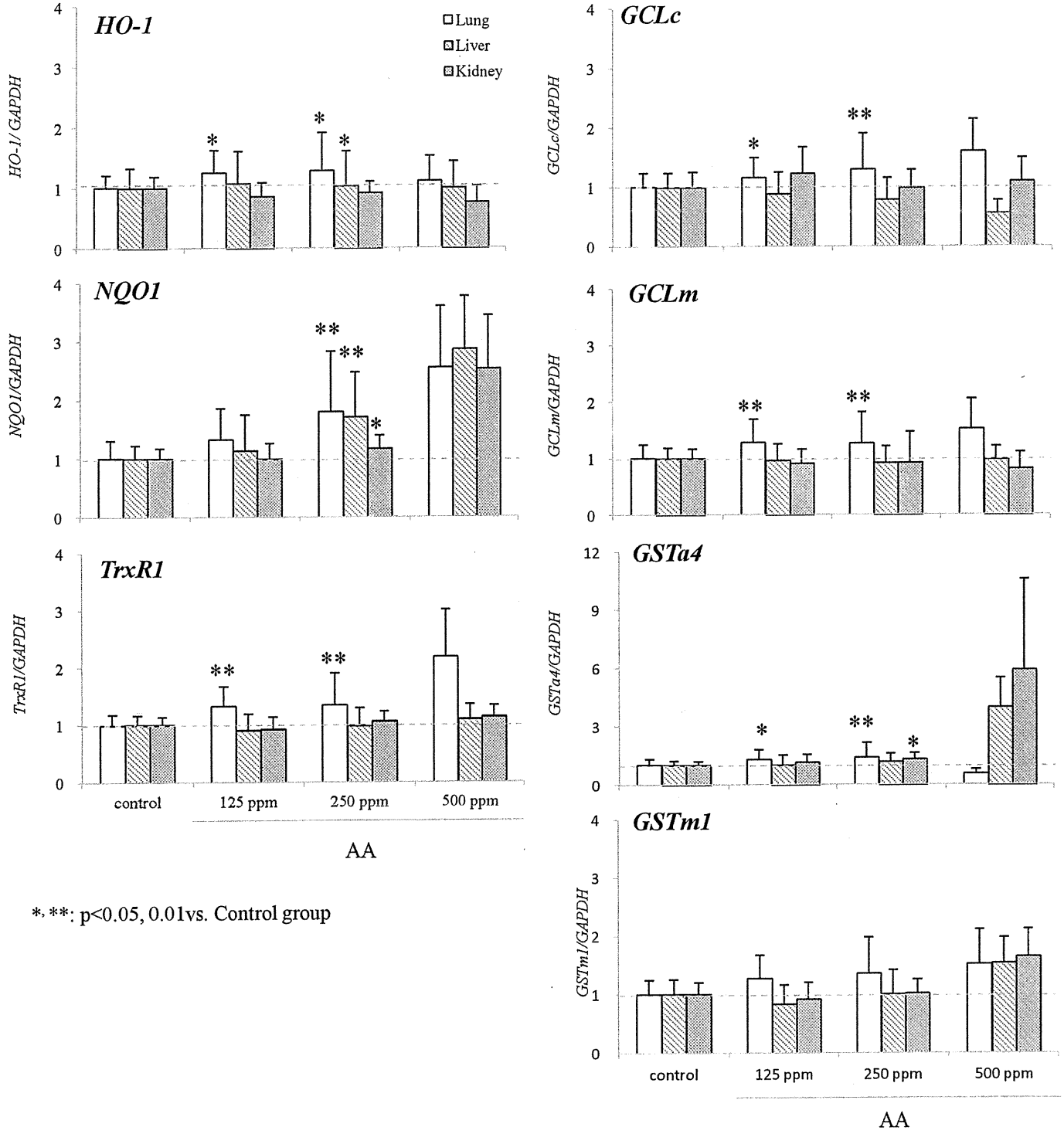

*, **: p<0.05, 0.01vs. Control group

Fig. 2 Expression of Nrf2 related-genes. Data represent means ± S.D. (n=5). **, *: p<0.01, 0.05 vs. Control group

