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.

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.

Acknowledgments

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Acrylamide genotoxicity in young versus adult *gpt* delta male rats

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The recent discovery that the potent carcinogen acrylamide (AA) is present in a variety of fried and baked foods raises health concerns, particularly for children, because AA is relatively high in child-favoured foods such as potato chips and French fries. To compare the susceptibility to AA-induced genotoxicity of young versus adult animals, we treated 3- and 11-week-old male gpt delta transgenic F344 rats with 0, 20, 40 or 80 p.p.m. AA via drinking water for 4 weeks and then examined genotoxicity in the bone marrow, liver and testis. We also analysed the level of N7-(2-carbamoyl-2-hydroxyethyl)-guanine (N7-GA-Gua), the major DNA adduct induced by AA, in the liver, testis and mammary gland. At 40 and 80 p.p.m., both age groups yield similar results in the comet assay in liver; but at 80 p.p.m., the bone marrow micronucleus frequency and the gpt-mutant frequency in testis increased significantly only in the young rats, and N7-GA-Gua adducts in the testis was significantly higher in the young rats. These results imply that young rats are more susceptible than adult rats to AA-induced testicular genotoxicity.

Introduction

Acrylamide (AA) is a low molecular weight vinyl compound commonly used in industries and laboratories. Because individuals are exposed to AA in the workplace, health concerns originally centred on occupational exposure (1). A recent study, however, reported that low levels of AA are formed in many heat-processed foods, especially starchy ones such as potato chips, crackers and French fries (2,3), as a result of asparagine reacting with sugars (Maillard reaction) (4,5). This finding raises concerns that AA poses health risks for the general population (6).

Many animal studies have demonstrated that AA induces neurotoxicity, testicular toxicity and reproductive toxicity (7–9). AA also causes cancers such as mammary fibroadenomas, thyroid follicular cell adenomas and testicular mesotheliomas in rats (10–12). In mice, it induces gene mutations in liver, micronuclei in haematopoietic cells (13,14) and chromosome aberrations in spermatids and spermatocytes (15,16). Thus, AA is clearly genotoxic *in vivo*, although its *in vitro* genotoxicity remains unclear because it is not metabolically activated in standard *in vitro* systems (17,18). AA is metabolised to glycidamide (GA), presumably by cytochrome P450 2E1 (CYP2E1), which quickly reacts with cellular DNA and protein (6,19,20). Two major 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 (21–23), with the level of N7-GA-Gua being 100 times as high as the level of N3-GA-Ade in the organs (22). Individual GA to AA ratios, which can be used as an indicator of the extent of AA metabolism, are highly variable, suggesting that some individuals or populations may be more susceptible than others to AA-induced genotoxicity (24). Other issues are AA intake and metabolism in children compared with adults. Children generally consume larger amounts of food relative to their body mass than adults and favour foods such as French fries and potato chips that have relatively high AA concentrations (25). These issues should be considered when evaluating the susceptibility of the paediatric population in genotoxic and carcinogenic risk assessments (26).

In the present study, to compare the susceptibility to AA-induced genotoxicity of young versus, adult age groups, we treated 3- and 11-week-old male *gpt* delta transgenic F344 rats with 0, 20, 40 or 80 p.p.m. of AA via drinking water for 4 weeks and examined genotoxicity in the bone marrow, liver and testis. We also analysed the level of N7-GA-Gua in the liver, testis and mammary gland.

Materials and methods

Animals, diet and housing

We purchased 20 male with 10-week-old and 15 pregnant female F344 *gpt* delta transgenic rats from Japan SLC (Shizuoka, Japan). The pregnant animals were time-mated at 10 weeks of age and arrived on gestational Day 12 or 13 to our facility. After delivery, we obtained >14 male pups from the pregnant rats. All animals were housed *three* to *five* rats in polycarbonate cage with sterilised wood chip bedding and maintained under specific pathogen-free standard laboratory conditions: room temperature, 24 ± 1°C; relative humidity, 55 ± 5%; 12-h light–dark cycle; basal diet (CRF-1; Oriental Yeast Company, Tokyo, Japan) and tap water *ad libitum* until parturition.

Treatments of animals

The protocol for this study was approved by the Animal Care and Utilisation Committee of the National Institute of Health Sciences. We randomly divided 14 and 20 of the 3- and 11-week-old rats into four groups of 3–5 animals, treated them for 4 weeks with AA (Wako Pure Chemical Co., Tokyo, Japan) at 0, 20, 40 or 80 p.p.m. in drinking water and monitored clinical signs, body weight and food and water consumption. At the end of the treatment period, we anaesthetized and killed the animals, and we excised organs for the *gpt* mutation assay (liver, testis), comet assay (liver), DNA adducts analysis (liver, testis, mammary gland and thyroid) and micronucleus (MN) test (bone marrow).

MN test

We removed bone marrow from the femur, mixed it with foetal calf serum, placed it on an acridine orange-coated glass slide, covered it with a coverslip and stained it supravitally (27). We analysed 2000 polychromatic erythrocytes per animal with a fluorescence microscope and recorded the number of micronucleated polychromatic erythrocytes, which fluoresced greenish yellow.

