Therefore, it is considered that the maternal malnutrition observed with ACR at ≥100 ppm could have greatly affected pup body weights in the present study. Although suppressed body weight persisted until PNW 11, differentiation of the effect of ACR toxicity from the influence of maternal malnutrition was difficult.

In the present study, neurotoxicity, represented by gait abnormalities in dams, was not found in offspring. Histopathologically, although findings suggesting developmental retardation and malnutrition, such as increase of retained external granular cells in the cerebellum, decrease of extramedullary hematopoiesis in the liver and spleen, and retardation of spermatogenesis, were found at weaning, it was difficult to separate direct effects of ACR on offspring from effects due to maternal toxicity. Increase of the axonal density and the proportion of small myelinated nerve fibers observed in the sciatic nerves at weaning might have been due to retarded growth accompanying maternal malnutrition rather than ACR-induced neurotoxicity, because the caliber of axons was reduced evenly in the ACR-treated groups. Moreover, degenerated axons in the sciatic nerves and aberrant dot-like SYP-immunoreactivity in the cerebellum, evident in dams, were not featured in the offspring at either weaning or PNW 11. Also, there were no other abnormalities indicating ACR toxicity in the nervous system at PNW 11. Therefore, the results suggest that ACR exposure through the maternal drinking water did not cause irreversible damage to the nervous system of offspring, at least under the present experimental conditions.

In a previous study, no signs of neurotoxicity were found in offspring after lactational ACR-exposure, and the authors considered that there was little possibility of ACR exposure to pups from the milk because of the maternal inanition (Friedman *et al.*, 1999). Similarly, it is likely that ACR exposure through milk was limited in our study, since maternal malnutrition and neurotoxicity might have greatly influenced lactation. On the other hand, ACR has been reported to produce developmental neurotoxicity, including decreased motor activity and auditory startle responses, in SD rat offspring at maternal neurotoxic doses of 15 mg/kg body weight/day by oral gavage from GD 6 through PND 10 (Wise *et al.*, 1995). Also, repeated oral doses of ACR up to 10 mg/kg body weight/day by gavage from GD 7 through delivery to maternal F344 rats and then to pups from PND 1 through PND 22 produced behavioral abnormalities and negative geotaxis performance at 10 mg/kg body weight/day with a linear trend in fall-time latencies on Rotarod performance on PND 21-22 (Garey *et al.*, 2005). Therefore, pups may certainly exhibit behavioral abnormalities if exposed to ACR. In the present study, the mean daily intakes of ACR of dams during the

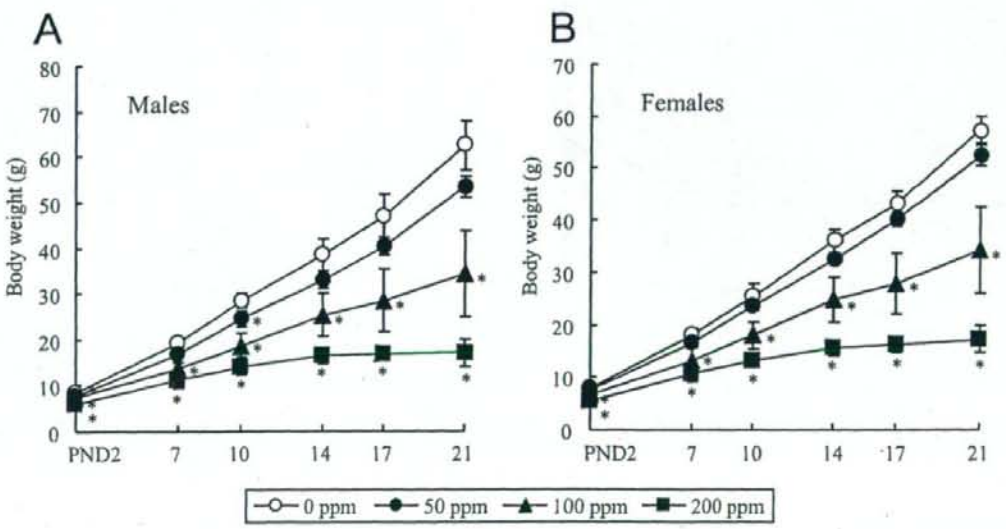

Fig. 4. Body weight changes of offspring before weaning. Data are mean ± SD. n=6 (0, 50 and 100 ppm group), n=4 (200 ppm group). *: $p<0.01$ vs. 0 ppm group. Abbreviation: PND, postnatal day.

Table 3. Data from histopathological analysis of F1 offspring at weaning.

	Males				Females			
	Acrylamide in the drinking water (ppm)				Acrylamide in the drinking water (ppm)			
Organ Findings	0	50	100	200	0	50	100	200
No. of animals	6	6	6	4	6	6	6	4
Cerebellum								
Increase of external granular cells (+/++/+++)[a]	6 (6/0/0)	6 (4/2/0)	6 (2/4/0)	4 (0/2/2) ††	6 (5/1/0)	6 (5/1/0)	6 (1/4/1)	4 (0/2/2) †
Liver								
Extramedullary hematopoiesis (±/+/++)[a]	6 (0/0/6)	6 (0/2/4)	6 (1/5/0) ††	2 (2/0/0) ††	6 (0/3/3)	6 (0/1/5)	5 (1/3/1)	4 (4/0/0) ††
Loss of cytoplasmic glycogen vacuoles, hepatocytes	0	0	0	3	0	0	0	2
Spleen								
Extramedullary hematopoiesis (±/+/++/+++)[a]	6 (0/0/0/6)	6 (0/0/2/4)	6 (0/3/3/0) ††	4 (4/0/0/0) ††	6 (0/0/5/1)	6 (0/0/6/0)	6 (1/3/2/0)	4 (3/1/0/0) ††
Testes								
Retardation of spermatogenesis (+/++/+++)[a]	0	0	6** (4/2/0) ††	4** (0/2/2) ††	–	–	–	–

[a]: Grade of change: ±: slight; +; mild, ++; moderate, +++: severe.
–: Not available.
**: $p<0.01$ vs. 0 ppm group (Fisher's exact test).
†, ††: $p<0.05$, $p<0.01$ vs. 0 ppm group (Mann-Whitney's *U*-test).

