Original Article

Lack of modifying effects of prepubertal exposure to acrylamide (AA) on *N*-methyl-*N*-nitrosourea (MNU)-induced multi-organ carcinogenesis in F344 rats

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ABSTRACT — Acrylamide (AA) has been reported to be formed in fried and baked foods with various concentrations, and exposure levels to AA from cooked foods in children are estimated to be higher than those in adults. In order to evaluate the carcinogenicity of AA exposure during childhood, we conducted a medium-term carcinogenicity study with prepubertal administration of AA followed by treatments of a multi-organ-targeted genotoxic carcinogen and a promoting agent for thyroid carcinogenesis in rats. A total of 36 postpartum F344 rats were given drinking water containing AA at 0, 20, 40 or 80 ppm for 3 weeks during the lactation period, and their weaned offspring received the same AA-containing water for 3 more weeks. Offspring were then injected with *N*-methyl-*N*-nitrosourea (MNU; 40 mg/kg body weight, i.p.) once at week 7 after birth. Half the animals of the 0 and 40 ppm groups were additionally treated with the anti-thyroid agent sulfadimethoxine (SDM; 125 ppm) in the drinking water thereafter. Offspring were subjected to complete necropsy at week 50. All the major organs and macroscopic abnormalities were excised and examined histopathologically. There was no significant difference in the incidences of hyperplastic and neoplastic lesions in the target organs of AA and/or MNU, such as the brain, spinal cord, pituitary gland, thyroid, adrenal glands, uterus, mammary glands, clitoral gland and tunica vaginalis. In conclusion, no significant modifying actions of AA on MNU-induced multi-organ carcinogenesis were exhibited in any organs of rats when exposed prepubertally under the present experimental conditions.

Key words: Acrylamide, Carcinogenesis, Prepubertal exposure, F344 rat

INTRODUCTION

Acrylamide (AA), a vinyl monomer, is industrially utilized for improvement of aqueous solubility, adhesion or cross-linking of polymers, and mobility control for oil recovery, flocculant for wastewater treatment and soil stabilization, and also as a reagent for scientific research (IARC, 1994). AA has also been reported to be spontaneously formed in fried and baked foods as a contaminant (Tareke *et al.*, 2002). There are many reports demonstrating various toxic effects of AA *in vitro* and *in vivo*, including neurotoxicity (Burek *et al.*, 1980; LoPachin *et al.*, 2003; Tyl *et al.*, 2000b), testicular toxicity (Burek *et al.*,

1980; Yang *et al.*, 2005), reproductive toxicity (Tyl *et al.*, 2000a, 2000b), carcinogenicity (Bull *et al.*, 1984a, 1984b; Friedman *et al.*, 1995; Johnson *et al.*, 1986) and genotoxicity (Manière *et al.*, 2005; Paulsson *et al.*, 2003; Yang *et al.*, 2005). Although AA is regarded as a potential mutagen based on experimental evidence that it can bind to DNA, the majority of genotoxicity data particularly from *in vitro* studies suggest that AA does not produce detectable genetic mutations (Dearfield *et al.*, 1988). AA however demonstrates clastogenicity, and glycidamide (GA), an epoxide metabolite of AA, evidently shows mutagenicity. It has been well documented that chronic AA exposure has been associated with increased incidence of mesothelio-

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mas in the tunica vaginalis and tumors of the central nervous system, thyroid, endocrine glands, mammary glands and in the reproductive tracts in adult rats (Friedman *et al.*, 1995; Johnson *et al.*, 1986).

