(Dybing *et al.*, 2005; Hartmann *et al.*, 2008; JECFA, 2005; Konings *et al.*, 2003). Neurotoxicity, genotoxicity and testicular toxicity have already been demonstrated in rats administered AA during young adult stages and a rat two-generation study (Tyl *et al.*, 2000a), as described above, but experimental data showing sensitivity or target organ/tissue differences in rats administered AA in fetal and/or infant stages are limited (Friedman *et al.*, 1999; Garey *et al.*, 2005; Takahashi *et al.*, 2008, 2009; Wise *et al.*, 1995).

In this study, to clarify the general toxicological profile of AA in the juvenile stage, male and female F344 rats were administered AA in their drinking water after birth for 12 weeks. The results were evaluated by referring to previously reported data in AA toxicological studies in young adult and adult rats, and suggested that juvenile rats are not more susceptible to AA-induced toxicity than young adults.

MATERIALS AND METHODS

Chemical and Antibody

AA (CAS no. 79-06-1, purity ≥ 99%) was purchased from Sigma-Aldrich (St Louis, MO, USA), and mouse monoclonal antibodies to proliferating cell nuclear antigen (PCNA; clone PC10) were purchased from DakoCytomation (Glostrup, Denmark).

Animal Treatments

For experiments 1 and 2, 12 and 10 specific pathogen-free pregnant F344 rats (F344/DuCrlCrlj), respectively, all synchronously mated at 10 weeks of age, were purchased from Charles River Laboratories Japan (Kanagawa, Japan). The animals were individually housed in clear polycarbonate cages with sterilized white wood chips (Sankyo Laboratory Service, Tokyo, Japan) for bedding in a standard air-conditioned room (24 ± 1 °C, 55 ± 5% relative humidity, 12 h light/dark cycle) and were given basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) and tap water *ad libitum* until parturition.

Experimental Protocol

Experiment 1

Three dams in four groups each were given free access to AA-containing drinking water at concentrations of 0 (control), 10, 20 and 40 ppm for the 3 weeks of lactation after parturition. Three days after birth, the F_1 litters were standardized by randomly selecting four males and four females per litter (in principle), to maximize the uniformity of growth rates of the offspring. The mean litter size at birth in the 0, 10, 20 and 40 ppm groups was 8.3, 8.6, 8.0 and 8.6, respectively. The sex ratio in the 0, 10, 20 and 40 ppm groups was 28, 54, 54 and 50%, respectively, for male, and 72, 46, 46 and 50%, respectively, for female. The number of offspring in the 0, 10, 20 and 40 ppm groups was 7, 12, 13 and 12, respectively, for male, and 17, 12, 11 and 12, respectively, for female. After weaning at 3 weeks of age, the dams were euthanized by exsanguination from the thoracic aorta under deep ether anesthesia, and the offspring were maintained on the same concentrations of AA in their drinking water as their dams for a further 9 weeks. The maximum dose level of AA for the present experiment was equivalent to those used in previous reports demonstrating that young adult rats receiving 5.0 mg kg^{-1} body weight per day AA for 13 weeks revealed slight peripheral nerve degeneration but not neurotoxicological symptoms (Burek *et al.*, 1980). Drinking water containing AA was replaced once a week.

During the experimental period, a check for clinical signs and mortality was performed at least once daily. Individual body weights and food and water consumption per cage were measured once a week. At the end of the experiment, all surviving animals were euthanized by exsanguination from the thoracic aorta under deep ether anesthesia, and subjected to a complete necropsy with the major organs and tissues examined macroscopically. Brain, thymus, lungs, heart, spleen, liver, adrenals, kidneys and testes were excised and weighed. In addition to these organs, the nasal cavity, trachea, aorta, pituitary, thyroids, parathyroids, salivary glands, tongue, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, pancreas, urinary bladder, epididymides, prostate, seminal vesicles, bulbourethral glands, ovaries, uterus, vagina, mammary glands, skin, cervical and mesenteric lymph nodes, sternum and femur including bone marrow, trigeminal nerve, sciatic nerve (left), spinal cord (cervical, thoracic and lumber cord), eyes, Harderian glands, thigh muscle and gross abnormalities were excised. All these organs and tissues were fixed in 10% buffered formalin, except for the testes, which were fixed in Bouin's solution. The thyroids were weighed after fixation. All fixed samples were trimmed for embedding in paraffin and routinely processed to hematoxylin and eosin (HE) stained sections. For histopathological assessment of axons in the peripheral nerves, the sciatic nerves (right) were exposed 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. Dissected sciatic nerves were further fixed with the same 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, Berkshire, UK). Semithin sections, 1 μm in thickness, were stained with toluidine blue. Histopathological examination was performed on all organs and tissues of animals in the control and high-dose groups of both sexes. If a chemical treatment-related change appeared at the high dose, the relevant organ/tissue(s) from the lower dose groups was then also examined. In addition, thyroid sections of all groups, including the controls, were immunohistochemically stained for PCNA using anti-PCNA mouse monoclonal antibodies diluted at 1:100 and a streptavidin–biotin–peroxidase complex kit (StreptABComplex/HRP, DakoCytomation), with the chromogen 3,3′-diaminobenzidine followed by counterstaining with hematoxylin. The numbers of cells positive for PCNA were counted to determine percentage values with at least 1000 follicular epithelial cells in each thyroid section.