Alkaline comet assay

We performed the comet assay using the procedure recommended by the comet assay working group of the International Workshop on Genotoxicity Testing (IWGT) (28,29), except that we used a MAS-coat type slide glass (Matsunami Glass *Ind.* Ltd, Tokyo, Japan) instead of a conventional agarose bottom layer (30). We prepared cell suspensions from the livers, mixed them with 0.5% w/v low-melting agarose, and spotted an aliquot of the mixture onto the slide. After electrophoresis, we stained the cells with SYBR-Gold (cat. # S-11494; Molecular Probes, Invitrogen, Tokyo, Japan), and examined at least 100 cells per animal using a fluorescence microscope (BX50 and BX51; Olympus Corporation, Tokyo, Japan) connected to the comet assay scoring system (Comet IV; Perceptive Instruments Ltd, Suffolk, UK), which quantified the result as %tail intensity.

gpt mutation assay

We extracted high molecular weight genomic DNA from the liver and testis using a Recover Ease DNA Isolation Kit (Stratagene, La Jolla, CA, USA), rescued lambda EG10 phages using Transpack Packaging Extract (Stratagene) and conducted the *gpt* mutation assay as previously published (31). We calculated the *gpt*-mutant frequency (*gpt*-MF) by dividing the number of 6-thioguanine-resistant colonies by the number of colonies with rescued plasmids.

DNA adduct assay

As a standard for liquid chromatography tandem mass spectrometry analysis, *N*7-GA-Gua and [^{15}N$_5$]-labelled *N*7-GA-Gua were synthesised as described previously (18,22). We extracted DNA from the liver, testis, mammary gland and thyroid using a DNeasy 96 Blood & Tissue Kit (QIAGEN, Düsseldorf, Germany), incubated it at 37°C for 48 h for deprination. We added an aliquot of the labelled standard to each sample and filtered through an ultrafiltration membrane to remove DNA. The eluted solution was evaporated thoroughly and dissolved in water and then the solutions were subsequently quantified by a Quattro Ultima Pt triple stage quadrupole mass spectrometer (Waters-Micromass, Milford, MA, USA) equipped with a Shimadzu LC system (Shimadzu, Japan). We analysed the liver and testis for each individual rat but pooled the mammary and thyroid glands for each treatment group because the tissue yields were too small to be examined individually.

Statistical analysis

We used the Student's *t*-test to determine the statistical significance of the difference in the results of the *gpt* mutation assay and the DNA adduct assay between the treated and negative control groups and between the young and adult groups. We examined variances in body weight and results of the MN and comet assays by one-way analysis of variance using the Dunnett's test to compare the differences between the control and treated groups.

Results

Clinical signs, body weight and AA intake

We observed no clinical abnormality in either the young or adult rats during the 28-day treatment period. We found no significant differences in body weight or food and water consumption between the adult treatment groups, although we did observe a slight but statistically insignificant suppression of body weight in the young, 80-p.p.m. treatment group (Table I).

The table shows average daily food, water and AA intake of the young and adult treatment groups and their mean body weights. The average daily intakes of AA are calculated as 3.01, 5.95 and 12.19 mg/kg body weight for 20, 40 and 80 p.p.m. group, respectively, in young rats and as 1.83, 3.54 and 7.05 mg/kg body weight for 20, 40 and 80 p.p.m. group, respectively, in adult rats.

MN test

While no AA dose induced MN in adult rat bone marrow, the highest dose (80 p.p.m.) significantly increased the MN frequency in young rat bone marrow (Figure 1a). Because of the large standard deviation, however, the difference between young and adult rats was not significant (Figure 1a).

Alkaline comet assay

DNA damage induced by AA in liver was evaluated by the comet assay under alkaline conditions (Figure 1b). The comet tail intensities increased in a dose-dependent manner in both young and adult rats with no statistically significant differences between the two groups. AA significantly induced DNA damage at 40 and 80 p.p.m. in the adult rat liver and at 80 p.p.m. in the young rat liver.

gpt mutation assay

Figure 2 shows the *gpt* mutation assay results. The *gpt*-MF of control (0 p.p.m.) young and adult rat livers was 1.57 ± 0.72 ($\times 10^{-6}$) and 3.66 ± 2.14 ($\times 10^{-6}$), respectively. The control *gpt*-MF of the young rat liver was lower than that of the adult rat liver, but not significantly. AA did not increase the *gpt*-MF in the liver of either age group at any dose; but at 80 p.p.m., it approximately doubled the *gpt*-MF in the testis of both young and adult rats, but the increase in adult rats was not statistically significant.

DNA adduct formation

Figure 3 shows *N*7-GA-Gua DNA adduct levels in the liver, testis and mammary glands and thyroid of the young and adult rats. The adduct level increased in a dose-dependent manner in all the tissues. In the mammary glands and thyroid, adduct levels did not differ significantly between young and adult rats. In the liver and testis, on the other hand, the level was higher in the young rats than in the adult rats. In the testis, the DNA adduct level of young rats was approximately six times that of adult rats at all treatment doses.