In 2002, new analytical data from a Swedish group indicated spontaneous formation of AA in fried and baked foods at various concentrations (Tareke *et al.*, 2002), and also demonstrated that risk due to dietary exposure may not be negligible. In recent epidemiological studies, however, controversial results have been reported indicating risks of renal, bladder, prostate, breast, endometrial, ovarian, and several other common cancers were not associated with dietary AA intake (Hogervorst *et al.*, 2007, 2008; Larsson *et al.*, 2009a, 2009b; Mucci *et al.*, 2003; Pelucchi *et al.*, 2006), while higher dietary AA intake increased risks of endometrial, ovarian and renal cell cancer (Hogervorst *et al.*, 2007, 2008). Thus, there is no consistent evidence indicating an association between dietary AA intake and the risk of several cancers. From these epidemiological reports, average estimated exposure levels to AA from cooked foods were 1 µg/kg body weight/day or lower in adults. However, since the exposure levels during childhood are estimated to be higher than those in adults (Dybing *et al.*, 2005; Hartmann *et al.*, 2008), the risk of dietary exposure to AA may therefore be more of a concern in children compared to adults. Epidemiological studies focused on dietary exposure to AA during childhood as well as experimental toxicological studies using juvenile animals are unfortunately limited.

Comparative studies of carcinogenic susceptibility between juvenile and adult exposure have been generally conducted based on long-term experiments using very large numbers of animals (Barton *et al.*, 2005; Chhabra *et al.*, 1992). However, some medium-term bioassay models using genotoxic chemical carcinogens have been introduced to detect modifying effects of prepubertal exposure to several test chemicals on multi-organ carcinogenesis, requiring smaller numbers of animals. For example, treatment with a mixture of aryl hydrocarbon-receptor agonists to rats from day 1 to 20 after birth was reported not to affect the development of *N*-methyl-*N*-nitrosourea (MNU)-induced mammary tumors (Desaulniers *et al.*, 2004), whereas a single postnatal administration of diethylstilbestrol followed by 7,12-dimethylbenz[*a*]anthracene (DMBA) treatment increased the incidence and multiplicity of mammary tumors as compared to DMBA alone (Ninomiya *et al.*, 2007). We have also reported that prepubertal exposure to an environmental chemical tetrabromobisphenol A (TBBPA) increased the susceptibility to thyroid and urinary bladder tumorigenesis induced by *N*-bis(2-hydroxypropyl)nitrosamine (DHPN) and DMBA

in rats (Imai *et al.*, 2009). In the present study, in order to evaluate carcinogenic effects of prepubertal administration of AA, we conducted a medium-term carcinogenicity study using MNU to target various organs and the promoting agent sulfadimethoxine (SDM), is a goitrogen caused by inhibition of thyroid peroxidase, to induce thyroid carcinogenesis in rats.

MATERIALS AND METHODS

Chemicals

AA, MNU and SDM were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animal treatments

Thirty-six pregnant F344 rats were obtained from Charles River Laboratories Japan (Kanagawa, Japan). The animals were time-mated at 10 weeks of age and arrived on gestational day 14 to our facilities. They 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 ($24 \pm 1^\circ\text{C}$, $55 \pm 5\%$ relative humidity, 12 hr light and dark cycle) and given a basal diet (CRF-1, Oriental Yeast, Tokyo, Japan) and tap water *ad libitum* until parturition. Offspring were also maintained under the same conditions, in group housing with 3 or 4 males or females each per cage.

Experimental protocol

Six dams in six groups each were given free access to AA-containing drinking water at concentrations of 0 (control), 20, 40 and 80 ppm for the 3 weeks of lactation after parturition. The dose levels of AA were determined according to our preliminary study, in which offspring in the 40 ppm group showed a transiently decreased body weight as compared to the controls. Eight pups per dam (4 males and 4 females) were randomly selected to maximize the uniformity of growth rates of the offspring at postnatal day 3. After weaning, the dams were euthanized and the offspring were maintained with the same drinking water as the dams for an additional 3 weeks. The drinking water containing AA was replaced weekly. Offspring were then intraperitoneally injected with 40 mg/kg body weight of MNU once at 7 weeks of age. Half of the animals of 0 and 40 ppm groups were additionally treated with SDM, an anti-thyroid agent, in the drinking water at a concentration of 125 ppm thereafter until week 50 (Fig. 1). The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Fig. 1. Experimental design. □: distilled water and basal diet (CRF-1), ■: 20, 40 or 80 ppm AA in drinking water, ▒: 40 ppm AA in drinking water, ◻: 125 ppm SDM in drinking water, ⬆: MNU (40 mg/kg, i.p.), S: sacrifice.