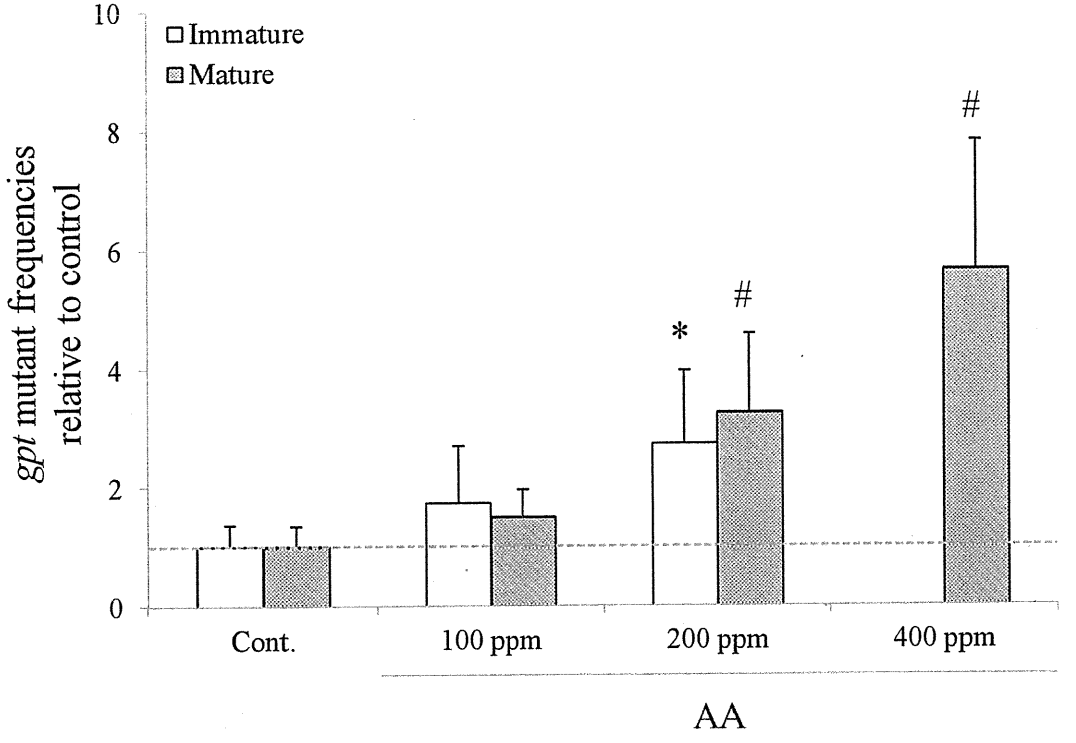

Fig. 3 *gpt* mutant frequencies in the lungs of AA-treated immature and mature *gpt* delta mice. Data represent means ± S.D. (n=5). *: $p<0.01$ vs. Control group (immature). #: $p<0.01$ vs. Control group (mature).