gestation and lactation periods were 16.7 ± 2.1 and 22.2 mg/kg body weight/day at 100, and 200 ppm, respectively, corresponding to the behaviorally neurotoxic dose to offspring in the above-mentioned study (Wise *et al.*, 1995). In man, fetal internal levels of ACR have been estimated to be at least equal to those achieved in mothers (Schettgen *et al.*, 2004). Therefore, the pups were likely to be exposed to ACR at a dose equivalent to that received by their dams during the gestation period at least. However, the duration of transplacental exposure in the present study from GD 10 through birth, within 2 weeks, might be too short to produce morphologically assessable lesions, since the dose used is known to induce neurotoxicity within 4 weeks in adult male SD rats (Lee *et al.*, 2005). Alternatively, the type and degree of neurotoxicity induced by developmental exposure of ACR may be different from the neurotoxic outcome apparent with progressive gait abnormalities observed in adult rats. Although the initial targets of ACR appear to be nerve terminals in both the central and peripheral nervous systems in adult animals (LoPachin *et al.*, 2003), these may be less sensitive to ACR-toxicity during development due to high neuronal plasticity.

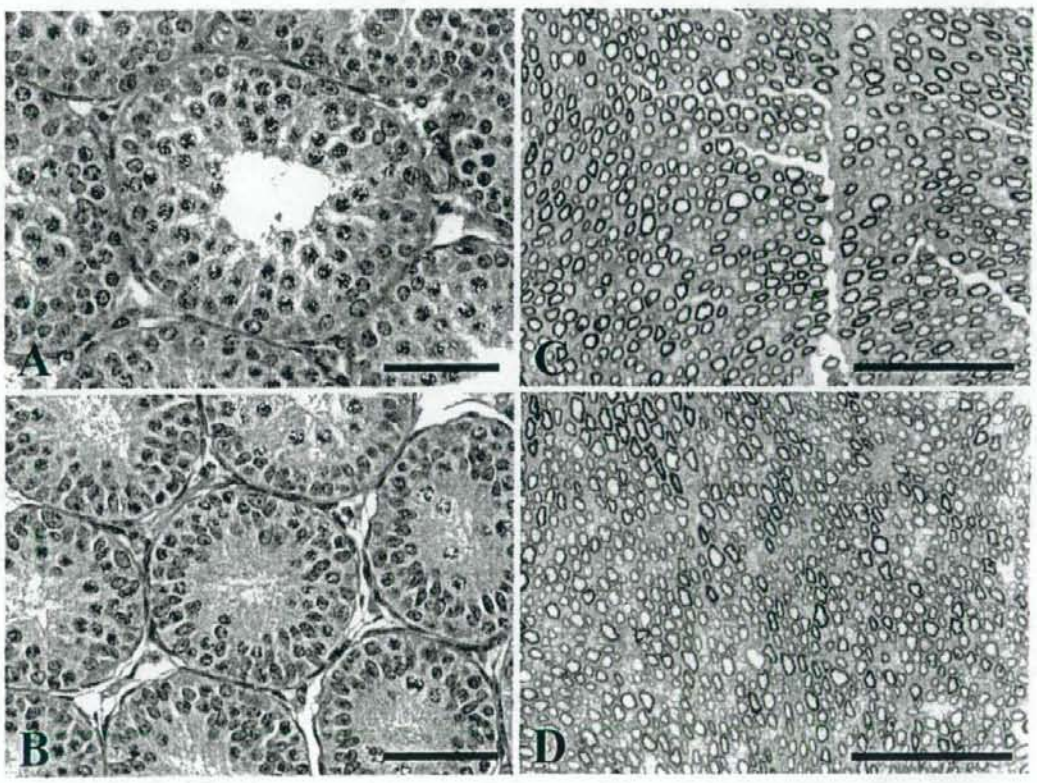

Fig. 5. Histopathology of the testis and sciatic nerve of pups at weaning exposed maternally to ACR at 0 (control) or 200 ppm for the gestation and lactation periods. (A) The seminiferous tubules showing 3–4 layers of spermatocytes in a male pup at 0 ppm. (B) The seminiferous tubules of a male pup at 200 ppm featured with 1–2 layers of gonocytes. Note reduced number of differentiated spermatocytes within the tubules as compared with the control animal. (C) The sciatic nerve of a male pup at 0 ppm. (D) The sciatic nerve of a male pup at 200 ppm. Note increase of nerve fiber density with small caliber as compared with the control animal. A, B: hematoxylin and eosin. C, D: resin-embedded semithin sections stained with toluidine blue. Bar=50 μm.

Regarding testicular toxicity, since it has been demonstrated that round spermatids are vulnerable to ACR (Sakamoto *et al.*, 1988), testes prior to spermatogenesis might be less sensitive. However, as with neurotoxicity, maternal malnutrition might have exerted a major effect, and further studies will be required to evaluate susceptibility to ACR-induced testicular toxicity in immature testes.

In conclusion, ACR administered in the maternal drinking water at concentrations of ≥100 ppm, while maternally neurotoxic, did not produce neurotoxicity or testicular toxicity in offspring under the experimental conditions applied here, despite decrease of pup weights at ≥50 ppm. Although there is a possibility that the target cells/cellular components of ACR in the adult case, such as the nerve

Table 4. Data for morphometry of lesions developing in the nervous systems of offspring at weaning and postnatal week 11.