Experiment 2

To confirm whether the apparent increase in the incidence and severity of myocarditis in the high-dose males and decrease in the incidence of calcification in renal tubular epithelium in the high-dose females in experiment 1 were truly AA-specific reactions, the second experiment was conducted. Five dams each in two groups were given free access to AA-containing drinking water at concentrations of 0 (control) or 40 ppm (high dose) during lactation (3 weeks) and then, once weaned, the offspring were maintained on the same concentrations of AA in their drinking water as their dams for 9 weeks (12 week total). The mean litter size at birth in the 0 and 40 ppm groups was 9.6 and 9.0, respectively. The sex ratio in the 0 and 40 ppm groups was 48 and 47% male,

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respectively. Twenty offspring of both sexes were obtained in the 0 and 40 ppm groups each. At the end of the experiment, all animals were fasted overnight and euthanized by exsanguination with blood sample collection from the abdominal aorta under deep ether anesthesia for serum biochemistry assays performed at SRL (Tokyo, Japan) for aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), creatine kinase (CK), calcium (Ca) and inorganic phosphorus (IP) as markers of cardiac and/or renal toxicities.

All animals were subjected to complete necropsy, and the major organs/tissues were all examined macroscopically. The heart and kidneys (females only) were excised and weighed. These organs were fixed in 10% buffered formalin, and fixed samples were trimmed for embedding in paraffin and routinely processed to HE-stained sections for histopathological examination.

The experimental protocols were reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Statistics

The sample unit throughout the experiment, including the lactation period, was an individual pup/animal not a litter, since the principal purpose of the present study is to clarify the general toxicological profile in juvenile rats, and the treatment with AA was not performed on the F_0 dam during the gestation. Variance in values for body weights and serum biochemistry, organ weights and PCNA-positivity of thyroids was checked for homogeneity using Bartlett's procedure. When the data were homogeneous, one-way analysis of variance (ANOVA) was used. In the heterogeneous cases, Kruskal–Wallis's test was applied. When statistically significant differences were indicated, Dunnett's multiple test was employed for comparison between control and treated groups. With histopathological changes, incidences were compared using Fisher's exact probability test and severity data were analyzed with Mann–Whitney's *U*-test.

RESULTS

Experiment 1

In-life parameters

One male animal in the 10 ppm group died on day 83. The cause of death was cachexia due to a leiomyosarcoma of the stomach, which could have been a spontaneous lesion. Therefore this case was excluded from the evaluation of organ weights and histopathology. No other obvious clinical abnormalities, including neurotoxicological symptoms, were found in any of the groups throughout the experimental period. A slight but statistically significant reduction of body weight was noted intermittently (3–9 weeks of experiment) in 20 and 40 ppm females as compared with the controls (Fig. 1). Food and water consumption was similar among the groups throughout the experimental period. The average daily intake of AA in the 10, 20 and 40 ppm groups was 1.0, 2.1 and 4.4 mg kg^{-1} body weight, respectively, for males, and 1.2, 2.5 and 4.9 mg kg^{-1} body weight for females, showing a good correlation with the expected doses.

Organ weights

Data for final body and organ weights are summarized in Tables 1 and 2. There were no significant differences in final body weight among the groups in both sexes. In males, no significant differences were observed in any organs among the groups. In females, a decrease was observed in absolute thyroid weights in the 10 ppm group, heart weights in the 20 and 40 ppm groups and brain weights in the 40 ppm group. In addition, the relative thyroid weights in the 10 ppm group were decreased, and the relative thyroid and spleen weights in the 40 ppm group were increased.

Necropsy/histopathology

There were no obvious macroscopic findings in either sex in any of the groups. The results of histopathological examination are summarized in Table 3. Increased incidences of focal degeneration and necrosis of the seminiferous epithelium in the testes and desquamated epithelial cells in the epididymal tubules were observed in 40 ppm males (Figs 2 and 3), along with a statistically significant increase in the incidence and severity of myocarditis in the heart (Fig. 4). In females, the incidence of calcification in the renal tubular epithelium was reduced with/without statistical significance in the treatment groups. In the brain, spinal cord and trigeminal and sciatic nerves, no treatment-related histopathological changes were observed in either sex in any of the groups. PCNA-labeling indices of thyroid follicular cells in the 0, 10, 20 and 40 ppm groups were 0.95 ± 0.95 (mean $\pm$ SD), 0.82 ± 0.50, 0.89 ± 0.42 and 0.51 ± 0.38%, respectively, in males and 0.16 ± 0.19, 0.29 ± 0.11, 0.33 ± 0.21 and 0.22 ± 0.10%, respectively, in females, showing statistical significance ($P < 0.05$) in the 20 ppm female group but lacking dose dependence.

Experiment 2

In-life parameters

One control male was excluded from the evaluation because of severe growth depression due to malocclusion of the incisors. No other obvious clinical abnormalities including neurotoxicological symptoms were observed throughout the experimental period. Lowered body weights were noted in both sexes of the 40 ppm group as compared with the controls (Fig. 5). Food and water consumption was similar among the groups throughout the experimental period. The average daily intake of AA in males and females was 5.0 and 5.5 mg kg^{-1} body weight, respectively, showing a slight increase as compared with the expected dose, most likely because of a slight body weight reduction in both sexes during this period.

Serum biochemistry

Serum biochemistry data are summarized in Table 4. There was no treatment-dependent variation in either sex.

Organ weights

Data for final body and organ weights are summarized in Table 5. Significant reduction of final body weight was observed in the 40 ppm group in both sexes. The absolute heart weights in males and the absolute heart and kidney weights in females were decreased in the 40 ppm group.

Necropsy/histopathology

There were no obvious macroscopic findings in either sex in either group. Histopathological findings are summarized in Table 6. Myocarditis in the heart in both sexes and calcification in the renal tubular epithelium in females were observed in both control and 40 ppm groups, but the incidences and severity of these lesions were comparable between the two groups.