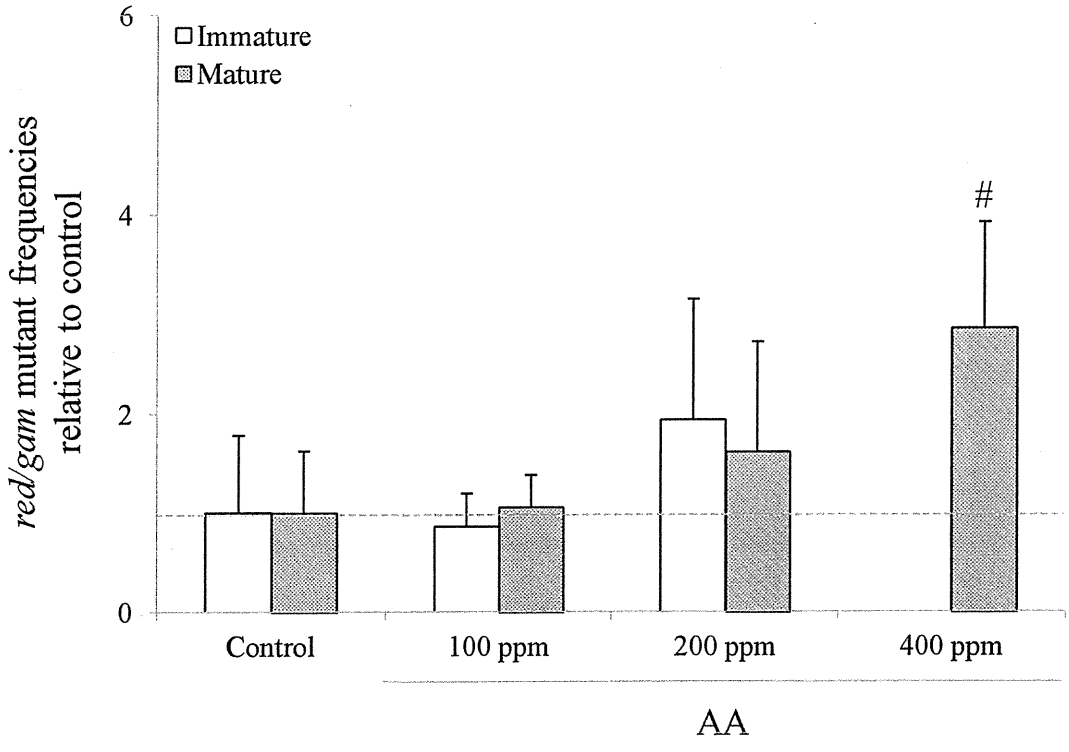

Fig. 4 *red/gam* mutant frequencies in the lungs of AA-treated immature and mature *gpt* delta mice. Data represent means ± S.D. (n=5). #: $p<0.01$ vs. Control group (mature).

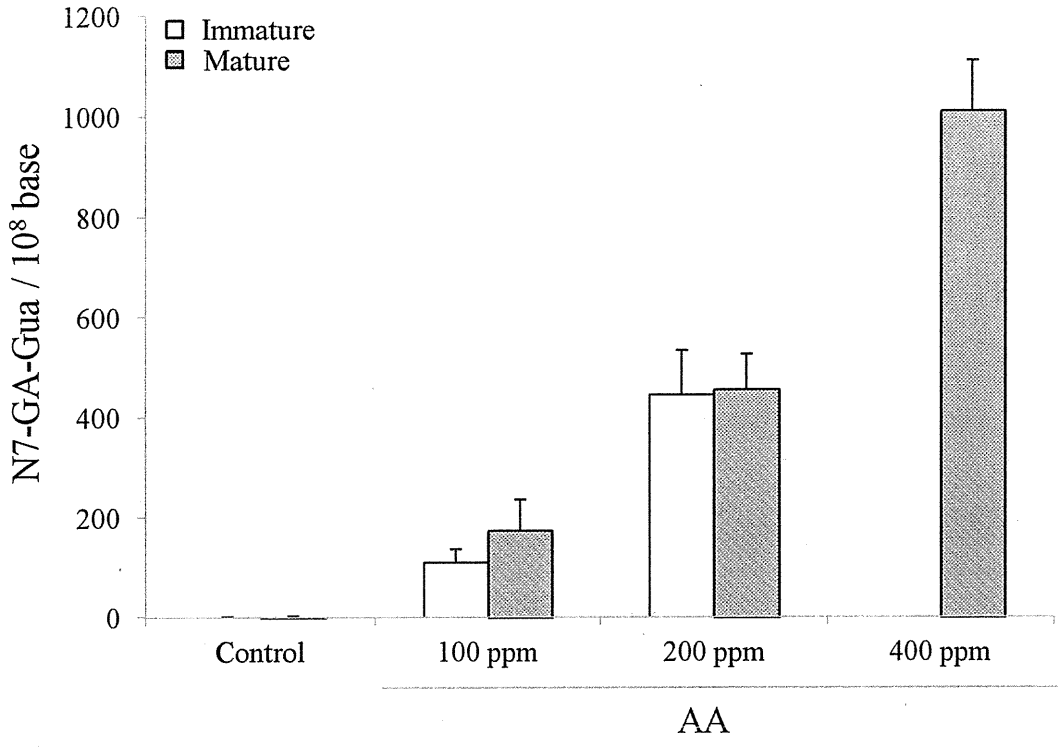

Fig. 5 N7-GA-Gua levels in the lungs DNA of immature and mature *gpt* delta mice. Data represent means ± S.D. (n=5).