During the experimental period, clinical signs and mortality were monitored at least once daily. Individual body weights, food and water consumption per cage were measured once a week during the first 20 weeks and once in 4 weeks from week 21 to the end of experiment. From week 12, after 5 weeks of MNU treatment, thoracic and abdominal tumor appearance was assessed by palpation, and the numbers and sizes were recorded once a week. Tumor volumes were calculated as follows (the formula for the volume of an ellipsoid):

$$\text{Tumor volume} = \text{length} \times \text{depth} \times \text{height} \times 0.52$$

At week 50, all surviving animals were euthanized by exsanguination from the thoracic aorta under deep ether anesthesia. The liver and kidney were excised and weighed. In addition to these organs, the brain, spinal cord, thyroid, thymus, lung, spleen, stomach, intestine, mesenteric lymph node, urinary bladder, mammary gland and any gross abnormalities were excised. All organs were fixed in 10% buffered formalin. The thyroid was weighed after fixation. All fixed samples were trimmed for embedding in paraffin and routinely processed for hematoxylin and eosin (HE) staining for histopathological examination. Animals that died or became moribund during the experiment were also necropsied and analyzed similarly to those sacrificed on schedule.

Statistics

Statistical analysis to compare survival rates was carried out using the Kaplan-Meier method. Variances in values for body and organ weights, multiplicity data,

and volumes of mammary tumors among groups without SDM treatment were checked for homogeneity by the Bartlett's procedure. When the data were homogeneous, a one-way analysis of variance (ANOVA) was used. In the heterogeneous cases, the Kruskal-Wallis test was applied. When statistically significant differences were indicated, Dunnett's multiple comparison test was employed for comparisons between control and treated groups. For comparison between groups 0 ppm and 0 ppm + SDM, 40 ppm and 40 ppm + SDM, and 0 ppm + SDM and 40 ppm + SDM, the quantitative data were analyzed by the Student's *t*-test or Welch's *t*-test following the *F* test for homogeneity of variance. The incidence data for preneoplastic and neoplastic lesions were compared using Fisher's exact probability test.

RESULTS

Effect of AA exposure on dams

One dam in the 40 ppm group was excluded from evaluation, since only one pup was born which was stunted and neglected.

Abnormal clinical signs and mortalities were not observed in dams during the treatment period. In dams, a slight tendency for decrease in body weight was observed in the 80 ppm group but without statistical significance. A slightly lowered food and water consumption was also observed in the 80 ppm group (Table 1). The average daily intake of AA in the 20, 40 and 80 ppm group dams was 4.1, 8.0 and 14.7 mg/kg body weight, respectively.

Effect of AA exposure and SDM treatment on offspring

As shown in Fig. 2, survival rates of the offspring were not affected by the AA treatments. Body weights were influenced by the treatments of AA and/or SDM. Final body weights were significantly lowered in the 80 ppm females as compared to the control group, the ratio being approximately 5% at the largest (Figs. 3 and 4). Mean food and water consumption per rat showed a tendency to decrease in the 80 ppm group; however, consumption per body weight increased in this group (Table 2). Intake of AA was almost concentration-dependent, as expected.

The incidence, multiplicity and volume of palpable mammary tumors were comparable among the female groups (Fig. 5).

Organ weights

Relative organ weights of the offspring are summa-

rized in Table 3. Kidney weights were decreased in the 40 and 80 ppm and SDM-only treated females as compared to the controls, and decreased and increased in the 40 ppm + SDM males and females, respectively, when compared to the 40 ppm groups. Thyroid weights were increased or showed a tendency to increase with SDM treatment in both males and females. As for liver weight, higher values were noted in males and females of the 40 ppm + SDM group as compared to 40 ppm or the SDM-only groups.

Histopathological examinations

Incidences of histopathologically detected preneoplastic and neoplastic lesions are summarized in Tables 4 and 5. Focal follicular cell hyperplasias and follicular adenomas and carcinomas in the thyroid were increased or showed a tendency to increase in SDM-treated groups in both sexes, as compared with non-treated controls.