		Acrylamide in the drinking water (ppm)			
		0	50	100	200
Weaning					
Males					
No. of animals		6	6	6	4
Sciatic nerve (distal portion)					
Density	(/100 μm²)	4.08 ± 0.34[a]	4.40 ± 0.22	4.47 ± 0.64	5.42 ± 0.37**
Degenerated axons	(%)	0.85 ± 0.17	0.67 ± 0.17	0.76 ± 0.20	0.72 ± 0.18
Myelinated axons, <3 μm in diameter	(%)	18.62 ± 2.32	19.11 ± 2.50	22.04 ± 5.08	25.51 ± 1.87*
Cerebellar cortex, molecular layer					
SYP-immunoreactive aberrant dots	(/mm cortex)	0.29 ± 0.09	0.26 ± 0.11	0.32 ± 0.16	0.44 ± 0.09
Females					
No. of animals		6	6	6	4
Sciatic nerve (distal portion)					
Density	(/100 μm²)	4.26 ± 0.21	4.14 ± 0.48	4.73 ± 0.70	5.28 ± 1.27
Degenerated axons	(%)	0.82 ± 0.33	0.71 ± 0.18	0.81 ± 0.31	0.86 ± 0.38
Myelinated axons, <3 μm in diameter	(%)	20.93 ± 1.64	20.1 ± 2.55	22.37 ± 2.48	23.92 ± 8.21
Cerebellar cortex, molecular layer					
SYP-immunoreactive aberrant dots	(/mm cortex)	0.55 ± 0.17	0.46 ± 0.17	0.55 ± 0.27	0.64 ± 0.33
Postnatal week 11					
Males					
No. of animals		6	6	6	4
Sciatic nerve (distal portion)					
Density	(/100 μm²)	1.87 ± 0.19	1.94 ± 0.19	1.83 ± 0.39	1.89 ± 0.16
Degenerated axons	(%)	1.06 ± 0.45	1.01 ± 0.55	0.93 ± 0.23	1.04 ± 0.19
Myelinated axons, <3 μm in diameter	(%)	9.50 ± 2.19	11.82 ± 1.75	11.38 ± 3.68	12.88 ± 1.03
Cerebellar cortex, molecular layer					
SYP-immunoreactive aberrant dots	(/mm cortex)	0.58 ± 0.17	0.63 ± 0.28	0.53 ± 0.20	0.51 ± 0.12
Females					
No. of animals		6	6	6	3
Sciatic nerve (distal portion)					
Density	(/100 μm²)	2.22 ± 0.14	2.01 ± 0.27	2.08 ± 0.09	1.99 ± 0.11
Degenerated axons	(%)	1.11 ± 0.84	0.89 ± 0.40	1.08 ± 0.71	1.30 ± 0.23
Myelinated axons, <3 μm in diameter	(%)	11.47 ± 0.76	10.99 ± 2.01	11.41 ± 1.90	12.03 ± 2.39
Cerebellar cortex, molecular layer					
SYP-immunoreactive aberrant dots	(/mm cortex)	0.74 ± 0.27	0.59 ± 0.36	0.61 ± 0.29	0.67 ± 0.38

[a]: Mean ± SD.
*, **: $p<0.05$, $p<0.01$ vs. 0 ppm group.
Abbreviation: SYP, synaptophysin.

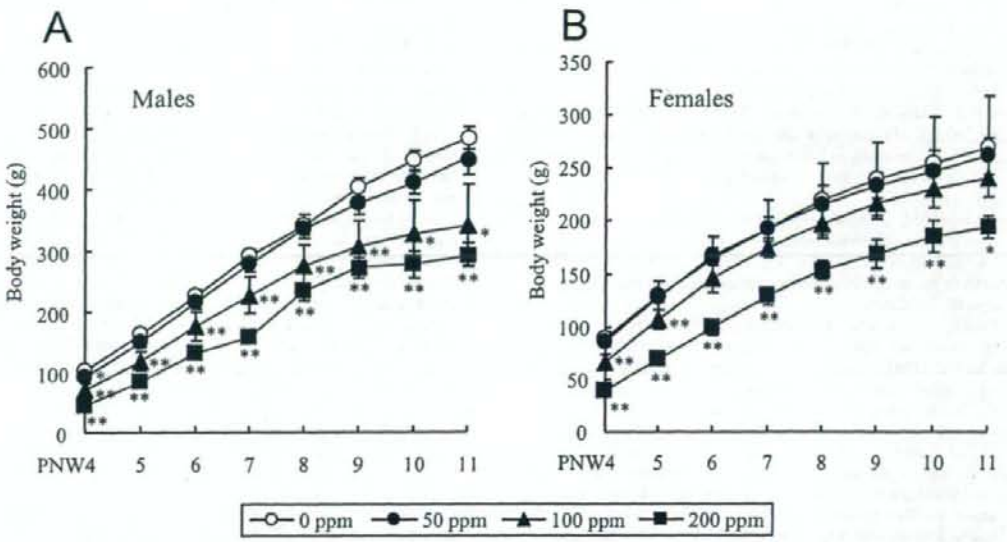

Fig. 6. Postweaning body weight changes of offspring. Data are mean ± SD. n=6 (0, 50 and 100 ppm group), n=4 or 3 (200 ppm group).*, **: $p<0.05$, $p< 0.01$ vs. 0 ppm group. Abbreviation: PNW, postnatal week.

terminals and seminiferous epithelium, might be less sensitive to ACR toxicity during development, limited exposure due to maternal toxicity during the lactational period may be the reason why pups did not show here any ACR-induced toxicity except for retarded body growth.

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TOXICOKINETICS AND METABOLISM

Limited lactational transfer of acrylamide to rat offspring on maternal oral administration during the gestation and lactation periods

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Abstract To evaluate the developmental exposure effects of acrylamide (ACR) on the nervous and male reproductive systems, pregnant Sprague-Dawley rats were given ACR at 0, 25, 50 or 100 ppm in the drinking water from gestational day 6 to postnatal day (PND) 21 and histopathological assessment was performed at PND 21. Exposure levels in offspring were examined by measurement of free ACR and hemoglobin (Hb)-ACR adducts on PND 14, and compared with maternal levels on PND 21. Additionally, a group of offspring that received ACR at 50 mg/kg by intraperitoneal injections directly three times a week from PND 2 to 21 was subjected to analysis for comparison with maternal exposure groups. Although maternal neurotoxicity was evident at 100 ppm, no changes suggestive of neurotoxicity or testicular toxicity were observed in their offspring except for growth retardation evident as lowered body weights. In contrast, offspring given ACR intraperitoneally exhibited obvious neurotoxicity, but not testicular damage. Free ACR in serum and milk was detected in neither dams nor their offspring. The level of ACR-Hb adducts in offspring was one tenth or less than that in dams. In summary, although preweaning rats have susceptibility to ACR-induced neurotoxicity, the internal level of ACR in offspring exposed through maternal oral administration is insufficient to induce neurotoxicity and testicular toxicity due to limited lactational transfer.