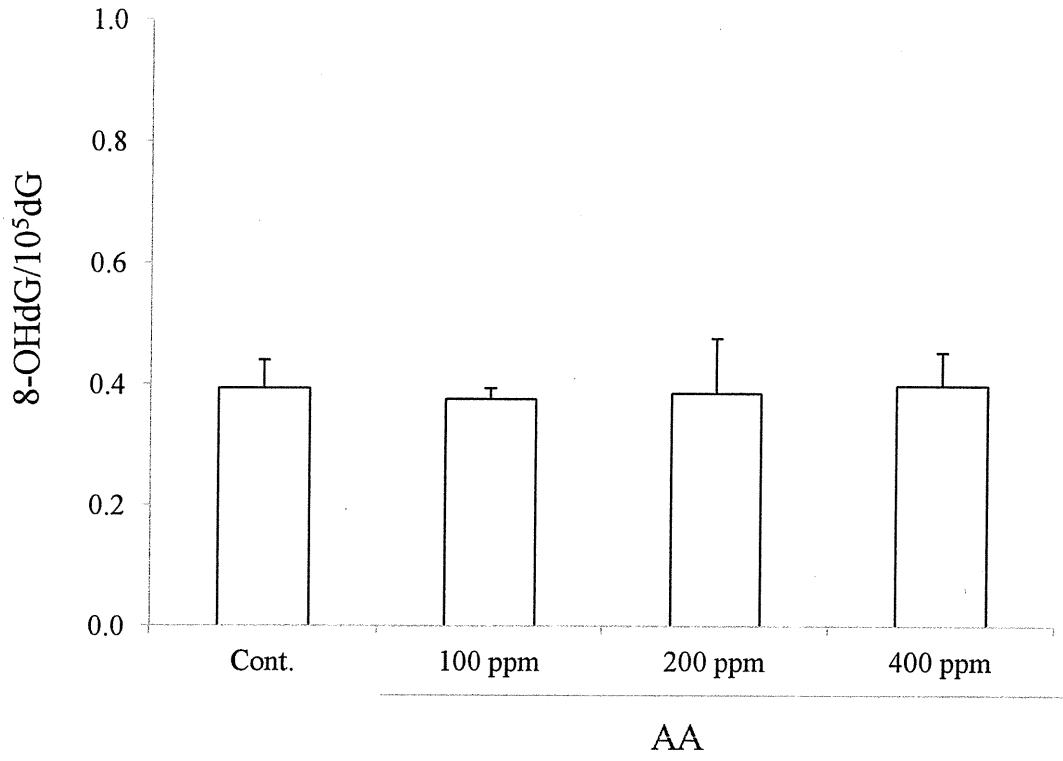

Fig. 6 8-OHdG levels in the lungs of immature *gpt* delta mice treated with AA for 4 weeks. Data represent means ± S.D. (n=5).

厚生労働科学研究費補助金・食品の安全確保推進研究事業
食品中成分から生成されるアクリルアミドのリスク管理対策に関する研究
平成23年度分担研究報告書

ライフステージを勘案したしたアクリルアミドの長期間低暴露の遺伝毒性影響に関する研究

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研究要旨

食品の安全性において、加熱調理等によって食品中に発生するアクリルアミド(AA)が問題となっている。特に、AAの小児への影響が懸念されている。これまでの我々のラットを用いた研究では、精巣において、幼若ラットは成熟ラットと比較し、突然変異の誘発と、顕著なDNAアダクトの蓄積が認められたことから、幼若期の精巣でのAAの遺伝毒性感受性が指摘された。この結果を検証するために、本年度はマウスを用いて、ライフステージの違いによるAAの遺伝毒性感受性の差を検討した。3週、および10週齢のgpt deltaマウス（雄）に0、100、200、400ppmのAAを28日間自由飲水経口摂取させ、赤血球のPig-a遺伝子突然変異と、精巣のgpt遺伝子突然変異を検討した。また、精巣ではDNAアダクト量も解析した。200ppm以上でPig-a（赤血球）、gpt（精巣）突然変異とも有意に増加したが、週齢間で差は見られなかったが。一方、DNAアダクト生成量（精巣）は、用量依存的に増加し、また幼若マウスでは蓄積量が顕著に高かった。体重当たりのAA摂取量に週齢間で差は見られなかったことから、幼若期の精巣でのAAの高蓄積性はライフステージに依存したAAの代謝に関連するものと考えられる。種間で共通して観察されたことから、ヒトに対する影響も考えられ、AAの発がんリスクの検討には、小児に対して特別の配慮が必要と考えられる。