Table 1. Initial and final body weights, food and water consumption and intake of AA in dams (from parturition until weaning)

Group	Initial body weight (g)	Final body weight (g)	Food consumption		Water consumption		Intake of AA (mg/kg/day)
			mg/rat/day	mg/kg/day	mg/rat/day	mg/kg/day	
0 ppm	175.3	189.2	28.8	147.9	41.3	212.7	
20 ppm	175.8	189.8	28.4	146.2	40.0	206.4	4.1
40 ppm	176.3	188.7	27.4	142.3	38.5	200.4	8.0
80 ppm	175.8	185.1	25.2	132.9	34.7	183.1	14.7

Fig. 2. Survival rates for male and female offspring administered AA followed by MNU/SDM treatment, respectively.

Fig. 3. Body weight curves of offspring administered AA followed by MNU/SDM treatment (from birth until 8 weeks of age).

Fig. 4. Body weight curves of offspring administered AA followed by MNU/SDM treatment (from 9 until 50 weeks of age).

Alveolar/bronchiolar adenocarcinomas in the lungs were increased in the 80 ppm males and showed a tendency to increase in all other AA-treated males as compared to the controls. On the contrary, the combined incidence of lung adenomas and adenocarcinomas was comparable as com-

pared to the non-treatment control group, and focal alveolar hyperplasias decreased in all AA-treated males. Alveolar/bronchiolar adenomas and adenocarcinomas in the lungs were increased or showed a tendency to increase, but again, incidences of focal alveolar hyperplasias were

Table 2. Food and water consumption and intake of AA in offspring (from 4 until 6 weeks of age)

Gender	Group	Food consumption		Water consumption		Intake of AA (mg/kg/day)
		mg/rat/day	mg/kg/day	mg/rat/day	mg/kg/day	
Male	0 ppm	7.7	96.4	12.6	162.0	
	20 ppm	7.5	99.2	12.6	169.2	3.4
	40 ppm	7.5	101.1	12.6	173.9	7.0
	80 ppm	6.9	103.4	11.4	176.6	14.1
Female	0 ppm	6.9	97.2	11.9	169.6	
	20 ppm	6.6	101.6	11.6	178.3	3.6
	40 ppm	6.7	101.2	11.7	179.7	7.2
	80 ppm	6.3	105.9	10.9	186.6	14.9

also decreased in the SDM-treated males. The incidence of focal alveolar hyperplasia, alveolar/bronchiolar adenomas and adenocarcinomas showed a tendency to decrease in the 40 ppm + SDM males as compared to the SDM-only males. Squamous cell hyperplasias in the oral cavity were increased in the SDM-only males, as compared with controls. No significant changes were noted in the incidences of preneoplastic and neoplastic lesions in other organs with the treatment of AA and/or SDM.

DISCUSSION

With regard to carcinogenicity, it has been reported that the incidence and multiplicity of lung tumors are increased by oral or intraperitoneal AA administration in A/J mice (Bull *et al.*, 1984b), and that oral AA treatment promotes the induction of dermal squamous cell papillomas and carcinomas with a 12-*O*-tetradecanoylphorbol 13-acetate promotion regimen in Swiss-ICR mice (Bull *et al.*, 1984a). In two long-term rat carcinogenicity studies, the incidences of mammary and thyroid tumors as well as mesotheliomas in tunica vaginalis were increased by AA administration in drinking water (Friedman *et al.*, 1995; Johnson *et al.*, 1986). In addition, increased incidences of tumors in the uterus, clitoral gland, pituitary gland, adrenal and oral cavity, and a tendency for increased incidence of brain and spinal cord tumors were also observed in one of the studies (Johnson *et al.*, 1986). Numerous *in vitro* and *in vivo* genotoxicity data and evidence showing formation of covalent adducts of AA and/or GA with DNA/hemoglobin in rodents and in humans support the classification for AA as a group 2A carcinogen; that is AA is probably carcinogenic to humans, by the International Agency for Research on Cancer (IARC, 1994). However, most epidemiologic studies of workers exposed to AA

have failed to demonstrate any relation between exposure to AA and either overall incidence of malignancy or incidence of specific cancers (Marsh *et al.*, 2007; Sobel *et al.*, 1986), except for one which showed increased rate of pancreatic cancer in AA workers as compared with an expected standardized mortality ratio (Swaen *et al.*, 2007).