Discussion

The *in vivo* genotoxicity of AA has been clearly demonstrated by various rodent genotoxicity tests including MN tests in peripheral blood (13,14,32) and gene mutation and comet assays in various organs (14,33,34). However, there has been no report for the comparison of genotoxicity between young and adult animals. In this study of the genotoxicity of AA in various organs of young (3-week-old) and adult (11-week-old) male rats, we showed that the testis were more vulnerable to AA genotoxicity in the young rat than in the adult rat. Especially, *N*7-GA-Gua DNA adduct was much higher accumulated in the testis of young rats than of adult rats (Figure 3). The daily intake of AA per weight in young rats was ~1.5-fold of the adult rats because the younger animals drank more water. It can explain the higher accumulation of adduct in the young rat liver, but the level in testis was

Table I. Body weight, food and water consumption and AA intake of young and adult rats

Group	AA dose (p.p.m.)	No. of animals	Initial body weight (g) mean ± SD	Final body weight (g) mean ± SD	Food consumption (mg/rat/day)	Water consumption (ml/rat/day)	Intake of AA (mg/kg/day)
Young	0	4	40.5 ± 2.7	168.9 ± 14.3	11.2	17.9	0
	20	3	37.1 ± 3.5	164.7 ± 17.8	11.3	16.9	3.01
	40	3	38.2 ± 2.1	165.1 ± 3.6	11.1	16.7	5.95
	80	4	40.6 ± 2.5	157.4 ± 8.2	11.0	16.9	12.19
Adult	0	5	249.8 ± 10.0	301.3 ± 11.5	16.4	25.8	0
	20	5	249.8 ± 8.2	299.9 ± 7.3	16.1	25.4	1.83
	40	5	250.5 ± 8.7	302.4 ± 12.2	16.2	24.8	3.54
	80	5	249.1 ± 7.7	306.6 ± 5.4	16.8	24.6	7.05
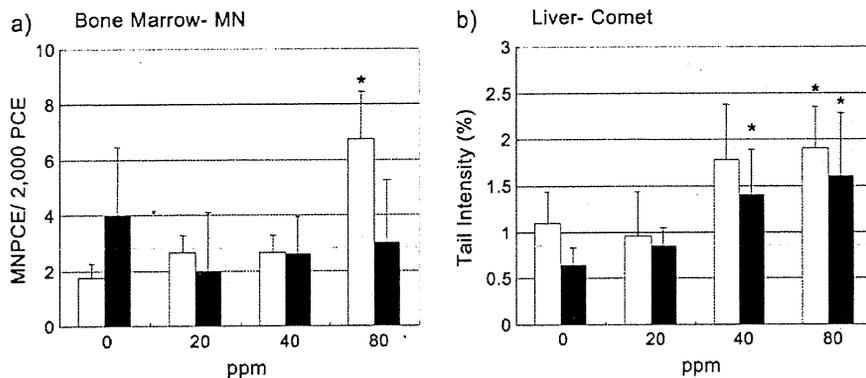
Fig. 1. (a) MN frequency in bone marrow of AA-treated young (open bars) and adult (closed bars) gpt delta rats. (b) Tail intensity (%) in the comet assay in liver of AA-treated young (open bars) and adult (closed bars) gpt delta rats. The values represent the mean of experiments ± standard deviations. *is statistically significant experiment compared with the untreated control ($P < 0.05$)discussion.
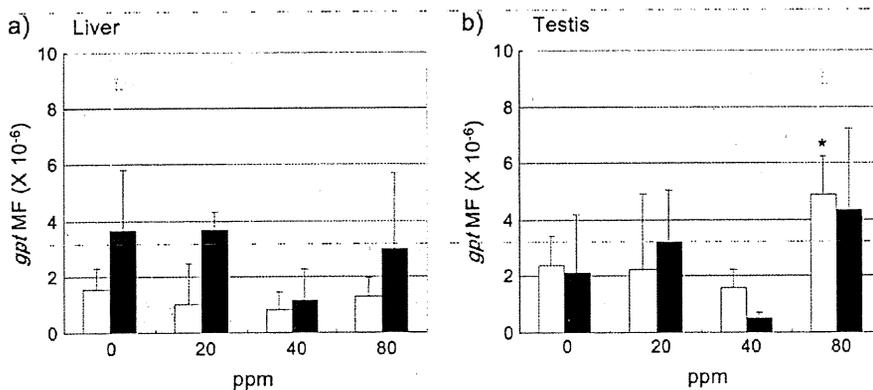
Fig. 2. gpt Mutation frequency in liver (a) and testis (b) of AA administered young (open bars) and adult (closed bars) gpt delta rats. The values represent the mean of experiments ± standard deviations. *is statistically significant experiment compared with the untreated control ($P < 0.05$).

approximately six times high in the young rats than in the adult rats, suggesting that AA metabolism in testis is different depending on animal age. Testis is one of the target organs of AA-induced genotoxicity (15,16,35–39). We believe that this is the first report of an age difference in the effect.