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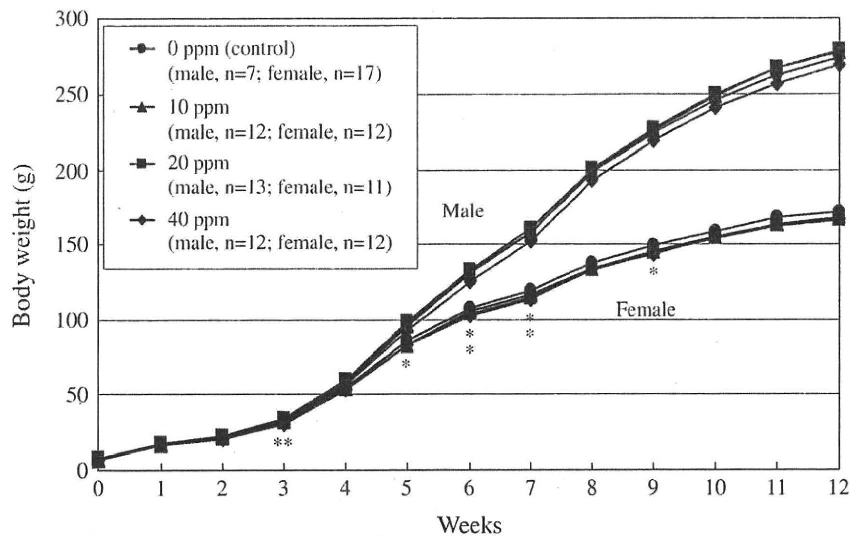

Figure 1. Experiment 1 – body weight curves for rats given drinking water containing acrylamide for 12 weeks. *, **Significantly different from the controls at $P < 0.05$ and 0.01, respectively.

Table 1. Experiment 1–Final body and organ weights of male rats administered water containing acrylamide for 12 weeks

	Dose level (ppm)			
	0 (control)	10	20	40
	No. of animals			
Items	7	11	13	12
Final body weights (g)	274.2 ± 10.0	276.3 ± 12.2	278.9 ± 10.7	268.4 ± 13.5
Absolute organ weights (g)				
Brain	1.91 ± 0.03	1.92 ± 0.01	1.93 ± 0.03	1.90 ± 0.04
Thyroids	0.016 ± 0.004	0.015 ± 0.002	0.014 ± 0.002	0.014 ± 0.004
Thymus	0.28 ± 0.02	0.27 ± 0.03	0.27 ± 0.03	0.27 ± 0.01
Lungs	0.96 ± 0.07	0.96 ± 0.08	1.01 ± 0.09	0.94 ± 0.07
Heart	0.80 ± 0.05	0.80 ± 0.04	0.83 ± 0.04	0.80 ± 0.04
Spleen	0.60 ± 0.20	0.60 ± 0.03	0.61 ± 0.03	0.60 ± 0.04
Liver	10.34 ± 0.40	10.20 ± 0.78	10.55 ± 0.94	10.26 ± 0.83
Adrenals	0.033 ± 0.005	0.032 ± 0.005	0.032 ± 0.004	0.031 ± 0.004
Kidneys	1.79 ± 0.06	1.84 ± 0.13	1.86 ± 0.12	1.82 ± 0.12
Testes	2.90 ± 0.07	2.87 ± 0.11	2.95 ± 0.06	2.83 ± 0.10
Relative organ weights (g 100 g^{-1} body weight)				
Brain	0.70 ± 0.03	0.70 ± 0.03	0.69 ± 0.02	0.71 ± 0.03
Thyroids	0.006 ± 0.001	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.001
Thymus	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.10 ± 0.01
Lungs	0.35 ± 0.02	0.35 ± 0.03	0.36 ± 0.02	0.35 ± 0.02
Heart	0.29 ± 0.01	0.29 ± 0.01	0.30 ± 0.01	0.30 ± 0.01
Spleen	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.00	0.23 ± 0.01
Liver	3.77 ± 0.16	3.68 ± 0.19	3.78 ± 0.28	3.82 ± 0.21
Adrenals	0.012 ± 0.002	0.012 ± 0.002	0.012 ± 0.001	0.011 ± 0.001
Kidneys	0.65 ± 0.02	0.66 ± 0.02	0.67 ± 0.03	0.68 ± 0.03
Testes	1.06 ± 0.04	1.04 ± 0.05	1.06 ± 0.03	1.06 ± 0.05

Data are mean ± SD values.

DISCUSSION

In the present study, toxicological findings related to AA administration in juvenile rats were limited to reduced body weight gain and histopathological abnormalities in the testes and epididymides. Reduction in body weight was observed intermittently in females given 20 and 40 ppm (2.5 and 4.9 mg kg^{-1} body weight per day, respectively) in experiment 1 (3–9 weeks of experiment) and males and females receiving 40 ppm (5.0 and 5.5 mg kg^{-1} body weight per day, respectively) in experiment 2 (1–12 weeks of experiment), without any change in their food consumption. These rats were treated with AA during the early

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Table 2. Experiment 1–Final body and organ weights of female rats administered water containing acrylamide for 12 weeks