MNU have widely shown to have carcinogenic potential to various rat organs, including thyroid, mammary gland and central nervous system, in which carcinogenic potentials of AA have also demonstrated. Therefore, in the present study, to evaluate additive/synergistic effects of the carcinogenic actions of prepubertal administration of AA, we conducted a medium-term carcinogenicity study using MNU and a promoting agent SDM for thyroid carcinogenesis in rats. In the present study, SDM increased the incidence of focal follicular cell hyperplasias, adenomas and/or carcinomas in the thyroid of both sexes, validating the experimental model applied. Moreover, enhancement of the tumorigenesis in the lung and oral cavity by SDM treatment was detected. It was reported that SDM increased the number of alveolar epithelial hyperplasias in a rat lung tumorigenesis model (Takegawa *et al.*, 1996) and SDM induced oxidative stress *in vitro* (Galati *et al.*, 2002). Therefore, the increased lung and oral cavity tumors might be associated with SDM treatment resulting at least partly in oxidative stress induction. Nevertheless, prepubertal AA administration at doses of 20, 40 or 80 ppm did not affect carcinogenicity in the multiple organs induced by MNU with or without SDM, except for lung adenocarcinomas in the 80 ppm males, which increased incidence. Regarding lung preneoplastic and neoplastic lesions, the combined incidence of adenomas and adenocarcinomas in males was comparable to the non-treatment control group, and the incidence of adenomas and adenocarcinomas showed a tendency to

Lack of modifying effects of prepubertal exposure to acrylamide

Fig. 5. Sequential changes in the incidence, multiplicity and volume of palpable mammary tumors in females administered AA followed by MNU/SDM treatment.

decrease in the 40 ppm + SDM males as compared to the SDM-only males. In addition, there were no treatment-related differences in the incidences of lung preneoplastic and neoplastic lesions in the female groups. Thus it can be stated from the overall data that prepubertal AA administration lacks any modifying effects on multi-organ carcinogenesis induced by MNU and SDM, though nevertheless, lung tumors have been induced by AA treatment in A/J adult mice (Bull *et al.*, 1984b).

We have previously reported that AA increases the development of mammary tumors induced by MNU in adult rats when given in the drinking water at doses of 20 and 40 ppm (Imai *et al.*, 2005). Thus it is likely in the present study that AA administration in juvenile rats does not affect mammary tumorigenesis induced by MNU, despite the significant effects seen in adult rats. Recently, we have also found under a similar experimental design using DHPN and DMBA that prepubertal exposure to

TBBPA enhances thyroid and urinary bladder tumorigenesis in rats (Imai *et al.*, 2009). In terms of the timing of treatment for tumor-initiation, the present experiment is similar to this recent study (Imai *et al.*, 2009) besides from the initiator itself. Therefore, it is also likely that AA exposed during prepubertal periods does not modulate thyroid carcinogenesis followed by the thyroid tumor-promoter SDM.

In conclusion, there were no treatment-related changes in the development of preneoplastic and neoplastic lesions in the target organs of AA and/or MNU such as the brain, spinal cord, oral cavity, pituitary, thyroid, adrenal gland, uterus, mammary gland, clitoral gland and tunica vaginalis. Prepubertal administration of AA followed by MNU and/or SDM did not influence multi-organ carcinogenesis in rats under the present experimental conditions. Taken together with our very recent data showing a limited lactational transfer of AA to rat offspring from maternal

Table 3. Final body and relative organ weights of rats administered AA followed by MNU/SDM treatment