Keywords Acrylamide · Hemoglobin adduct · Neurotoxicity · Testicular toxicity · Rat

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Introduction

Acrylamide (ACR), a widely used chemical in many industries, is known to be a neuro- and reproductive toxicant, and to act as a carcinogen in animals. Recently, it was found to be generated in foods containing carbohydrate and amino acids by heating, and risk assessment studies of ACR in foodstuff are now being conducted globally (Exon 2006; Parzefall 2008). Mean daily intake of ACR for adults is estimated as 1 μg/kg body weight/day. The values for infants and children are estimated to be 2- to 3-fold higher than for adults when expressed on a body weight basis (WHO/IPCS 2006). However, because toxicity studies of ACR have hitherto mainly been performed using adult animals, information on effects during fetal, infantile and pubertal periods and the relationship between the age and susceptibility to ACR-induced toxicity is insufficient.

In our previous study, to evaluate the developmental effects of exposure to ACR on the nervous and male reproductive systems, pregnant Sprague–Dawley (SD) rats were given ACR at 0, 50, 100 or 200 ppm in the drinking water during the gestation and lactation periods and histopathological assessment of offspring was performed at weaning and postnatal week 11 (Takahashi et al. 2008). As a result, maternal neurotoxicity was observed from 100 ppm, but no

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changes suggestive of neurotoxicity or testicular toxicity except for growth retardation were observed in offspring at any dose. Although there is a possibility that the target cells/cellular components of ACR in adults, such as the nerve terminals and seminiferous epithelium, are less sensitive to ACR toxicity during development than in the adult case, limited exposure due to maternal toxicity during the lactational period may be operated for the lack of ACR toxicity except for retarded body growth. Placental transfer of ACR has been reported in experimental animals and humans (Ikeda et al. 1983; Schettgen et al. 2004). In human, the concentration of ACR-adducts in the red blood cells (RBC) of neonates is approximately 50% of the adult levels, and in view of the shorter life span of the RBC and the rather small body size of neonates, internal level of ACR in neonates by transplacental exposure was estimated to be at least equal to that of mothers (Schettgen et al. 2004). On the other hand, although a few studies have shown the potential for exposure to ACR through human milk (Sörgel et al. 2002; Fohgelberg et al. 2005), the actual extent and impact of trans-breast milk exposure remain unknown.

Formation of hemoglobin (Hb)-ACR adducts in RBCs is known to be a valuable marker of ACR exposure (Bergmark et al. 1991; Bergmark et al. 1993; Sumner et al. 2003). To test our working hypothesis that lack of offspring toxicity might mainly be due to limited exposure by the lactational route, the present study was performed employing histopathological assessment of the nervous and male reproductive systems in offspring exposed maternally to ACR, in combination with measurement of free ACR in the serum and Hb-ACR adducts in the RBC in both dams and offspring, as well as free ACR containing in the stomach milk. For comparison, a group of preweaning animals were directly injected ACR intraperitoneally during the lactation period as positive controls for susceptibility of infant rats to ACR.

Materials and methods

Chemicals and animals

Acrylamide was purchased from Sigma (St Louis, MO, USA; CAS #79-06-1) as a white powder with a purity of >98%. A total of 18 pregnant CD® (SD) IGS rats were obtained from Charles River Japan Inc. (Kanagawa, Japan) at gestational day (GD) 1 (the day when a vaginal plug was observed was designated as GD 0) and housed individually in polycarbonate cages with wood chip bedding, in an air-conditioned animal room (temperature 24 ± 1°C, relative humidity 55 ± 5%) with a 12-h light/dark cycle. They received powdered basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water ad libitum during the 5-day acclimatization period.

Experimental design

On GD 6, dams were randomly divided into four groups of four dams each and given ACR at 0, 25, 50, 100 ppm in the drinking water from GD 6 to postnatal day (PND) 21. The highest dose was selected as that inducing progressive neurotoxicity to dams in our previous study (Takahashi et al. 2008). Two dams were maintained untreated until delivery, and delivered neonates directly received ACR at 50 mg/kg/day by intraperitoneal injections three times a week from PND 2 to 21. This dosing regimen is known to induce degeneration of the sciatic nerve in adult male SD rats within 3 weeks (Saita et al. 1996).

All dams were housed individually, and body weights and food and water consumption were measured regularly. Litter size, sex and body weight of pups were checked at PND 2, and litters were culled randomly to preserve eight pups, mostly four per sex per litter on PND 4. Daily observation for clinical signs, including gait abnormalities, and mortality of dams and offspring, was conducted throughout the experimental period. On PND 14, blood samples of one to two pups per litter in the maternally exposed groups were collected using heparinized syringes from the abdominal aorta under deep ether anesthesia. To avoid the effects of self feeding/drinking of pups at the late lactation period on the levels of free-ACR or Hb-ACR adducts in offspring, PND 14 was selected because pups were nourished solely from maternal milk at this time point. After centrifuging, plasma and blood cells were stored separately in brown glass bottles at −80°C. Milk in the stomach was also collected and stored in brown glass bottles at −80°C until analysis. From dams, blood samples were collected at PND 21, and plasma and blood cells were stored in the same manner as described above. On PND 21, all dams and remaining offspring were subjected to autopsy, and the brain, testes, epididymides and the trigeminal nerves were removed. The brains were fixed in methacarn solution at 4°C, and the testes in Bouin's solution at room temperature, in both cases overnight. The other organs were fixed in neutrally-buffered 10% formalin. All were then routinely processed for paraffin embedding, sectioned at 3 μm, and stained with hematoxylin and eosin (HE). For histopathological assessment of axonal changes in the peripheral nerves, 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. The portion located at the ankle position was carefully excised and further fixed with fresh fixative overnight, postfixed in 1% osmium tetroxide in the same buffer for 2 h at 4°C, and embedded in epoxy resin (TAAB Laboratories Equipment

Ltd., Berkshire, UK). Semithin sections, 1 μm in thickness, were stained with toluidine blue for light microscopic assessment. The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Morphometric assessment

Morphometric assessment was performed on the sciatic nerves and cerebellum of the dams and offspring given ACR at 0 or 100 ppm. Offspring given ACR intraperitoneally were also assessed. To evaluate aberrant dot-like structures immunoreactive with synaptophysin (SYP) in the cerebellar molecular layer, sections obtained from methacarn-fixed brain slices including the cerebellum were subjected to immunohistochemistry for SYP. Rabbit polyclonal antibody Ab-4 (1: 200, Lab Vision Corp., Fremont, CA, USA) was used as the primary antibody and immunodetection was conducted with the horseradish peroxidase–avidin–biotin complex method utilizing a VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3′-diaminobenzidine/H_2O_2 as the chromogen, as described previously (Takahashi et al. 2008).