	Dose level (ppm)			
	0 (control)	10	20	40
	No. of animals			
Item	17	12	11	12
Final body weights (g)	170.7 ± 8.1	168.9 ± 6.2	164.7 ± 5.1	166.3 ± 6.6
Absolute organ weights (g)				
Brain	1.78 ± 0.04	1.76 ± 0.02	1.78 ± 0.02	1.73 ± 0.02**
Thyroids	0.012 ± 0.002	0.010 ± 0.001*	0.011 ± 0.001	0.014 ± 0.002
Thymus	0.23 ± 0.01	0.23 ± 0.02	0.22 ± 0.02	0.22 ± 0.01
Lungs	0.73 ± 0.04	0.71 ± 0.05	0.73 ± 0.04	0.72 ± 0.06
Heart	0.56 ± 0.03	0.54 ± 0.02	0.53 ± 0.02*	0.53 ± 0.03*
Spleen	0.42 ± 0.03	0.40 ± 0.02	0.41 ± 0.02	0.42 ± 0.01
Liver	5.04 ± 0.32	5.19 ± 0.40	5.11 ± 0.15	4.99 ± 0.36
Adrenals	0.040 ± 0.007	0.037 ± 0.007	0.042 ± 0.005	0.035 ± 0.005
Kidneys	1.16 ± 0.08	1.13 ± 0.06	1.11 ± 0.07	1.11 ± 0.05
Relative organ weights (g 100 g^{-1} body weight)				
Brain	1.05 ± 0.05	1.04 ± 0.04	1.08 ± 0.02	1.04 ± 0.04
Thyroids	0.007 ± 0.001	0.006 ± 0.000*	0.007 ± 0.001	0.009 ± 0.001*
Thymus	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.13 ± 0.01
Lungs	0.43 ± 0.02	0.42 ± 0.03	0.44 ± 0.02	0.43 ± 0.03
Heart	0.33 ± 0.02	0.32 ± 0.01	0.32 ± 0.01	0.32 ± 0.01
Spleen	0.244 ± 0.012	0.238 ± 0.011	0.247 ± 0.011	0.254 ± 0.008*
Liver	2.96 ± 0.15	3.07 ± 0.19	3.10 ± 0.09	3.00 ± 0.14
Adrenals	0.024 ± 0.004	0.022 ± 0.004	0.025 ± 0.003	0.021 ± 0.003
Kidneys	0.68 ± 0.03	0.67 ± 0.04	0.67 ± 0.03	0.67 ± 0.03

Data are mean ± SD values.
*,**Significantly different from the control values at $P < 0.05$ and 0.01, respectively.

stage of life including lactational and prepubertal stages and were evaluated by referring to previously reported data on AA toxicology in young adult and adult rats. In a previous two-generation reproduction study, a decrease in body weight was observed in F_0 male and female F344 rats exposed to AA in their drinking water during their pre-breeding young adult and adult stages for 10 weeks at doses of 0.5, 2.0 and 5.0 mg kg^{-1} per day (Tyl *et al.*, 2000a). Therefore, reduction in body weight might be a typical toxicological change with AA, but juvenile rats cannot be considered more sensitive as compared with young adults. Because the lactational transfer of AA to Sprague–Dawley rat offspring upon maternal oral administration was limited (Takahashi *et al.*, 2009), maternal inanition resulting in a lactation and/or a nursing disorder, as reported previously (Friedman *et al.*, 1999), may be one of the causes of reduced body weight gain in this experiment.

Focal degeneration and necrosis of the seminiferous epithelium in the testes and desquamated epithelium in the ducts of epididymides were observed in juvenile males given 40 ppm (4.4 mg kg^{-1} body weight per day) of AA. Exposure to 5 mg kg^{-1} body weight per day of AA in young adult male F344 rats in their drinking water for 13 weeks, from 6 to 19 weeks of age, has been reported to induce seminiferous tubule atrophy (Burek *et al.*, 1980). Thus, it is possible that the testes in juvenile rats are more susceptible to AA-induced toxicity than those in young adult and adult rats but, if so, this is only marginal.

Previously, histopathological alterations such as demyelinated and/or degenerated axons in the sciatic nerves were reported in male and female rats in a 13-week study with administration of AA at a dose of 5.0 mg kg^{-1} body weight per day started in the young adult stage (Burek *et al.*, 1980) and a neurological symptom, leg splay, was detected in F_0 female rats given 5 mg kg^{-1} per day in a two-generation reproduction study (Tyl *et al.*, 2000a). However, in the present study, no histopathological changes in the sciatic nerves or neurological symptoms were detected in either sex of the 40 ppm group (males, 4.4 mg kg^{-1} body weight per day; females, 4.9 mg kg^{-1} body weight per day). Therefore, juvenile female rats might be more resistant to neurotoxicity caused by AA exposure than young adults.

The thyroid has been considered one of the targets of AA carcinogenicity based on long-term rat studies, in which AA was administered in drinking water at doses up to 2.0 or 3.0 mg kg^{-1} body weight per day (Friedman *et al.*, 1995; Johnson *et al.*, 1986). In the previous literature, controversial results were reported in young adult rats. In one study, AA was administered to 8-week-old male F344 rats at doses of 2.0 and 15 mg kg^{-1} body weight per day for 7, 14 and 28 days, and an increase in DNA synthesis analyzed by BrdU-labeling of thyroid follicular cells was observed (Lafferty *et al.*, 2004). Another study examined possible evidence for disruption of the hypothalamic–pituitary–thyroid axis. Male F344 rats (from 21 to 70 days of age) were treated with AA in drinking water at doses of 2.5, 10 and 50 mg kg^{-1} body weight per day for 14 days, followed by evaluation of serum levels of thyroid and pituitary hormones; target tissue expression of genes involved in hormone synthesis, release and receptors; neurotransmitters in the CNS that affect hormone homeostasis; and histopathological changes in target tissues. The results showed no evidence for systematic alteration of the hypothalamic–pituitary–thyroid axis; Ki-67 gene expression level, a marker of cell proliferation, was

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Table 3. Experiment 1 – Histopathological findings in rats administered water containing acrylamide for 12 weeks