AA is primarily metabolised in animals via two competing pathways: oxidation by CYP2E1 to form GA (activation) and conjugation by glutathione S-transferase (GST) with reduced glutathione (detoxification) (19,40,41). GA may subsequently

undergo conjugation or hydrolysis catalysed by epoxide hydrolase. The balance between activation and detoxification probably determines AA genotoxicity *in vivo*. Rat testis shows CYP2E1 activity (42). Wang *et al.* (43) reported that the treatment of 1.4 and 7.0 mM of AA or GA via drinking water for 4 weeks induced the testicular *cII* mutation in Big Blue mice. The *cII* mutation spectra significantly differed between testis and liver, suggesting that testis may have different pathway to metabolise AA and GA. However, the
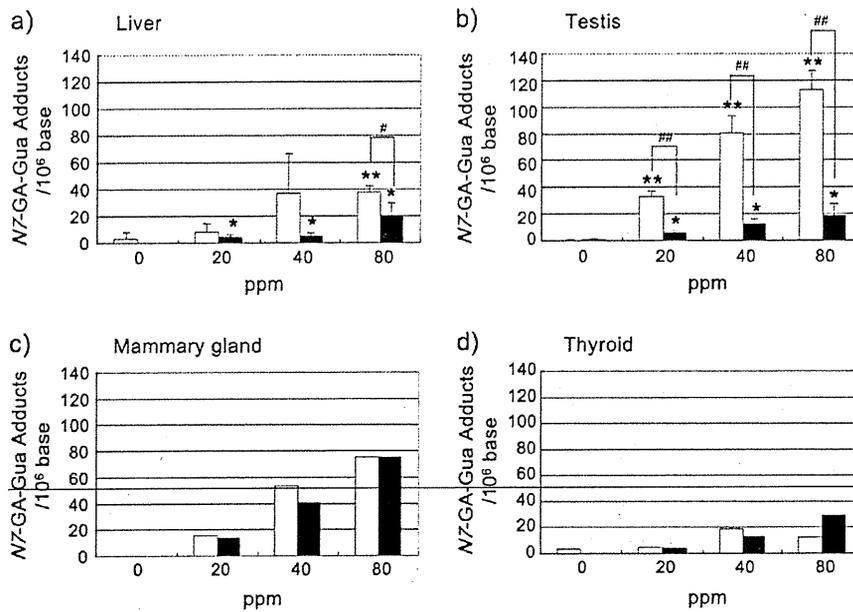
Fig. 3. Levels of *N*7-GA-Gua in the liver (a) testis (b) mammary gland (c) thyroid and (d) administered AA young (open bars) and adult (closed bars) *gpt* delta rat. The mammary gland and thyroid were pooled and analysed in the treatment group. Data are expressed as the number of adducts in 10^6 nucleotides. * and **are statistically significant experiment compared with the untreated control (*$P < 0.05$, **$P < 0.05$). # and ##are statistically significant experiment compared between young and adult *gpt* delta rat (#$P < 0.05$, ##$P < 0.05$).

developmental changes were not studied. Recently, Takahashi *et al.*. (44) showed that GST activity in the testis was significantly lower in young rats than in adult rats and that could explain the different age-related *N*7-GA-Gua adduct levels and *gpt*-MFs in the present study. The greater mutagenicity of aflatoxin B1 in liver of neonatal mice than of adult mice corresponds to liver GST levels (45). The GST level in the organs could be responsible for the expression of genotoxicity of AA and aflatoxin B1.

While the *N*7-GA-Gua adduct level in liver and testis clearly increased in a dose-dependent manner and significantly differed between young and adult rats, the *gpt* mutation results were not clear. We treated the rats with doses that were lower than those used in other studies (14,34,43), and these doses may have been insufficient to induce gene mutations in our study. Indication of the DNA adduct must be good biomarker to demonstrate genotoxic insult under low-dose exposure condition.

In conclusion, this finding that young rats were more susceptible than adult rats to AA-induced genotoxicity, especially in the testis, suggests that we should be concerned about the risk to children exposed to AA via ordinary foods.

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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 TGIyT 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 mM δ-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 umuC 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 microrosomes 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).

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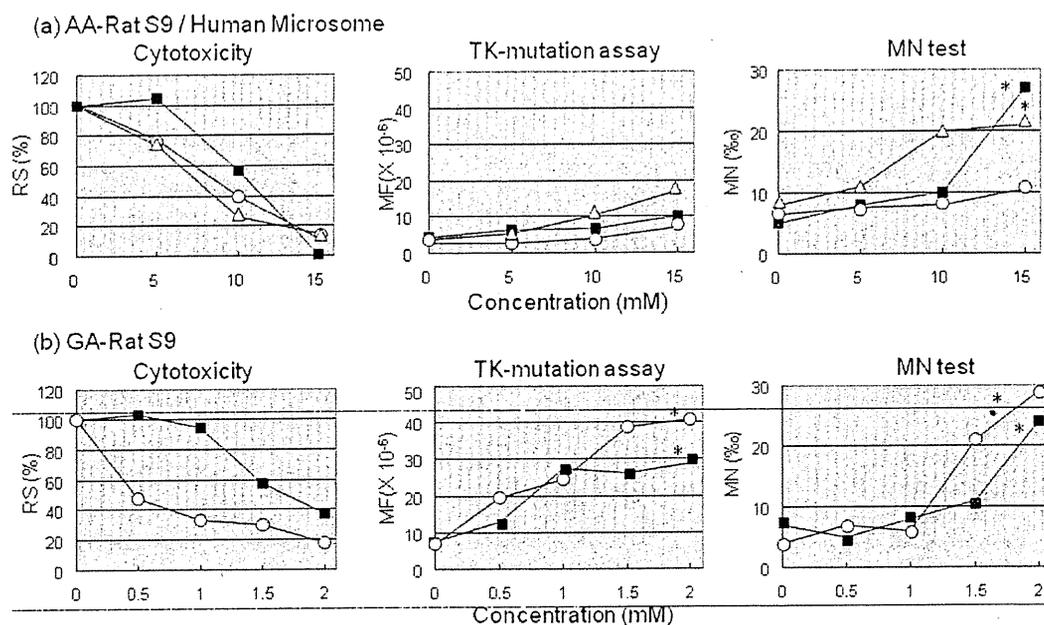
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Fig. 1.   Cytotoxic (relative survival, RS) and genotoxic (TK and MN assays) responses of TK6 cells treated with AA or GA for 4 hr with or without metabolic activation. (a) TK6 cells were treated with AA without (■) or with (○) rat liver S9 or human microsomes (△). (b) TK6 cells were treated with GA without (■) or with (○) rat liver S9. *P < 0.05 (Omori method for TK-mutation assay, trend test for MN assay).