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 microscope attached digital camera (DP71, Olympus Corp., Tokyo, Japan). Measurement was then performed using an image analysis software (WinROOF, Version 5.7.1, Mitani Corp. Tokyo, Japan). The total number of axons/unit area and the number of degenerated axons and the diameter of axons were counted in one cross sectional area at 400× magnification of toluidine blue-stained specimens from each animal, and the density, the percentage of degenerated axons and the 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.

Measurement of free ACR in serum and milk

The serum was mixed with acetonitrile by vortexing, and centrifuged to remove the supernatant. Extraction was again performed in the same manner, and acetonitrile was removed from the pooled supernatants. The residue was then reconstituted with distilled water, filtered through a 0.22-μm polytetrafluoroethylene syringe filter and analyzed by high performance liquid chromatography (HPLC). The milk was subjected to ultrasonic extraction instead of vortexing.

The analyses were carried out using an Agilent 1100 Series (Agilent Technologies, Inc., Santa Clara, CA, USA). Separation was performed on an analytical column (CAPCEL-PAK C18 MGII S-5 4.6 × 250 mm; Shiseido Co., Ltd., Tokyo, Japan), and with a guard column (CAPCEL-PAK C18 MGII S-5 4.6 × 35 mm; Shiseido Co., Ltd.). Detection was performed with a monitoring absorbance at 210 nm. The analysis of the milk was with Develosil TM packing C30-UG-3 4.6 × 250 mm (Nomura Chemical Co., Ltd., Seto, Japan) as an analytical column.

Measurement of ACR-Hb adducts in red blood cells

Red blood cell samples were mixed with saline by vortexing. After removing cellular debris by centrifugation, the supernatant was added to acetone containing 1% HCl, and the resulting precipitate was washed twice with acetone, then once with ether, and air-dried.

Globin was mixed with 6 M HCl and heated at 120°C for 15 h, and after removal of HCl the residue was dissolved in distilled water and incubated at 37°C for 1 h. After adjusting the pH to 9.0 with 0.1 M NaOH, the obtained solutions were applied to an Oasis Max (150 mg/6 cc, 60 μm) column (Waters Corp., Milford, MA, USA) and eluted with 100 mM formic acid in methanol. Solvents were then removed, and the resultant residues were reconstituted with 0.5 M HCl in methanol and heated at 85°C for 2 h. After removal of solvents, the residues were reconstituted with acetonitrile and heptafluorobutanoic anhydride, heated again at 85°C for 20 min, and mixed with ethyl acetate and phosphate buffer (pH 6.8). The ethyl acetate phase was concentrated.

The analyses were carried out using a Agilent 6890N gas chromatograph linked to a Agilent 5973N mass spectrometer (Agilent Technologies, Inc.) with the monitoring mass at $m/z = 204$. Separation was performed on 30-m HP-5 (0.32 mm i.d., 0.25-μm phase thickness) fused silica capillary column (J&W Scientific, Inc., Folsom, CA, USA).

Statistical analysis

Variance in data for body weights and ACR-Hb adduct levels was 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. Values for morphometric assessment in the sciatic nerves and cerebellar molecular layer were analyzed by the Student's *t*-test when the variance was proven to be homog-

enous among the groups using a test for equal variance. If a significant difference in variance was observed, Welch's *t*-test was performed.

Results

In-life parameters and assessment of neurotoxicity

Dams in the 100 ppm group exhibited gait abnormality from PND 2, which progressed to moderate and severe degrees at PND 21. Their body weights were suppressed in parallel with the progression of neurotoxic symptoms. At 50 ppm, a slightly abnormal gait appeared from PND 18. Tendencies for decreased food and water consumption were observed at 100 ppm during the lactation period. Mean daily intake of ACR by dams during the gestation and lactation periods was 3.72 ± 0.28, 7.89 ± 1.70 and 14.56 ± 2.47 mg/kg body weight/day at 25, 50, and 100 ppm, respectively. ACR did not affect the gestation period, number of implantations, live birth ratio and male pup ratio. On PND 8-12, deaths of offspring were sporadically found in all groups, including the control group. No apparent abnormalities were found on clinical observation in offspring exposed to ACR maternally at each dose. In contrast, offspring given ACR intraperitoneally revealed gait abnormalities similar to the adult cases from PND 15.

At PND 21, body weights of dams in the 100 ppm group were decreased, although without statistically significance, and body weights of offspring in both sexes exposed to ACR maternally were significantly decreased at 100 ppm, as compared with those of the 0 ppm groups, as in the previous report (Takahashi et al. 2008; Table 1). Also, significant lowering of body weight was observed in offspring given ACR intraperitoneally.

On histopathological analysis of dams, central chromatolysis of ganglion cells in the trigeminal nerves was observed from 50 ppm (Table 2). In offspring exposed to ACR maternally, although increase of retained external granular cells in the cerebellum and retardation of spermatogenesis were found, no histopathological changes suggestive of neurotoxicity and testicular toxicity were observed (Fig. 1). On the other hand, central chromatolysis of ganglion cells in the trigeminal nerves was evident in offspring given ACR intraperitoneally, in addition to increase of retained external granular cells in the cerebellum and retardation of spermatogenesis.