Sex	Organ	Findings	Grade[a]	Dose level (ppm) 0	10	20	40
Male	No. of animals			7	11	13	12
	Heart						
		Myocarditis, focal and/or zonal	+	2 (29%)	4 (36%)	6 (46%)	5 (42%)*
			++	0	2 (18%)	1 (8%)	4 (33%)*
	Pituitary						
		Cyst, pars distalis	+	0	0	0	2 (17%)
		Cyst, pars intermedia	+	0	1 (9%)	0	1 (8%)
	Testis						
		Tubular atrophy, partial	+++	0	1 (9%)	0	0
		Degeneration and necrosis of seminiferous epithelium, focal	+	0	1 (9%)	0	8 (66%)**
	Epididymis						
		Desquamated seminiferous epithelial cells	+	0	0	0	8 (66%)**
	Prostate						
		Mononuclear cell infiltration, focal	+	1 (14%)	2 (18%)	3 (23%)	3 (25%)
			++	1 (14%)	0	1 (8%)	0
		Atrophy, focal	+	0	1 (9%)	1 (8%)	1 (8%)
Female	No. of animals			17	12	11	12
	Kidney						
		Calcification, renal tubular epithelium	+	16 (94%)	7 (58%)*	7 (64%)	6 (50%)*
	Heart						
		Myocarditis, focal	+	2 (12%)	2 (17%)	1 (9%)	4 (33%)
	Submandibular gland						
		Basophilic focus	+	1 (6%)	0	0	0
	Pituitary						
		Cyst, pars distalis	+	1 (6%)	1 (8%)	0	0

[a] +, Slight; ++, moderate; +++, severe.
*,** Significantly different from the control values at $P < 0.05$ and 0.01, respectively.

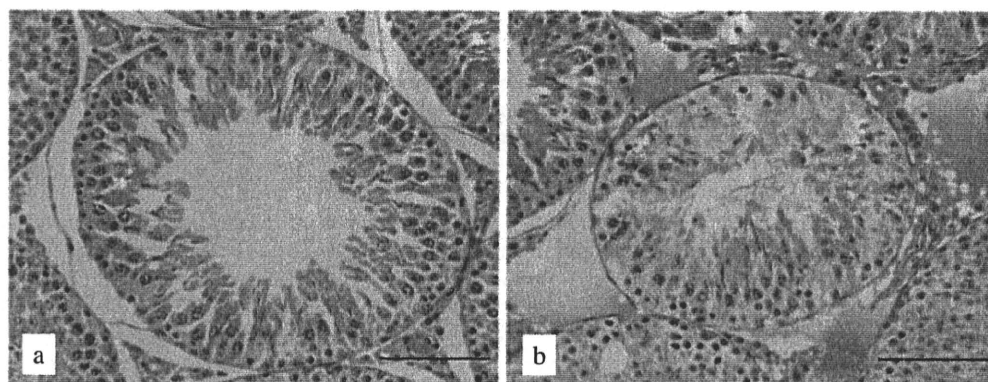

Figure 2. Experiment 1 – testis. (a) Control, a male. The seminiferous tubules of the testis are normal. The tubule in the central zone is in stage XIII. (b) The 40 ppm group, a male. Note the degeneration and necrosis of seminiferous tubules. The tubule in the central zone is in stages XII–XIII. Bar = 100 µm. HE staining.

not changed, and immunohistochemical Ki-67-positivity was decreased in the pituitary and thyroid by AA treatment (Bowyer *et al.*, 2008). In the present investigation, giving 4.4 mg kg^{-1} body weight per day of AA in male F344 rats for 12 weeks produced no obvious effects of AA on histopathology and a cell proliferative marker, PCNA-positivity, in thyroid follicular epithelial cells. Thus, juvenile rats may not be more susceptible to thyroid carcinogenesis after AA exposure.

An increase in the incidence and severity of myocarditis in 40 ppm males (9/12, 75%, vs 2/7, 29%, in the control) and a decrease in the incidence of calcification in the renal tubular epithelium in the treatment females (6/12, 50%, vs 16/17, 94%,

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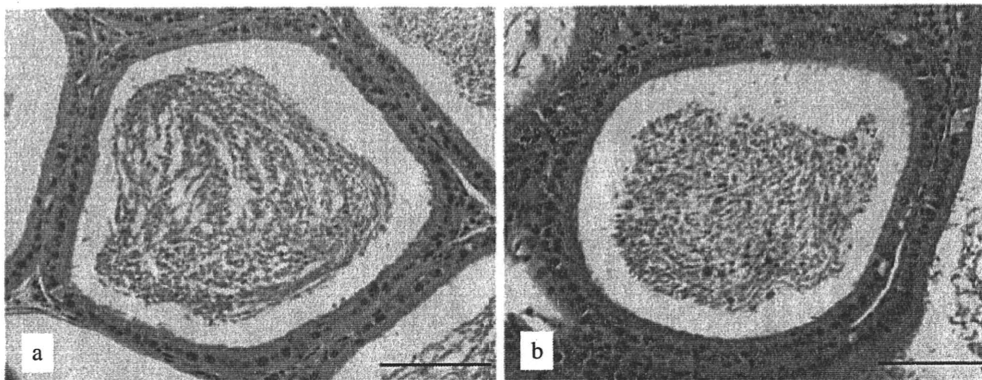

Figure 3. Experiment 1 – epididymis. (a) Control, a male. The epididymal ducts of the epididymis are normal. (b) The 40 ppm group, a male. Note desquamated epithelial cells in the epididymal ducts of the epididymis. Bar = 100 μm. HE staining.