We also seeded cells into the 96-well plates in the absence of TFT to determine plating efficiency (PE3). TK6 cells were seeded at 40,000 cells/well and 1.6 cell/well for TFT and PE plates, respectively. AHH-1 and h2E1v2 cells were seeded at 5,000 cells/well and 3.2 cells/well for TFT and PE plates, respectively. All plates were incubated at 37°C in 5% CO_2 in a humidified incubator. We scored for the colonies in the PE plates at 14th day after plating, and scored for the colonies in the TFT plate on the 28th day after plating. Mutation frequencies were calculated according to the Poisson distribution [Furth et al., 1981]. The data were statistically analyzed by Omori's method, which consists of a modified Dunnett's procedure for identifying clear negative, a Simpson-Margolin procedure for detecting downturn data, and a trend test to evaluate the dose-dependency [Omori et al., 2002]. We evaluated cytotoxicity for TK6 by relative survival (RS), which is calculated from plating efficiency (PE0), and for AHH-1 and h2E1v2 by relative suspension growth (RSG), which is calculated from cell growth rate during 3 days expression period.

Western Blot Analysis

A goat polyclonal anti-rat CYP2E1 antibody (Daiichi Pure Chemical, Tokyo) and rabbit anti-rat actin (Sigma, St. Louis, MO) were used as primary antibodies. AP-conjugated secondary antibody (Cappel, Organon Technika Corp., West Chester, PA) was used to detect primary antibody signals.

DNA Adduct Assay

As a standard for LC/MS/MS analysis. N7-GA-Gua and [$^{15}N_5$]-labeled N7-GA-Gua were synthesized as described previously [Gamboa da Costa et al., 2003]. DNA was extracted from the cells by using DNeasy 96 Blood &Tissue Kit (QIAGEN, Düsseldorf) and incubated at 37°C for 48 hr for deprination. An aliquot of the [$^{15}N_5$]-labeled N7-GA-Gua standard was added to each sample and filtered through an ultrafiltration membrane to remove DNA. The eluted-solution was evaporated thoroughly and dissolved in water, and then the solutions were subsequently quantified by LC/MS/MS.

RESULTS

Cytotoxicity and Genotoxicity of AA and GA Under Metabolic Activation

We used human microsomal preparation and phenobarbital- and 5,6-benzoflavone-treated rat liver S9 for metabolic activation. CYP2E1 activity of the human microsomal preparation was more than twice that of the rat liver S9 preparations (2,917 vs. 1,295 pmol/mg/min).

Figure 1 shows the cytotoxicity (RS; relative survival), MN, and TK-mutations induced by AA (a) and GA (b) with and without rat liver S9 or human microsomes. Rat liver S9 or human microsomes enhanced cytotoxicity (RS) of AA and GA. On the other hand, AA showed weak genotoxicity only at relatively high concentrations (>10 mM) without S9, but neither activating system enhanced the weak genotoxicty. GA induced TK-mutations dose-dependently from the low concentration (0.5 mM) and induced MN from 1.5 mM both with and without S9. Thus, neither the rat nor human metabolizing system activated AA or inhibited the expression of GA genotoxicity.