キーワード；アクリルアミド(AA)、遺伝毒性、DNAアダクト、精巣、小児影響

A. 研究目的

食品の高温調理により自然発生することが報告されているアクリルアミド(AA)が、人の健康にどれだけの影響を与えるかが問題となっている。

AAはヒトでの発がん性の証拠は不十分であるものの、ラット、マウスにおいて発がん性が認められることからIARCではグ

ループ 2A にランクされている。遺伝毒性に関しては細菌を用いた遺伝子突然変異試験（エームス試験）ではすべて陰性を示し、真核生物を用いたほとんどの試験系では in vitro、in vivo 試験とも陽性結果が得られている。我々のこれまでの研究から、AA の代謝物であるグリシダミド(GA)が、ほ乳類細胞において、強い遺伝子突然変異誘発性を示すことが明らかとなり、AA の生体摂取は遺伝毒性、発がんリスクを増加させることを示した。このようなことから、日常生活において、できるだけ AA の摂取量を減らすこと、また、AA が生体内で速やかに解毒させることが、そのリスクを低減させることに重要であることが示唆された。

AA は生体内で薬物代謝酵素 CYP2E1 によって GA に変換され、これが、遺伝毒性、発がん性の本体とされている。また、GA はグルタチオン転移酵素のよって解毒される。従って両酵素のバランスによって発がんリスクが決定されると考えられるが、これら酵素は新生時期と成熟期では、活性に差があることが知られていることから、小児と成人ではその発がんリスクの程度が異なることが考えられる。これまでの我々のラットを用いた研究で、AA を幼若、および成熟ラットに飲水投与、もしくは強制経口投与すると、精巣において、幼若ラットは成熟ラットと比較し、突然変異の誘発と、顕著な DNA アダクトの蓄積が認められた。

本年度はこの結果を検証するために、トランスジェニックマウスを用いて、ライフステージの違いによる AA の遺伝毒性感受性の差を検討した。

B．研究方法

i) 動物

gpt delta マウス（雄）を国立医薬品食品研究所内で繁殖し、3 週、および 10 週齢の動物を使用した。

ii) 投与

3 週、および 10 週齢の gpt delta マウス(雄)をそれぞれ 4 群各 10 匹ずつに分けた。それぞれの群に蒸留水で調整した 0、100、200、400ppm の AA（M.W. 71、 シグマ）を、給水ビンで 28 日間飲水経口摂取させた。

iii) Pig-a 遺伝子突然変異試験

Pig-a 遺伝子突然変異試験は Miura らの方法に従った。蛍光標識抗体（抗 TER119/Erythroid Cells- PE/Cy7、及び抗 CD24-FITC）で赤血球を標識した。フローサイトメータによる解析には BD 社の FACS Canto II を使用した。

iv) gpt 遺伝子突然変異試験

精巣の一部を採取し、DNA を抽出し、遺伝子突然変異用のサンプルとした。gpt 遺伝子試験は Masumura らの方法に従って行った。

v) DNA アダクトの定量

精巣一部を、液体窒素を用いて急速凍結し冷凍保存した。後日 DNA を抽出し、DNA アダクトの定量用サンプルとした。AA による主たる DNA アダクトである N7-GA-Gua を LC/MS/MS により測定した。LC/MS/MS は Waters-Micromass 社の Quattro Ultima Pt を用い、HPLC のカラムは Shim-pack XR-ODS(75×3.0mm)を用いた。

*N*7-GA-Gua およびその安定同位体は Gamboa da Costa らの方法に従い合成した。LC/MS/MS は Waters-Micromass 社の Quattro Ultima Pt を用い、HPLC のカラムは Shim-pack XR-ODS(75×3.0mm)を用いた。

（倫理面への配慮）

本研究で特に倫理上問題になる実験はない。また、動物実験を含む全ての実験は本研究所倫理規定に準拠して行った。

C．研究結果

3、10 週齢 gpt マウスに 100、200、400ppm の AA を自由飲水させ屠殺した。屠殺時の体重と組織重量を表 1、2 に示す。また、AA の摂取量を表 3 に示す。10 週齢では 400ppm で強い毒性と体重抑制が観察され、3 週齢では 200ppm 以上から強い毒性と顕著な体重抑制が観察された。幼若マウスでの 400ppm は毒性強く、一部のマウスが死亡したため、その後の投与量を 300ppm へ減弱した。個体当たり、体重当たりの AA 摂取量は 200ppm までは 3、10 週齢では差が認められなかった。400ppm では 10 週齢マウスで AA 摂取量が高かったが、これは 3 週齢マウスでは強に毒性により飲水行動が困難だったためと考えられる。