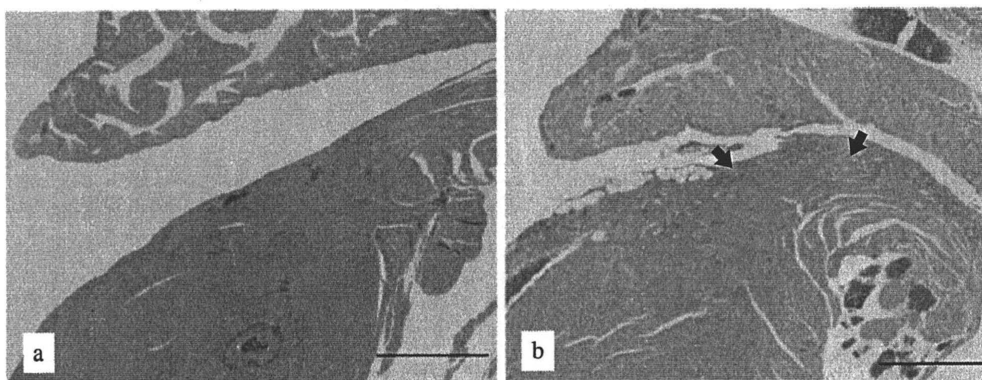

Figure 4. Experiment 1 – heart. (a) Control, a male. The side of the right ventricle of the heart is normal. (b) The 40 ppm group, a male. Note moderate infiltration of lymphocytes and macrophages with degeneration of myofibers (arrows) in the heart. Bar = 500 μm. HE staining.

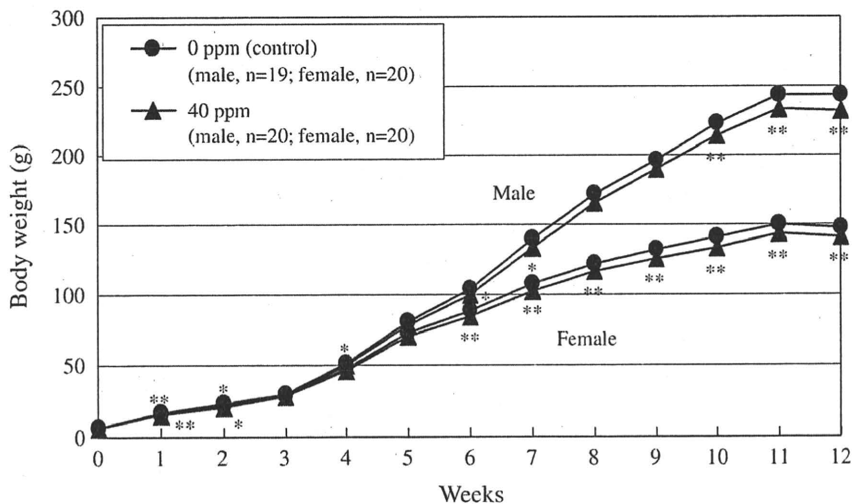

Figure 5. Experiment 2 – body weight curves for rats given drinking water containing acrylamide for 12 weeks. *, **Significantly different from the controls at $P < 0.05$, 0.01, respectively.

in the control) observed in experiment 1 were concluded to be incidental and not toxicologically significant for the following reasons. The incidence rates of myocarditis in control males and calcification in the kidneys in control females in this study may be low and high, respectively, compared with another study using male and female F344 control rats at our institute, in which the incidence of myocarditis in males was 40% and calcification in the kidneys in females was 10% (Takami *et al.*, 2008). Moreover, in a confirmatory study of experiment 2, there were no significant differences in the incidence and

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Table 4. Experiment 2–Serum biochemistry data for rats administered water containing acrylamide for 12 weeks

Sex	Item	Dose level(ppm)	
		0	40
Male	No. of animals	19	20
	AST (IU L^{-1})	91.0 ± 8.8	93.3 ± 6.7
	ALT (IU L^{-1})	38.8 ± 3.3	39.0 ± 2.9
	CK (U L^{-1})	676 ± 90	684 ± 127
	LDH (IU L^{-1})	1142 ± 170	1218 ± 262
Female	No. of animals	20	20
	AST (IU L^{-1})	80.7 ± 6.8	81.3 ± 5.4
	ALT (IU L^{-1})	37.1 ± 2.9	38.2 ± 3.9
	CK (U L^{-1})	486 ± 134	446 ± 108
	LDH (IU L^{-1})	775 ± 247	754 ± 207
	Ca (mg dL^{-1})	10.0 ± 0.5	9.9 ± 0.3
	IP (mg dL^{-1})	7.4 ± 0.9	7.1 ± 0.8

Data are mean ± SD values.

Table 5. Experiment 2–Final body and organ weights of rats administered water containing acrylamide for 12 weeks

Sex	Item	Dose level (ppm)	
		0	40
Male	No. of animals	19	20
	Final body weight	244.2 ± 8.9	232.5 ± 8.7**
	Absolute heart weight (g)	0.80 ± 0.05	0.76 ± 0.04**
	Relative heart weight (g 100 g^{-1} body weight)	0.33 ± 0.01	0.33 ± 0.01
Female	No. of animals	20	20
	Final body weight	147.7 ± 6.3	140.6 ± 5.5**
	Absolute organ weight (g)		
	Heart	0.55 ± 0.04	0.51 ± 0.03**
	Kidneys	0.95 ± 0.04	0.91 ± 0.05**
	Relative organ weight (g 100 g^{-1} body weight)		
	Heart	0.37 ± 0.02	0.37 ± 0.01
	Kidneys	0.65 ± 0.02	0.65 ± 0.02

Data are mean ± SD values.
**Significantly different from the control values at $P < 0.01$.