umu Assay Using Strains Expressing Human CYP2E1

We used S. typhimurium OY1002/2E1 strain to assess the cell toxicity and genotoxicity of AA at exposures up to 10mM (Fig. 2c). We also examined AA and GA with
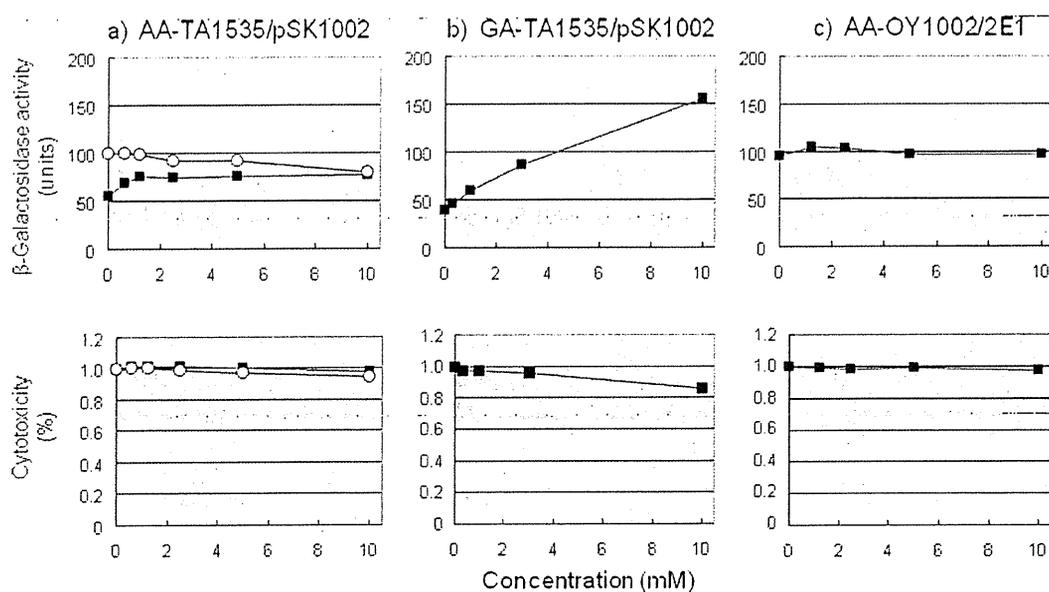
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Fig. 2. Induction of *umuC* gene expression and cytotoxic response by AA (a, c) or GA (b) in *S. typhimurium* tester strains TA1535/pSK1002 (a, b) and OY1002/2E1 (c). The *umu* tests were conducted without (■) or with rat S9 (○). β-Galactosidase activity (units) was determined as described in Materials and Methods. Cytotoxic activities are expressed as % optical density change at 600 nm.

or without rat S9 using TA1535/pSK1002 strain. Although GA clearly produced a dose-related increase in response to DNA damage (Fig. 2b), AA elicited no genotoxic or cell toxic response with and without S9 (Fig. 2a). Thus, we could not demonstrate any in vitro genotoxicity of AA in the bacterial system.

Cytotoxic and Genotoxic Responses to AA in Transgenic Cell Lines

Western blot analysis revealed that h2E1v2 accumulated more CYP2E1 than either of its parental cell lines (Fig. 3). Both the h2E1v2 and AHH-1 cells exhibited weak responses (TK-gene mutations and MN) to AA at ≤3 mM with little difference in cytotoxicity (RSG, relative suspension growth) (Fig. 4a). h2E1v2 differed from AHH-1, however, in that it showed clear genotoxic and cytotoxic responses (RSG) to DMN, which is a representative substrate for CYP2E1 (Fig. 4b). Thus, the h2E1v2 cell line had CYP2E1 activity but did not activate AA.

DNA Adduct Formation by AA and GA in the Cell Lines

AA induced trace amounts of N7-GA-Gua adduct in TK6 cells (with and without S9) (Fig. 5a) and in AHH-1 and h2E1v2 cells (Fig. 5b). GA, on the other hand, induced a substantial number of N7-GA-Gua adducts in TK6 cells (Fig. 5c). These results suggest that the expression of genotoxicity may be dependent on N7-GA-Gua
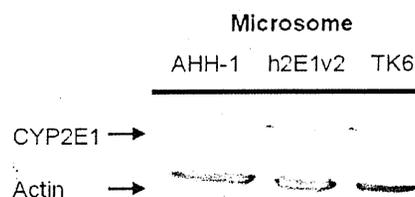
Microsome

AHH-1   h2E1v2   TK6

Fig. 3. Western blot analysis of CYP2E1 in AHH-1, h2E1v2, and TK6 cells. Equal amount of materials were loaded for each sample. CYP2E1 protein was stained with the anti-CYP2E1 antibody. Actin was used as a loading control.

adduct formation, and the in vitro metabolic activation system did not metabolize AA into GA.

DISCUSSION

A large number of studies about the in vitro genotoxicity of AA have been reported [Dearfield et al., 1995; Besaratinia and Pfeifer, 2005]. AA was negative in Ames assay in both the presence and absence of S9 [Zeiger et al., 1987; Knaap et al., 1988; Tsuda et al., 1993]. In mammalian cell assays, cytogenetic tests such as chromosome aberration test and sister chromatid exchange tests were positive [Sofuni et al., 1985; Tsuda et al., 1993]. AA also induced *Tk* mutation in the MLA but did not induce *Hprt* mutation in V79 cells [Moore et al., 1987;

ORGAN TOXICITY AND MECHANISMS

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

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Kaoru Irie · Tomomi Morikawa · Makoto Shibutani · Masamitsu Honma ·
Akiyoshi Nishikawa

Abstract In order to assess age-dependence of suscepti-
bility 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, regard-
less 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 sus-
ceptible 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

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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 eval-
uate toxicity targeting children is important for risk
assessment of ACR exposure in man.