Morphometric assessment of the nervous tissues of dams showed significant increases of degenerated axons and myelinated nerves of <3 μm in diameter at 100 ppm (Table 3; Fig. 2). In the cerebellar molecular layer, significant increase of dot-like SYP-immunoreactive structures was also detected at 100 ppm. No differences between the 0 and 100 ppm were noted in parameters examined in the sciatic nerves and in the cerebellum in offspring exposed to ACR maternally (Table 4; Fig. 2). In offspring given ACR intraperitoneally, significant increases of degenerated axons and myelinated nerves of <3 μm in diameter were observed. However, an increase of dot-like SYP-immunoreactive structures in the cerebellar molecular layer was not apparent.

Free ACR and Hb-ACR adducts in serum and milk

On HPLC analysis, free ACR in serum was detected in neither dams nor their offspring at each dose. In addition, free ACR was not detected in the stomach milk of offspring in the 100 ppm group. The data for ACR-Hb adducts analyzed

Table 1 Body weights of dams and offspring at PND 21

	ACR in the drinking water (ppm)				ACR 50 mg/kg per day, i.p.
	0	25	50	100	
Dams					
No. of animals	4	4	4	4	2
(g)	316.3 ± 18.6[a]	323.9 ± 21.2	315.9 ± 26.2	291.0 ± 18.3	317.5
Offspring					
Males					
No. of animals	3	4	8	5	4
(g)	57.6 ± 4.9	58.2 ± 9.1	52.7 ± 7.3	33.3 ± 2.6**	31.3 ± 9.0**
Females					
No. of animals	7	6	6	3	5
(g)	58.8 ± 3.9	49.6 ± 18.3	53.5 ± 7.2	31.9 ± 1.7*	36.8 ± 4.6*

ACR acrylamide, *PND* postnatal day

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

[a] Mean ± SD

Table 2 Data from histopathological analysis of dams and offspring at PND 21

	ACR in the drinking water (ppm)				ACR 50 mg/kg per day, i.p.
	0	25	50	100	
Dams					
Number of animals examined	4	4	4	4	2
Trigeminal nerve					
Central chromatolysis of ganglion cells (+/++/+++)[a]	0	0	4 (3/1/0)	4 (0/3/1)	0
Offspring					
Males					
Number of animals examined	3	4	8	5	4
Cerebellum					
Retained external granule layer (+/++/+++)	3 (3/0/0)	4 (3/1/0)	8 (1/7/0)	5 (1/3/1)	4 (0/3/1)
Trigeminal nerve					
Central chromatolysis of ganglion cells (+/++/+++)	0	0	0	0	4 (3/1/0)
Testis					
Retardation of spermatogenesis (+/++/+++)	0	0	0	5 (3/2/0)	4 (2/2/0)
Females					
Number of animals examined	7	6	6	3	5
Cerebellum					
Retained external granule layer (+/++/+++)	7 (5/2/0)	6 (3/3/0)	6 (3/3/0)	3 (0/3/0)	5 (1/3/1)
Trigeminal nerve					
Central chromatolysis of ganglion cells (+/++/+++)	0	0	0	0	5 (4/1/0)

ACR acrylamide, *PND* postnatal day

[a] Grade of change: +, mild; ++, moderate; +++, severe

with GC/MS are summarized in Table 5. The levels in dams and offspring at 0 ppm were 0.19 ± 0.05 and 0.12 ± 0.05, respectively, consistent with a previous report (Bergmark et al. 1991). In dams, the level of ACR-Hb adducts was increased in a dose dependent manner and highly correlated with daily intake of ACR. The level of ACR-Hb adducts was also increased in relation to maternal dose in offspring. However, the level in offspring was one tenth or less than that in dams.

Discussion

As with our previous study (Takahashi et al. 2008), although ACR-induced neurotoxicity in maternal animals was evident at 100 ppm, as indicated by gait abnormalities and morphometric assessment, no changes suggestive of neurotoxicity or testicular toxicity were observed in their offspring except for lowered body weights. Since postnatal undernutrition is known to induce retained external granular cells in the cerebellum and retardation of spermatogenesis (Sima and Persson 1975; Jean-Faucher et al. 1982), these findings observed in the offspring exposed maternally were regarded as dependent on growth retardation due to decreased body weight, rather than being direct effects of ACR. On the other hand, offspring given ACR intraperitoneally exhibited obvious neurotoxicity similar to the adult cases in the present study. Therefore, it is recognized that preweaning rats have susceptibility to ACR-induced neurotoxicity, when they are administered ACR directly. In the intraperitoneally administered group, an increase of dot-like SYP-immunoreactive structures in the cerebellar molecular layer was not apparent, compared to dams given ACR in the drinking water. Although various factors such as differences in age, route of administration and the given dose are possible causes, we could not specify the reason for this difference. In the testes, retardation of spermatogenesis was observed in offspring exposed maternally and intraperitoneally, whereas no histopathological changes suggestive of testicular toxicity were obtained in either group. Since it has been demonstrated that round spermatids are vulnerable to ACR (Sakamoto et al. 1988), testes prior to spermatogenesis might be less sensitive.

Free ACR was not detected in the serum of dams and their offspring up to 100 ppm in the present study. Free ACR is known to be very short-lived in blood, but forms a stable reaction product with hemoglobin (ACR-Hb adducts), which can be used as a valuable marker of ACR exposure (Bergmark et al. 1991; Bergmark et al. 1993; Sumner et al. 2003). It has been reported that ACR administration in the drinking water to rats (1 mg/kg per day) results in a steady state level of serum ACR (average concentration:

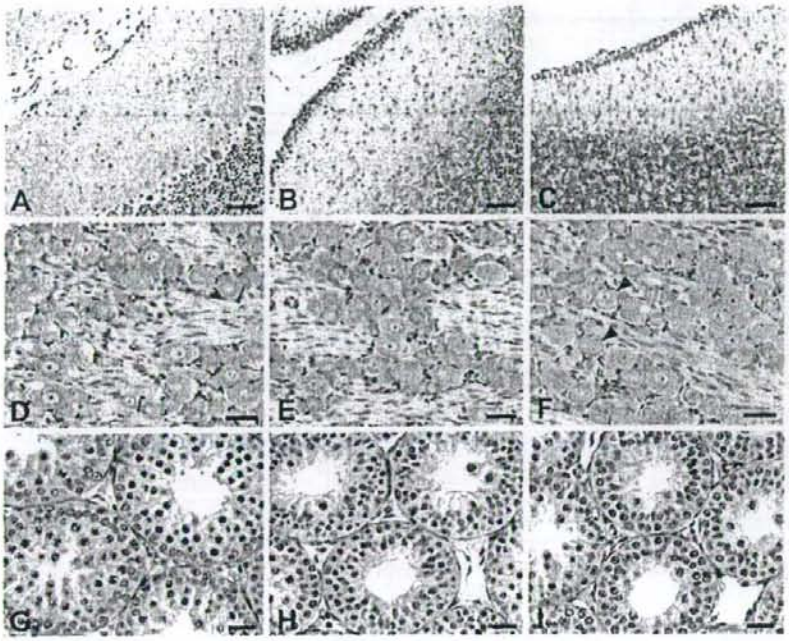

Fig. 1 Histopathological changes in the cerebellum (**a–c**), trigeminal nerve ganglia (**d–f**) and testis (**g–i**) of male offspring at PND 21 that were exposed to ACR through maternal drinking water or by direct intraperitoneal injections. **a, d, g** 0 ppm ACR in the drinking water (untreated controls). **b, e, h** 100 ppm ACR in the drinking water. **c, f, i** 50 mg ACR/kg per day by intraperitoneal injections. Sparse distribution of the external granular cells retained in the outermost layer of the cerebellar cortex of an untreated control (**a**). In contrast, increase of retained external granular cells is apparent by exposure to ACR maternally or intraperitoneally (**b, c**). No distinct alterations in the trigeminal nerve ganglia by exposure to ACR maternally (**e**), similarly to the case of untreated controls (**d**). Central chromatolysis of ganglion cells (*arrowheads*) is evident by direct intraperitoneal ACR injections (**f**). The seminiferous tubules showing 3–4 layers of developing spermatocytes in a male offspring of untreated controls (**g**). Retardation of spermatogenesis as manifested by a few spermatocytes developed inside of the 2–3 layers of gonocytes in a tubule by exposure to ACR maternally or intraperitoneally (**h, i**). HE stain, *Bars* = 50 μm (**a–c**), 20 μm (**d–i**)

Table 3 Data from morphometry of lesions developing in the nervous tissues of dams

	ACR in the drinking water (ppm)	
	0	100
No. of animals examined	4	4
Sciatic nerve (distal portion)		
Density (/100 μm^2)	1.82 ± 0.04[a]	1.97 ± 0.12
Degenerated axons (%)	0.39 ± 0.09	1.93 ± 0.64*
Myelinated axons, <3 μm in diameter (%)	10.41 ± 1.14	13.03 ± 1.32*
Cerebellar cortex, molecular layer		
SYP-immunoreactive aberrant dots (/mm cortex)	0.38 ± 1.50	1.50 ± 0.56*

ACR acrylamide, *SYP* synaptophysin

* $P < 0.05$, ** $P < 0.01$ vs. 0 ppm group (Student's *t*-test)

[a] Mean ± SD

approximately 0.6 μM) (Doerge et al. 2005). Since the detection limit of analysis method used in the present study is relatively high, it is very possible that a certain level of free ACR might have been contained in the serum. The same can be postulated for the milk. However, since the concentration of ACR in the milk was apparently much lower than the concentration of ACR in the maternal drinking water, it can be estimated that ACR intake of offspring from milk was very low.

Acrylamide-Hb adducts were detected even in the 0 ppm group, similar to a previous report (Bergmark et al. 1991). It has been reported that a measurable amount of Hb-ACR adducts is observed in the untreated animals due to a low but measurable content of ACR in commercial rodent diets (Twaddle et al. 2004; Tareke et al. 2006). In the present study, the level of ACR-Hb adducts in dams increased depending on the dose, and was highly correlated with daily intake level of ACR. In offspring, although the adduct level was also increased in relation with the maternal ACR

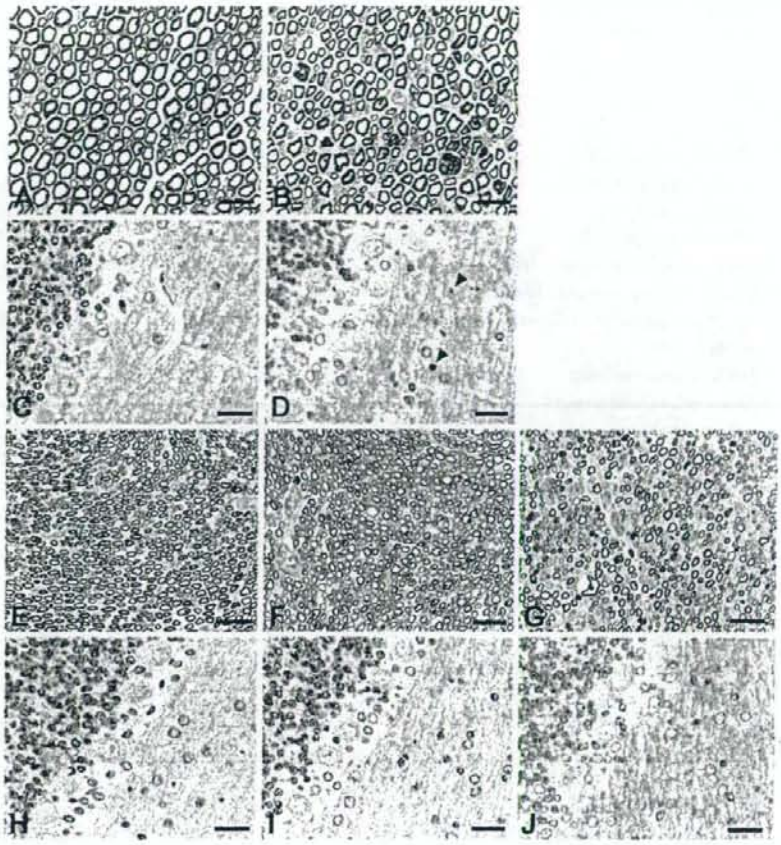

Fig. 2 Morphometrically assessed histopathological changes in the nervous system of dams (**a–d**) and male offspring (**e–j**). **a, c, e, h** 0 ppm ACR group (untreated controls). **b, d, f, i** 100 ppm ACR group. **g, j** male offspring given ACR at 50 mg/kg per day by intraperitoneal injections. Increases of degenerated axons and atrophic fibers in the sciatic nerve (**b**) and dot-like SYP-immunoreactive structures (*arrowheads*) in the cerebellar molecular layer (**d**) are evident in dams treated with 100 ppm ACR in the drinking water, while no such alterations are detected in untreated dams (**a, c**). In offspring exposed to ACR maternally, no distinct alterations are evident in the sciatic nerve and cerebellar SYP-immunoreactivity as with offspring of untreated controls (**e, f, h, i**). On the other hand, increase of degenerated axons and atrophic fibers are evident in the sciatic nerve of offspring given ACR intraperitoneally (**g**), while changes in cerebellar SYP-immunoreactivity are not detected in these cases (**j**). **a, b, e–g** Toluidine blue stain. **c, d, h–j** Immunohistochemical staining for SYP. All bars = 20 μm