Table 6. Experiment 2–Histopathological findings in rats administered water containing acrylamide for 12 weeks

Sex	Organ	Findings	Grade[a]	Dose level (ppm)	
				0	40
Male	No. of animals			19	20
	Heart				
		Myocarditis, focal and/or zonal	+	10 (53%)	9 (45%)
			++	3 (16%)	3 (15%)
Female	No. of animals			20	20
	Heart				
		Myocarditis, focal and/or zonal	+	3 (15%)	4 (20%)
			++	0	1 (5%)
	Kidney				
		Calcification, renal tubular epithelium	+	11 (55%)	9 (45%)

[a]+, Slight; ++, moderate.

severity of myocarditis and serum AST, ALT, CK and LDH levels between 40 ppm and control males, nor in the incidence of calcification in the kidneys and serum Ca and IP levels between 40 ppm and control females. Other findings for organ weights in experiments 1 and 2 were without any toxicological significance or dose relationship.

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In conclusion, the present toxicity study of administered AA after birth for 12 weeks to juvenile F344 rats showed reduced body weights at 40 ppm in males and at 20 and 40 ppm in females. Histopathologically, focal degeneration and necrosis of seminiferous epithelium in the testes and desquamated epithelial cells in the epididymal tubules in 40 ppm males were observed; however, no significant lesions in other organs including the sciatic nerves were apparent. The results thus suggest that juvenile rats should not be considered more susceptible to AA-induced general toxicity, including neuro- and testicular toxicity, than young adult rats.

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Research Article

Genotoxicity of Acrylamide In Vitro: Acrylamide Is Not Metabolically Activated in Standard In Vitro Systems

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The recent finding that acrylamide (AA), a genotoxic rodent carcinogen, is formed during the frying or baking of a variety of foods raises human health concerns. AA is known to be metabolized by cytochrome P450 2E1 (CYP2E1) to glycidamide (GA), which is responsible for AA's in vivo genotoxicity and probable carcinogenicity. In in-vitro mammalian cell tests, however, AA genotoxicity is not enhanced by rat liver S9 or a human liver microsomal fraction. In an attempt to demonstrate the in vitro expression of AA genotoxicity, we employed *Salmonella* strains and human cell lines that overexpress human CYP2E1. In the *umu* test, however, AA was not genotoxic in the CYP2E1-expressing *Salmonella* strain or its parental strain. Moreover, a transgenic human lymphoblastoid cell line overexpressing CYP2E1 (h2E1v2) and its parental cell line (AHH-1) both showed equally weak cytotoxic and genotoxic responses to high (>1 mM) AA concentrations. The DNA adduct N7-GA-Gua, which is detected in liver following AA treatment in vivo, was not substantially formed in the in vitro system. These results indicate that AA was not metabolically activated to GA in vitro. Thus, AA is not relevantly genotoxic in vitro, although its in vivo genotoxicity was clearly demonstrated. Environ. Mol. Mutagen. 52:12–19, 2011. © 2010 Wiley-Liss, Inc.

Key words: acrylamide; glycydamide; cytochrome P450 2E1 (CYP2E1), in vitro tests; *Salmonella*

INTRODUCTION

Recently, low levels of acrylamide (AA), a synthetic chemical widely used in industry, were detected in a variety of cooked foods [Tareke et al., 2000; Mottram et al., 2002]. It has been proposed that AA forms during frying and baking principally by the Maillard reaction between asparagine residues and glucose [Stadler et al., 2002; Tornqvist, 2005]. This finding raised concerns about a health risk for the general population [Tareke et al., 2002; Rice, 2005].

The International Agency for Research on Cancer classifies AA as 2A, a probable human carcinogen [IARC, 1994]. Because AA clearly induces gene mutations and micronuclei in mice, it could be a genotoxic carcinogen [Cao et al., 1993; Abramsson-Zetterberg, 2003; Manjanatha et al., 2005]. AA is metabolized by cytochrome P450 2E1 (CYP2E1) to glycidamide (GA), which can react with cellular DNA and protein [Sumner et al., 1999; Ghanayem et al., 2005a; Rice, 2005]. Two major

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GA-DNA adducts, N7-(2-carbamoyl-2-hydroxyethyl)-guanine (N7-GA-Gua) and N3-(2-carbamoyl-2-hydroxyethyl)-adenine (N3-GA-Ade), have been identified in mice and rats treated with AA or GA [Segerback et al., 1995; Gamboa da Costa et al., 2003; Doerge et al., 2005], with the level of N7-GA-Gua being 100 times as high as the level of N3-GA-Ade in the test organ [Gamboa da Costa et al., 2003]. It is likely that these DNA adducts are responsible for AA's in vivo genotoxicity [Carere, 2006; Ghanayem and Hoffler, 2007]. In our previous study, however, AA did not induce micronuclei in human lymphoblastoid TK6 cells in the presence of rat liver S9, although the genotoxicity of *N*-di-*N*-butylnitrosamine (DBN), which is also metabolized by CYP2E1, was enhanced under the same conditions [Koyama et al., 2006]. Other in vitro genotoxicity studies have also failed to demonstrate the metabolic activation of AA in the presence of S9 [Knaap et al., 1988; Tsuda et al., 1993; Dearfield et al., 1995; Friedman, 2003]. It may be because most S9 preparations have low CYP2E1 activity [Calleman et al., 1990; Hargreaves et al., 1994].

In an attempt to demonstrate the genotoxicity of AA in vitro, we tested the compound using bacteria and mammalian cell lines that express CYP2E1. *S. typhimurium* OY1002/2E1 strain expresses respective human CYP2E1 enzyme and NADPH-cytochrome P450 reductase (reductase), and bacterial *O*-acetyltransferase [Oda et al., 2001]. Using the strain, as well as its parental strain not expressing these enzymes, we conducted an *umu* assay to evaluate induction of cytotoxicity and DNA damage by AA relative to that induced by its metabolite GA. The principle of the *umu* assay is based on the ability of the DNA-damaging agents inducing the *umu* operon. Monitoring the levels of *umu* operon expression enables us to quantitatively detect environmental mutagens [Oda et al., 1985]. In addition, we evaluated the relative mutagenicity of AA vs. GA in assays using transgenic human lymphoblastoid cell lines. Induction of gene mutation at the *TK* locus and of chromosome damage leading to micronucleus (MN) formation were assessed in the h2E1v2 which overexpress human CYP2E1 [Crespi et al., 1993a], vs. its parental cell line, AHH-1. We also investigated the relationship between AA genotoxicity and the formation N7-GA-Gua (derived from GA) in the in vitro mammalian cell system.