Even the limited studies on susceptibility to neurotox-
icity of ACR in relation with the life stage have not gen-
erated 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.
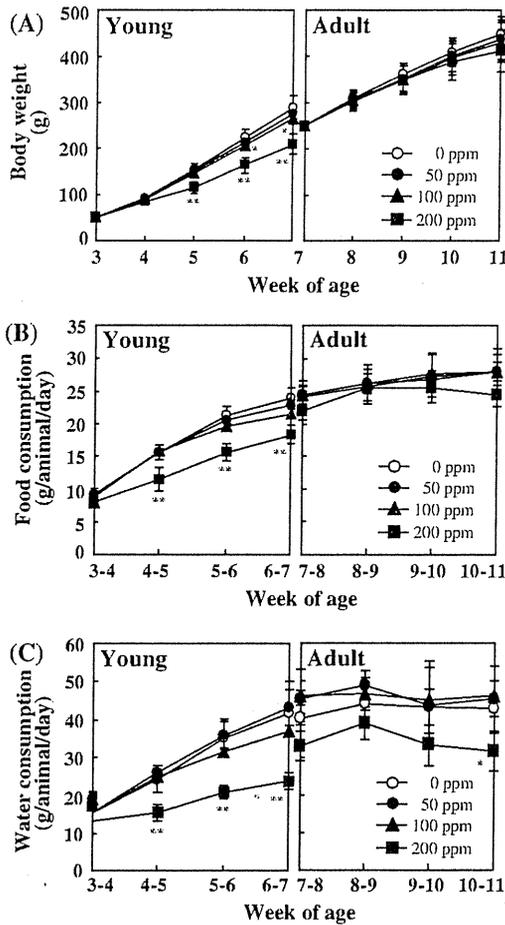
Statistical analysis

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

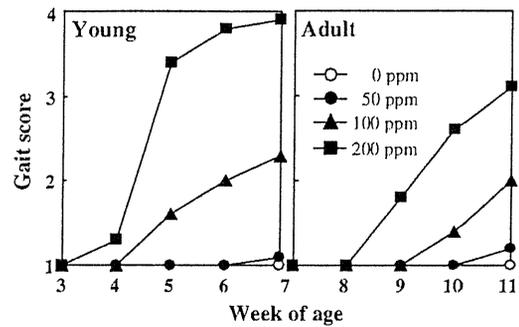Suppression of body weight gain was observed in the young groups at 100 and 200 ppm from week 2 (Fig. 1a).

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

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

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

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.