Gender	Items	Group	0 ppm (control)	20 ppm	40 ppm	80 ppm	0 ppm + SDM	40 ppm + SDM
Male	No. of animals	22		22	20	19	22	20
	Final body weight (g)		372.0 ± 22.8 ^a	358.6 ± 31.3	377.4 ± 22.0	358.7 ± 20.6	384.5 ± 24.2	375.9 ± 20.6
	Relative organ weights							
	Liver (g/100 g B.W.)		2.71 ± 0.21	3.08 ± 1.90	2.74 ± 0.14	2.79 ± 0.21	2.72 ± 0.15	2.85 ± 0.15 ^{##,§}
	Kidneys (g/100 g B.W.)		0.74 ± 0.90	0.63 ± 0.37	0.56 ± 0.02	0.55 ± 0.03	0.54 ± 0.02	0.54 ± 0.02 ^{§§}
Female	Thyroids (g/100 g B.W.)		0.005 ± 0.001	0.005 ± 0.001	0.006 ± 0.003	0.005 ± 0.001	0.011 ± 0.022	0.007 ± 0.003
	No. of animals	22		22	22	20	23	17
	Final body weight (g)		200.8 ± 7.8	193.4 ± 10.4 [*]	196.3 ± 7.6	191.5 ± 9.3 ^{**}	202.7 ± 13.0	194.1 ± 10.4 [#]
	Relative organ weights							
	Liver (g/100 g B.W.)		2.69 ± 0.18	2.66 ± 0.17	2.56 ± 0.15 [*]	2.90 ± 0.22 ^{**}	2.78 ± 0.22	2.93 ± 0.27 ^{§§}
	Kidneys (g/100 g B.W.)		0.64 ± 0.04	0.64 ± 0.04	0.60 ± 0.03 ^{**}	0.61 ± 0.03 [*]	0.60 ± 0.06 [*]	0.63 ± 0.04 ^{§§}
	Thyroids (g/100 g B.W.)		0.006 ± 0.001	0.007 ± 0.001	0.006 ± 0.001	0.006 ± 0.001	0.014 ± 0.020	0.009 ± 0.001 ^{§§}

^a: Mean ± S.D.^{*} ^{**}: Significantly different from the control values at $P < 0.05$ and 0.01, respectively.[#] ^{##}: Significantly different from the values of 0 ppm + SDM group at $P < 0.05$ and 0.01, respectively.[§] ^{§§}: Significantly different from the values of 40 ppm group at $P < 0.05$ and 0.01, respectively.

Lack of modifying effects of prepubertal exposure to acrylamide

Table 4. Hyperplastic and neoplastic lesions in male rats administered AA followed by MNU/SDM treatment

Organ	Findings	No. of animals						MNU/SDM treatment			
		Group	0 ppm (control)	20 ppm	40 ppm	80 ppm	0 ppm + SDM	23	21	23	40 ppm + SDM
			23	23	23	23					20
Thyroid											
	Focal follicular cell hyperplasia	2 (9)	2 (9)	1 (4)	2 (10)	5 (22)	17 (74)***				14 (70)
	Follicular adenoma	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	10 (43)***				4 (20)
	Follicular carcinoma	0 (0)	0 (0)	0 (0)	1 (5)	0 (0)	1 (4)				3 (15)
Mammary gland											
	Fibroadenoma	1 (4)	1 (4)	0 (0)	0 (0)	1 (4)	0 (0)				0 (0)
Testis/Epididymis											
	Mesothelioma	10 (43)	10 (43)	5 (22)	7 (33)	10 (43)	5 (22)				5 (25)
Spinal cord											
	Malignant meningioma	1 (4)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)				0 (0)
	Malignant astrocytoma	0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)				0 (0)
Pituitary											
	Adenoma, pars distalis	0 (0)	0 (0)	0 (0)	1 (5)	1 (4)	0 (0)				0 (0)
Oral cavity											
	Squamous cell hyperplasia	2 (9)	2 (9)	3 (13)	2 (10)	3 (13)	8 (35)*				3 (15)
	Papilloma	1 (4)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)				0 (0)
Lung											
	Focal alveolar hyperplasia	20 (87)	20 (87)	13 (57)*	13 (62)	14 (61)*	9 (39)***				5 (25)
	Alveolar/bronchiolar adenoma	13 (57)	13 (57)	18 (78)	16 (76)	17 (74)	22 (96)**				17 (85)
	Alveolar/bronchiolar adenocarcinoma	6 (26)	6 (26)	12 (52)	10 (48)	14 (61)*	16 (70)**				9 (45)
	Adenoma + Adenocarcinoma	15 (65)	15 (65)	20 (87)	17 (81)	18 (78)	23 (100)**				20 (100)
Skin											
	Keratoacanthoma	2 (9)	2 (9)	4 (17)	3 (14)	2 (9)	5 (22)				3 (15)
Forestomach											
	Squamous cell hyperplasia	9 (39)	9 (39)	8 (35)	9 (43)	6 (26)	7 (30)				3 (15)
Small intestine											
	Adenocarcinoma	1 (4)	1 (4)	2 (9)	4 (19)	0 (0)	0 (0)				0 (0)
Large intestine											
	Adenocarcinoma	2 (9)	2 (9)	3 (13)	1 (5)	2 (9)	2 (9)				1 (5)