赤血球の Pig-a 突然変異結果を図 1 に示す。200ppm 以上で 3、10 週齢とも顕著な遺伝子突然変異の誘発が観察されたが、週齢による差は認められなかった。また、400ppm による突然変異頻度の抑制は細胞毒性に起因するものと考えられる。

精巣での gpt 突然変異の結果を図 2 に示す。

表 1　体重と組織重量（10 週齢）

Item	Control	AA 100 ppm	AA 200 ppm	AA 400 ppm
No. of animals	10	10	10	10
Body weight (g)	30.57 ± 1.43	28.57± 1.69	28.36 ± 1.84	19.68 ± 2.21**
Absolute (g)				
Lungs	0.16 ± 0.02	0.17 ± 0.01	0.16 ± 0.01	0.13± 0.01 **
Liver	1.51 ± 0.15	1.47 ± 0.13	1.43 ± 0.16	0.78 ± 0.12 **
Kidneys	0.43 ± 0.05	0.43 ± 0.06	0.41 ± 0.05	0.27 ± 0.02 **
Testis	0.18 ± 0.06	0.20 ± 0.02	0.15 ± 0.03 *	0.09 ± 0.02 **
Relative (g%)				
Lungs	0.52 ± 0.04	0.58 ± 0.05	0.55 ± 0.04	0.65 ± 0.05 **
Liver	4.94 ± 0.31	5.12 ± 0.62	5.05 ± 0.35	3.94 ± 0.27 **
Kidneys	1.42 ± 0.12	1.51 ± 0.21	1.45 ± 0.09	1.39 ± 0.10
Testis	0.60 ± 0.21	0.68 ± 0.07	0.54 ± 0.10	0.43± 0.09 **

*, **: p<0.05, 0.01vs. Control group

表 2　体重と組織重量（3 週齢）

Item	Control	AA 100 ppm	AA 200 ppm	AA 400 ppm [a]
No. of animals	10	10	10	4 [b]
Body weight (g)	23.21 ± 0.76	21.52 ± 1.07 *	18.08 ± 1.62 **	7.63 ± 0.53 **
Absolute (g)				
Lungs	0.13 ± 0.01	0.13 ± 0.01	0.11 ± 0.02 **	0.07 ± 0.01 **
Liver	1.20 ± 0.07	1.10 ± 0.11	0.96 ± 0.16 **	0.32 ± 0.04 **
Kidneys	0.34 ± 0.01	0.32 ± 0.03	0.27 ± 0.05	0.13 ± 0.02 **
Testis	0.17 ± 0.01	0.13 ± 0.03 **	0.08 ± 0.03 **	0.03 ± 0.01 **
Relative (g%)				
Lungs	0.58 ± 0.04	0.60 ± 0.06	0.63 ± 0.08	0.93 ± 0.11
Liver	5.19 ± 0.24	5.10 ± 0.39	5.29 ± 0.53	4.26 ± 0.62
Kidneys	1.45 ± 0.05	1.48 ± 0.06	1.50 ± 0.14	1.63 ± 0.10
Testis	0.72 ± 0.04	0.62 ± 0.10	0.45 ± 0.11 *	0.33 ± 0.06 **

*, **: p<0.05, 0.01vs. Control group
*: In the group treated with 400 ppm AA, dose was changed into 300 ppm at 3 weeks.
[b]: Of the mice treated with 400 ppm AA, six animals died at 2 to 4 weeks.