MATERIALS AND METHODS

Bacterial Strains, Cell Lines, Chemicals, and Human Liver Microsomal Fraction

For the bacterial tests, we used *umu* strain *S. typhimurium* OY1002/2E1, which expresses human CYP2E1, reductase, and bacterial *O*-acetyltransferase, and its parental strain, *S. typhimurium* TA1535/pSK1002 that does not express these enzymes [Oda et al., 2001].

For the mammalian cell tests, we used human lymphoblastoid cell lines, TK6, AHH-1, and h2E1v2. The TK6 cell line has been described previously [Honma et al., 1997]. The AHH-1 and h2E1v2 cell lines were kindly gifted from Dr. Charles Crespi (BD Bio Sciences, Bedford, MA). AHH-1 is a clonal isolate, derived from RPMI 1788 cells, which was selected for sensitivity to benzo[*a*]pyrene [Crespi and Thilly, 1984]. AHH-1 shows high activity of endogenous CYP1A1. Heterozygosity of AHH-1 cells at thymidine kinase (TK) locus was derived in a two-step selection process utilizing the frameshift mutagen, ICR-191. The AHH-1 cell line was then transfected with plasmids encoding human CYP2E1 enzymes, generating h2E1v2 cell line. AHH-1 expresses CYP1A1 and h2E1v2 expresses both CYP1A1 and CYP2E1 [Crespi et al., 1993a,b].

We purchased AA (CAS No. 79-06-1) and GA (CAS No. 5694-00-8) from Wako Pure Chemical (Tokyo) and dissolved them in phosphate-buffered saline just before use. We purchased *N*-di-*N*-methylnitorosamine (DMN) (CAS No. 62-75-9) from Sigma Aldrich Japan (Tokyo) and dissolved it in DMSO as a positive control for use. We purchased liver S9 prepared from SD rats treated with phenobarbital and 5,6-benzoflavone from the Oriental Yeast (Tokyo). The human liver S9 (HLS-104) was prepared from a human liver sample, which was legally procured from the NDRI (National Disease Research Interchange) in Philadelphia, USA, with permission to use for research purpose only. HLS-104 showed high activity of CYP2E1 [Hakura et al., 2005]. We prepared microsomal fraction from the S9 according to an established procedure [Suzuki et al., 2000]. We prepared the S9- or microsome-mix by mixing 4 ml S9 or microsomal fraction with 2 ml each of 180 mg/ml glucose-6-phosphate, 25 mg/ml NADP, and 150 mM KCl. CYP2E1 activity of the S9 and microsomal fractions were determined as the activity of chlorzaxazone 6-hydroxylation according to the method of Ikeda et al. [2001].

We grew the cell lines in RPMI1640 medium (Gibco-BRL, Life Technology, Grand Island, NY) supplemented with 10% heat-inactivated horse serum (JRH Biosciences, Lenexa, KS), 200 μg/ml sodium pyruvate, 100 U/ml penicillin, and 100 μg/ml streptomycin, and we maintained the cultures at 10^5-10^6 cells/ml at 37°C in a 5% CO_2 atmosphere with 100% humidity.

umu Assay

The *umu* assay was carried out by the method of Aryal et al. [1999, 2000] with slight modification. Overnight cultures of tester strains were diluted 100-fold with TGlyT medium (1% Bactotryptone, 0.5% NaCl (w/v), 0.2% glycerol (v/v), and 1 μg of tetracycline/ml, 1.0 mM IPTG, 0.5 mMδ-ALA, and 250 ml of trace element mixture/l) [Sandhu et al., 1994]. The culture was incubated for 1 hr at 37°C and then 0.75 ml aliquots of TGA culture (OD_{600}: 0.25–0.3) and human. Induction of the *umu*C gene by HCAs in different strains was determined by measuring cellular β-galactosidase activity, as described by Oda et al. [1985]. Cell toxicity was determined in reaction mixture by measuring the optical density change at 600 nm.

Mammalian Cell Assays Measuring Gene Mutation and Chromosome Damage

We incubated 20-ml aliquots of TK6, AHH-1, or h2E1v2 cell suspensions (5.0×10^5 cells/ml) treated with serially diluted AA, GA, or DMN in the presence or absence of S9 or micorosomes at 37°C for 4 hr, washed them once, resuspended them in fresh medium, and cultured them in new flasks for the MN and TK assays. For TK6 cells, we also seeded cells into the 96-well plates (1.6 cells/well) to determine plating efficiency (PE0).

Forty-eight hours after treating the cells, we prepared the MN test samples as previously reported [Koyama et al., 2006]. At least, 1,000 intact interphase cells for each treatment were examined, and the cells containing MN were scored. The MN frequencies between nontreated and treated cells were statistically analyzed by Fisher's exact test. The concentration–response relationship was evaluated by the Cochran-Armitage trend test [Matsushima et al., 1999].

We maintained the cultures another 24 hr to allow phenotypic expression prior to plating for determination of the mutant fractions. After the expression time, to isolate the TK deficient mutants, we seeded the cells into 96-well plates in the presence of 3.0 μg/ml trifluorothymidine (TFT).