(), %: *, **, ***, Significantly different from the control values at $P < 0.05$, 0.01 and 0.001, respectively.

Table 5. Hyperplastic and neoplastic lesions in female rats administered AA followed by MNU/SDM treatment

Organ	Findings	No. of animals						MNU/SDM treatment			
		Group		0 ppm (control)		20 ppm		40 ppm	80 ppm	0 ppm + SDM	40 ppm + SDM
		No. of animals		22	24	24	24	24	22	24	19
Thyroid											
	Focal follicular cell hyperplasia			0 (0)	0 (0)	0 (0)	1 (4)	14 (58) ***	0 (0)		12 (63)
	Follicular adenoma			0 (0)	0 (0)	0 (0)	0 (0)	2 (8)	0 (0)		4 (21)
	Follicular carcinoma			0 (0)	0 (0)	0 (0)	1 (4)	2 (8)	0 (0)		1 (5)
Mammary gland											
	Fibroadenoma			0 (0)	1 (4)	1 (4)	0 (0)	1 (4)	1 (5)		0 (0)
	Adenocarcinoma			9 (41)	9 (38)	7 (29)	16 (67)	7 (37)	8 (36)		
Brain											
	Malignant astrocytoma			0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	0 (0)		1 (5)
Spinal cord											
	Oligodendroglioma			0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (5)		0 (0)
Pituitary											
	Focal hyperplasia, pars distalis			0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)		1 (5)
Uterus											
	Cystic endometrial hyperplasia			4 (18)	3 (13)	3 (13)	3 (13)	4 (17)	4 (18)		4 (21)
	Endometrial stromal polyp			1 (5)	3 (13)	1 (4)	1 (4)	4 (17)	1 (5)		1 (5)
	Focal glandular hyperplasia			0 (0)	2 (8)	2 (8)	2 (8)	2 (8)	1 (5)		4 (21)
	Endometrial adenoma			0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)		0 (0)
Clitoral gland											
	Adenoma			2 (9)	1 (4)	0 (0)	0 (0)	2 (8)	0 (0)		0 (0)
	Carcinoma			2 (9)	1 (4)	2 (8)	1 (4)	1 (4)	1 (5)		1 (5)
Oral cavity											
	Squamous cell hyperplasia			0 (0)	1 (4)	4 (17)	2 (9)	2 (8)	2 (9)		2 (11)
Lung											
	Focal alveolar hyperplasia			12 (55)	8 (33)	7 (29)	11 (50)	13 (54)	11 (58)		
	Alveolar/bronchiolar adenoma			12 (55)	10 (42)	12 (50)	10 (45)	9 (38)	9 (47)		
	Alveolar/bronchiolar adenocarcinoma			0 (0)	3 (13)	2 (8)	4 (17)	3 (16)	1 (5)		
	Adenoma + Adenocarcinoma			12 (55)	10 (42)	13 (54)	11 (50)	12 (50)	10 (53)		
Forestomach											
	Squamous cell hyperplasia			10 (45)	7 (29)	11 (46)	9 (41)	9 (38)	8 (42)		

(), %; *** , Significantly different from the control values at $P < 0.001$.

Lack of modifying effects of prepubertal exposure to acrylamide

administration during gestational and lactation periods (Takahashi *et al.*, 2009), the present results suggest little evidence that prepubertal exposure to AA causes more susceptibility than pubertal exposures in rats.

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