表 3　AA 摂取量

Group	Water consumption (ml/mouse/day)	AA intake (mg/mouse)	AA intake (mg/kg/day)
Young			
Control	4.9	-	-
100 ppm	4.2	11.7	21.8
200 ppm	3.0	17.0	41.2
400 ppm	1.2	11.1	46.2
Adult			
Control	8.2	-	-
100 ppm	6.4	17.92	22.5
200 ppm	5.4	30.13	38.60
400 ppm	3.8	42.11	59.21

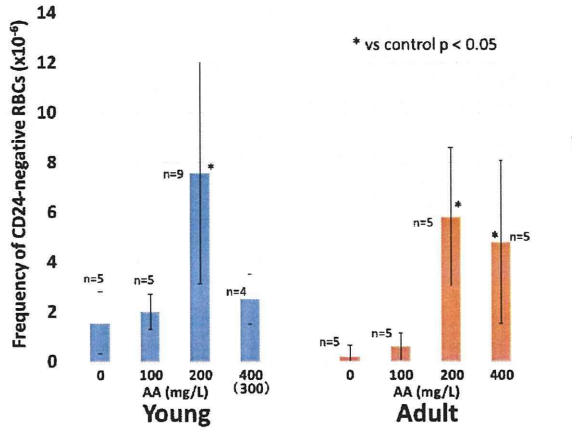

図 1　赤血球の Pig-a 突然変異

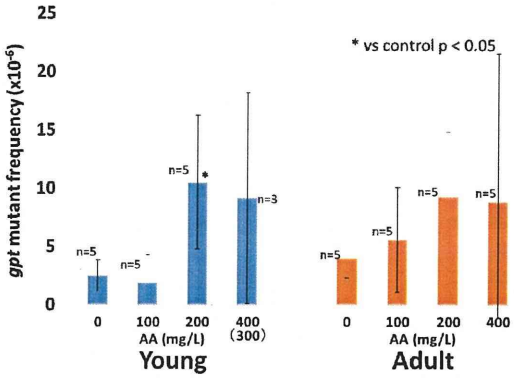

図２　精巣の gpt 突然変異

200ppm 以上で 3、10 週齢とも遺伝子突然変異の誘発が観察されたが、10 週齢では統計的有意差が無かった。また、週齢による差も認められなかった。

精巣での N7-GA-Gua アダクト量を図３に示す。

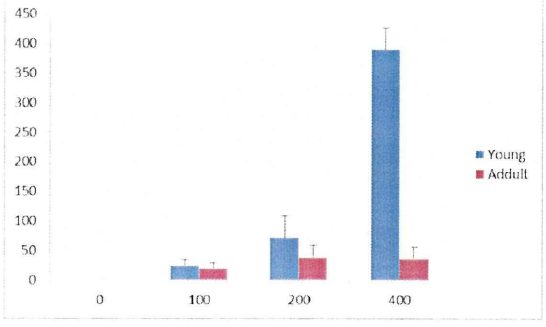

図３　精巣の N7-GA-Gua アダクト量

DNA アダクト量は 3、10 週齢とも用量依存的に増加した。200ppm 以上で週齢差が観察され、400ppm では 3 週齢の方が 10 週齢と比較して 10 倍程度の N7-GA-Gua の蓄積が観察された。

D．考　察

これまでの我々のラットを用いた研究では、AA を幼若、成熟ラットに飲水投与もしくは強制経口投与すると、多くの組織では遺伝毒性が観察されたが、幼若、成熟間での差は認められなかった。しかしながら精巣において、幼若ラットは成熟ラットと比較し、突然変異の誘発と、顕著な DNA アダクトの蓄積が認められた。

今年度は、このライフステージの違いによる AA の精巣での遺伝毒性感受性の差を確認する目的で、トランスジェニックマウスを用いて、AA の遺伝毒性感受性の差を検討した。3 週、および 10 週齢の gpt delta マウス（雄）に 0、100、200、400ppm の AA を 28 日間自由飲水経口摂取させ、赤血球の Pig-a 遺伝子突然変異と、精巣の gpt 遺伝子突然変異を検討した。また、精巣では AA の主たる DNA アダクトである N7-GA-Gua 量も解析した。

200ppm 以上で Pig-a（赤血球）、gpt（精巣）突然変異とも有意に増加したが、週齢間で差は見られなかったが。一方、DNA アダクト生成量（精巣）は、用量依存的に増加し、また幼若マウスでは蓄積量が顕著に高かった。体重当たりの AA 摂取量に週齢間で差は見られなかったことから、幼若期の精巣での AA の高蓄積性はライフステージに依存した AA の代謝に関連するものと考えられる。