dose, the level was only one tenth or less of that in dams. Since Hb adducts accumulate over the life span of erythrocytes and during chronic exposure, the adduct level reflects the average exposure/internal level during the last month (Hagmar et al. 2005). In addition, because the life-span of erythrocytes of foetuses and neonates is shorter than that of adults (Landaw and Guancial 1977; Linderkamp et al. 1986), it is considered that half-time for loss of ACR adducts in offspring is shorter than adult cases. Therefore, the adduct level in offspring would be lower than the adult cases, even when the internal level of exposure was equivalent for both. In the present study, the exposure period of offspring subjected to this assay was 1 week shorter than that for dams. However, the adduct level of offspring was considered to be too low to be caused by the difference in the exposure period and shorter life span of erythrocytes. It is considered that ACR intake of offspring from milk was very low, because free ACR in milk was under the detection limit, suggesting that insufficiency in lactational exposure to ACR may be the main reason for the low level of ACR adducts in offspring. These results suggest that the exposure level of offspring through maternal oral administration is low and insufficient to induce neurotoxicity, even allowing for the possibility that the offspring took ACR for themselves after PND 14. Hb-adducts detected in offspring might be derived not only from milk but also from transplacental exposure. No signs of neurotoxicity were earlier reported in offspring of dams gavaged with ACR at 25 mg/kg body weight/day throughout lactation, and it was suggested that there was little or no possibility of ACR exposure to offspring from the milk because of short half-life of free ACR (Friedman et al. 1999). In the present study, worsening of maternal general conditions due to neurotoxicity, rather than direct effects of ACR, might have greatly affected the body weights of offspring exposed to ACR maternally at 100 ppm. In contrast, difficulty in taking milk due to neurotoxicity might account for decreased body weight of offspring exposed to ACR intraperitoneally.

In summary, although preweaning rats have susceptibility to ACR-induced neurotoxicity, the internal level of ACR in

Table 4 Data from morphometry of lesions developing in the nervous tissues of offspring

	ACR in the drinking water (ppm)		ACR 50 mg/kg per day i.p.
	0	100	
Males			
No. of animals examined	3	5	4
Sciatic nerve (distal portion)			
Density (/100 μm^2)	4.35 ± 0.34[a]	4.21 ± 0.39	4.00 ± 0.27
Degenerated axons (%)	0.59 ± 0.53	0.44 ± 0.21	2.99 ± 0.63**
Myelinated axons, <3 μm in diameter (%)	28.45 ± 4.34	31.23 ± 4.17	48.97 ± 4.34**
Cerebellar cortex, molecular layer			
SYP-immunoreactive aberrant dots (/mm cortex)	0.31 ± 0.12	0.31 ± 0.08	0.50 ± 0.24
Females			
No. of animals examined	5	3	5
Sciatic nerve (distal portion)			
Density (/100 μm^2)	4.46 ± 0.19	4.32 ± 0.18	4.39 ± 0.50
Degenerated axons (%)	0.53 ± 0.27	0.33 ± 0.01	2.02 ± 0.22**
Myelinated axons, <3 μm in diameter (%)	30.19 ± 3.62	28.03 ± 2.72	44.24 ± 4.87**
Cerebellar cortex, molecular layer			
SYP-immunoreactive aberrant dots (/mm cortex)	0.23 ± 0.06	0.18 ± 0.06	0.26 ± 0.13

ACR acrylamide, *SYP* synaptophysin

* $P < 0.05$, ** $P < 0.01$ vs. 0 ppm group (Student's *t*-test)

[a] Mean ± SD

Table 5 Measurement of ACR-Hb adducts in dams and offspring after maternal exposure to ACR

	ACR in the drinking water (ppm)				R^d
	0	25	50	100	
Dams					
No. of animals examined	4	4	4	4	
ACR adducts/Hb (μmol/Hb g)	0.19 ± 0.05[b,c]	3.34 ± 0.16	4.68 ± 0.55	5.74 ± 1.15*	0.862
ACR concentration/RBC[a] (μmol/g)	0.04 ± 0.01	0.88 ± 0.09	1.18 ± 0.32*	1.15 ± 0.18*	0.798
Offspring					
No. of animals examined	5	6	7	8	
ACR adducts/Hb (μmol/Hb g)	0.12 ± 0.05	0.22 ± 0.08	0.28 ± 0.12*	0.34 ± 0.08**	0.669
ACR concentration/RBC[a] (μmol/g)	0.02 ± 0.01	0.06 ± 0.01	0.07 ± 0.03*	0.08 ± 0.04*	0.616

ACR acrylamide, *Hb* hemoglobin, *RBC* red blood cell

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

[a] Calculated as ACR monomer per 1 g red blood cell

[b] Mean ± SD

[c] Measurable level of Hb adduct was observed in untreated animals [0.15 (0.14–0.17) μmol/Hb g; Bergmark et al. 1991]

[d] Correlation coefficients between the mean daily intake of ACR by dams and the levels of ACR adducts or ACR concentration per red blood cells

offspring exposed maternally to the compound is insufficient to induce neurotoxicity due to limited lactational exposure. Regarding testicular toxicity, although there is a possibility that testes prior to spermatogenesis might be less sensitive, further studies will be required to evaluate susceptibility to ACR in immature testes in view of the dosage.

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