obtained from simple morphometry as used in the DNT study conducted by Biesemeier et al. [2] may provide some evidence of subtle alterations of the brain components; however, it may sometimes be difficult to identify the target mechanism by this type of morphometry as well as by the conventional histopathological analysis. Therefore, interpretation of such morphometric changes in relation with cellular morphology and function becomes difficult, and toxicological relevance of such data may sometimes be judged to be low or negligible. Also it should be noted that the lack of changes just provides evidence of no effects on the parameters examined. Thus, there still is a room for improving the DNT testing guidelines by adding mechanism-based histopathological analysis, such as for thyroid hormone-disrupting chemicals.

On the other hand, Biesemeier et al., in their DNT study of DBDE [2], showed statistically significant decreases in the hemisphere height from the lowest dose (1 mg/kg body weight-day) in male offspring on PND 72 (Supplementary data: Table 4). At the dose of 1000 mg/kg body weight-day, male offspring also showed statistically significant decrease in the pons vertical thickness at PND 21 and cortex vertical thickness at PND 72 (Supplementary data: Table 4). Authors have concluded that these morphometric changes were not adverse, because all of statistically significant values were within the range of the laboratory's historical control data, and, in contrast, some of the control values were higher than the historical control values. Although this kind of morphometric data should have been discussed firstly in relation with cellular morphology and function of the brain area measured, such kind of discussion was lacking in their report. Moreover, they did not show historical control data as supplementary information. Although the morphometric method applied does not clarify the target cellular component(s), reduction in the height or thickness may be the reflection of the volume reduction of the cortex or white matter. It may be reasonable to apply morphometric analysis regarding oligodendroglial development in the brain tissue samples using immunohistochemistry with 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNPase) that has been previously validated by us using a model of developmental hypothyroidism [28]. We have found reduction in the area of corpus callosum stained with CNPase as well as the number of CNPase-positive oligodendrocytes distributed in the cingulate deep cortex in our DBDE study [1], similarly to the hypothyroidism cases [28]. Moreover, because thyrotoxic effect is reported with DBDE [29] as observed in our DBDE study, Biesemeier et al. should have examined thyroid parameters in their study, such as weight measurement and histopathology of the thyroid and serum levels of thyroid-related hormones, in offspring; however, their study lacked such information.

With regard to the third comment on the effect of data variability between studies conducted in our laboratory in relation with small sample size, Biesemeier et al. pointed out variability in the control values of our already published studies regarding offspring (male) body and relative liver weights on postnatal day (PND) 20 at weaning; serum levels of thyroid-related hormones (triiodothyronine, thyroxine, and thyroid-stimulating hormone) in male offspring at both PND 20 and postnatal week 11; and corpus callosum area and CNPase-positive oligodendrocytes. There may be minor but unavoidable multifactorial influence operating on these differences. We think variability of data between studies may preferentially arise in the absolute values, such as in absolute body weight, levels in thyroid-related hormones, area of corpus callosum in our studies, and use of different animal lots between studies may be operated on such variability. With regard to serum levels of thyroid-related hormones, measurement at different time points may also cause variability between the studies. In terms of relative liver weights, we do not think there is variability between the studies. With regard to the counts of CNPase-positive oligodendrocytes, in which values were normalized by the area measured, difference in the immunohistochemistry conditions, especially of the visualization of immunoreactivity, may cause variability between the studies. As Biesemeier et al. pointed out, litter effects may arise in the offspring values of our data; however, as shown in Supplementary data (Tables 1–3), we found that the litter effect was negligible in numerical values, even with small sample size (i.e., $n = 8$ dams/group).

With regard to the fourth comment on the lack of further studies regarding the brain changes in relation with functional abnormalities, we could say our study purpose was different from that of large scale DNT study. As mentioned above, the purpose of our studies was to establish a small scale efficient screening system of environmental chemicals, based on the pathological mechanism of DNT. As one approach for this scope, we focused on the responses in parameters related to neuronal migration and oligodendroglial development utilizing a model of developmental hypothyroidism [1,28,30–32]. In addition, using the same animal samples of the study by Fujimoto et al. [1], we found distribution changes of the subpopulation of interneurons in the hilus of the dentate gyrus by DBDE, suggestive of aberration of neuronal migration probably due to aberration of neurogenesis in the subgranular zone of the dentate gyrus, at doses of 100 ppm and higher (Y. Saegusa et al., data submitted), similarly to the development of mild white matter hypoplasia as reported by Fujimoto et al. [1]. However, the effect on neuronal development itself was found to be reversible, while a sustained increase of mature neurons was detected in the dentate hilus.

In summary, as Biesemeier et al. pointed out, litter effects could have been operated on the observed changes in the study by Fujimoto et al. [1]; however, recalculation of the data using the litter as the experimental unit revealed no changes in items showing statistically significant difference. With regard to the citation of study reports that have been judged to fail to control for litter effect by others, the study by Xing et al. [19] has actually failed to control for potential litter effects; however, a potential litter effect derived from prenatal environment in a series of postnatal exposure studies by Eriksson and/or Viberg was considered to be rather low as compared with the exposure cases from prenatal periods. We have cited them as an example of the direct brain effect of DBDE; however, our results supported the possibility of hypothyroidism-related effect as described in the conclusion of the study by Fujimoto et al. [1], despite the recent study results suggestive of the direct effect on the brain. With regard to the possible effect of variability of data between studies conducted in our laboratory in relation with the use of small number of animals, we think that minor but unavoidable multifactorial influence may be operated. We here judge that the litter effects were negligible in our experimental system even with small sample size. With regard to the lack of further studies regarding the brain changes in relation with functional abnormalities, we could say that our study was aimed at different endpoint from that of large-scale DNT study. The purpose of our studies was to establish a new screening system in a small scale animal study applying morphometrically measurable parameters selected based on the mechanism of DNT.

In closing, comments raised by Biesemeier et al. on our recently published study regarding developmental toxicity of DBDE were revealed to be rather minor ones or on the point out of the scope of our study. We rather recommend them to perform additional assessment on their recently published DNT study of DBDE regarding histopathological parameters on neuronal migration and oligodendroglial development that have been proven to fluctuate in relation with developmental hypothyroidism [28,32].

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.reprotox.2011.06.074.

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Makoto Shibutani[a,b,*]
[a] *Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan*
[b] *Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan*

Hitoshi Fujimoto
Gye-Hyeong Woo
Kaoru Inoue
Miwa Takahashi
Akiyoshi Nishikawa

Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

* Corresponding author at: Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan. Tel.: +81 42 367 5874; fax: +81 42 367 5771.
E-mail address: mshibuta@cc.tuat.ac.jp (M. Shibutani)

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