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 μm²)	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 μm²)	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
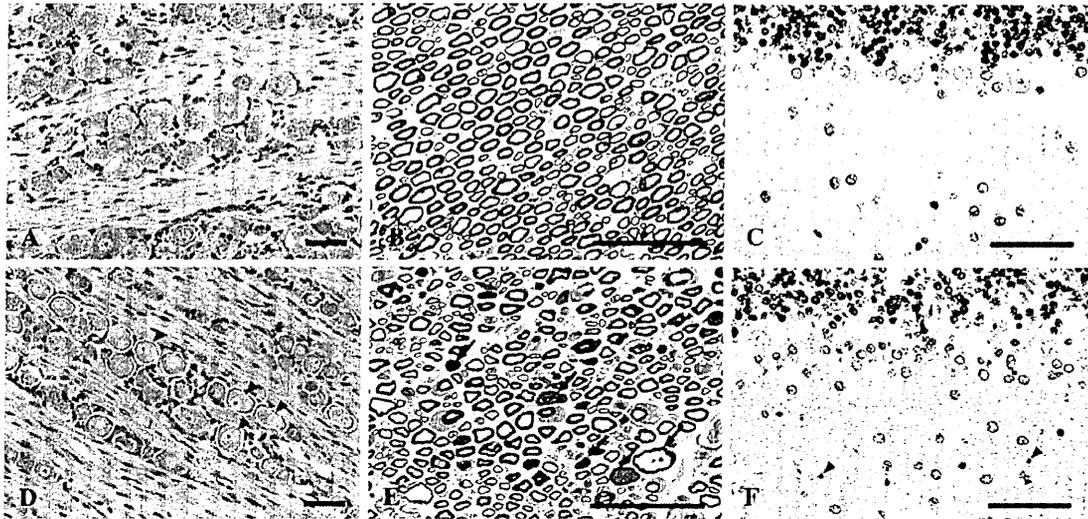
Fig. 3 Histopathology of the trigeminal nerve (**a**, **d**), sciatic nerve (**b**, **e**), and cerebellar molecular layer (**c**, **f**) in young rats given ACR at 0 or 200 ppm for 4 weeks. (**a–c**) Normal tissues of a young rat from the 0 ppm group. (**d–f**) At 200 ppm, central chromatolysis of ganglion 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
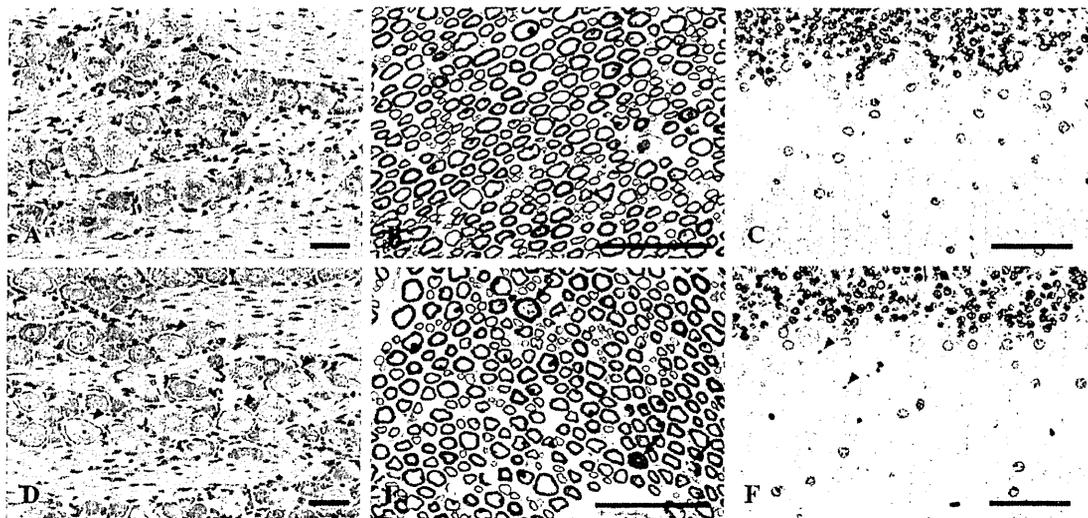
Fig. 4 Histopathology of the trigeminal nerve (**a**, **d**), sciatic nerve (**b**, **e**), and cerebellar molecular layer (**c**, **f**) of adult rats given ACR at 0 or 200 ppm for 4 weeks. (**a–c**) Normal tissues of an adult rat from 0 ppm group. (**d–f**) Similar to the young group, central chromatolysis 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
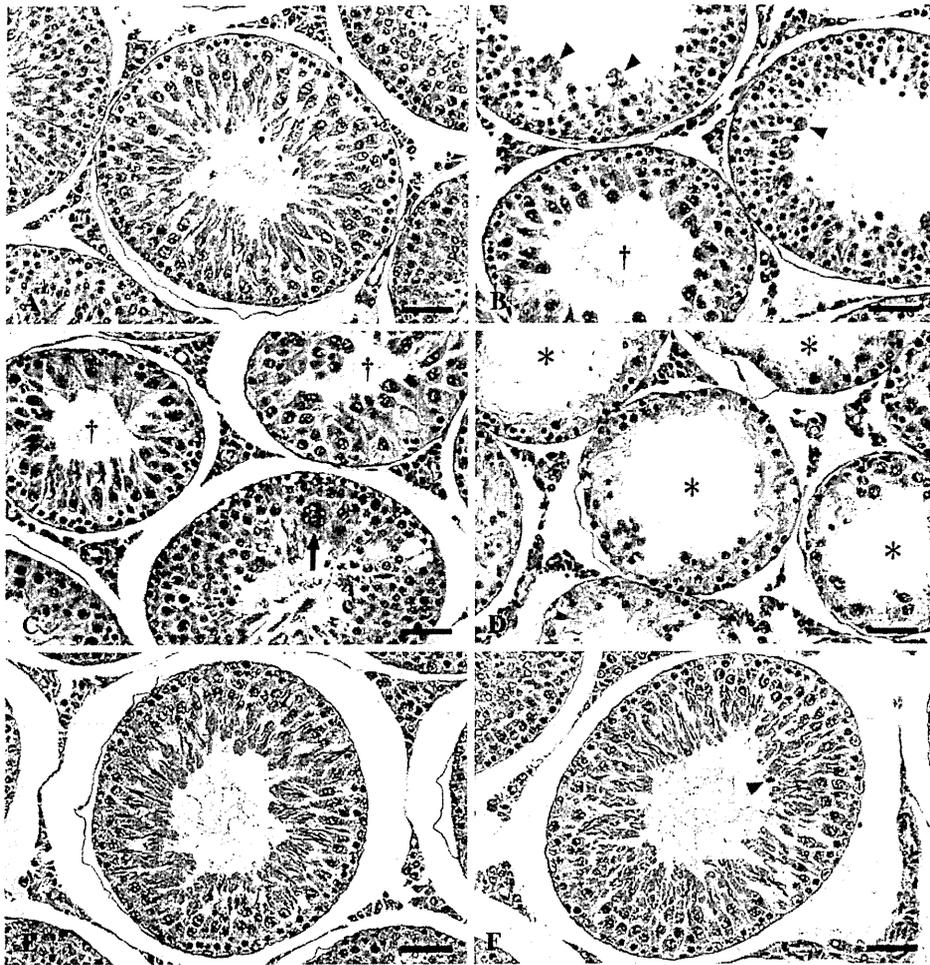
Fig. 5 Histopathology of the testis of young and adult rats given ACR at 0 or 200 ppm for 4 weeks. **a** Normal seminiferous tubules of a young rat from the 0 ppm group. (**b–d**) Degeneration of spermatids (*arrowheads*), loss of or decreased in elongated spermatids (†), and multinucleated giant cells (*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