幼若期の精巣での AA の高蓄積性はラットでも観察され、推定された摂取量を考慮しても幼若期では N7-GA-Gua アダクトが特異的に多く生成されるものと考えられる。AA は生体内で CYP2E1 によって代謝活性化を受け、グリシダミド（GA）に変化する。GA は N7-G-GA、N3-A-GA、N1-A-GA の 3 種類の DNA アダクトを生成することが知られている。一方、AA および GA はグルタチオントランスフェラーゼ

（GST）による抱合反応により解毒される。従って、アダクト生成量はCYP2E1による代謝活性化と、GSHによる解毒反応のバランスによって決定される。幼若ラットでのこれら代謝反応の寄与に関しては明らかではないが、最近Takahashiらが精巣中のGST活性が、幼若動物で有意に低いことを報告している。GSH反応の低下が、幼若動物の精巣でのアダクト量の増加を説明できるかもしれない。

DNAアダクト量が成熟、幼若動物で顕著である一方、他の遺伝毒性マーカの差は顕著ではない。この原因は不明であるが、今回の実験では幼若マウスの方が、同じAA摂取量であるにもかかわらず、より低濃度で毒性が発現したことから、幼若動物ではAAの毒性に対して感受性が高く、細胞死に引き起こしやすいのかもしれない。N7-GA-Guaアダクト量はこれを反映しているため、毒性評価の有効なバイオマーカーとなり得るかもしれない。

幼若期の精巣でのAAの高蓄積性はライフステージに依存したAAの代謝に関連するものと考えられる。種間で共通して観察されたことから、ヒトに対する影響も考えられ、AAの発がんリスクの検討には、小児に対して特別の配慮が必要と考えられる。

E．結　論

3週、および10週齢のgpt deltaマウス（雄）に0、100、200、400ppmのAAを28日間自由飲水経口摂取させ、赤血球のPig-a遺伝子突然変異と、精巣のgpt遺伝子突然変異とDNAアダクト量を解析した。200ppm以上でPig-a（赤血球）、gpt（精巣）突然変異とも有意に増加したが、週齢間で差は見られなかったが、DNAアダクト生成量（精巣）は、用量依存的に増加し、また幼若マウスでは蓄積量が顕著に高かった。幼若期の精巣でのAAの高蓄積性はライフステージに依存したAAの代謝に関連するものと考えられ、ヒトでも同様の影響も考えられる。AAの発がんリスクの検討には、小児に対して特別の配慮が必要かもしれない。

F.　研究発表

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小山直己、安井学、木村葵、高見成昭、鈴木拓也、増村健一、能美健彦、増田修一、木苗直秀、松田知成、、今井俊夫、本間正充；gptトランスジェニックラットを用いたライフステージ（週齢）を考慮したアクリルアミドの遺伝毒性評価 第38回日本トキシコロジー学会(2011.7)

G．知的所有権の取得状況

なし

別添５

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
	該当なし						

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Takami S, Imai T, et al.	Juvenile rats do not exhibit elevated sensitivity to acrylamide toxicity after oral administration for 12 weeks.	J Appl Toxicol	(in press)		
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Research Article

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Juvenile rats do not exhibit elevated sensitivity to acrylamide toxicity after oral administration for 12 weeks

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ABSTRACT: Acrylamide (AA), a neurotoxic, testicular toxic, genotoxic and carcinogenic chemical, has been reported to be formed in processed food, and sensitivity to AA intoxication in childhood is a concern. In the present study, to clarify the general toxicological profile of AA in juvenile rats, subchronic toxicity was evaluated in F344 rats administered AA in the drinking water at 0 (control), 10, 20 and 40 ppm, presented to the dams (three per group) immediately after the birth of their litters, through lactation (3 weeks), and directly to the offspring in their drinking water after weaning for a further 9 weeks (12 weeks total). Treatment with AA caused a decrease in body weights in 20 and 40 ppm F_1 females, compared with the controls. Average AA intake throughout the treatment period for the 10, 20 and 40 ppm groups after weaning was equivalent to 1.0, 2.1 and 4.4 mg kg^{-1} body weight per day, respectively, in males and 1.2, 2.5 and 4.9 mg kg^{-1} body weight per day, respectively, in females. No toxicologically significant organ weight changes were observed. AA-induced histopathological changes were limited to focal degeneration and necrosis of the seminiferous epithelium in the testes and desquamated epithelium in the ducts of epididymides, noted only in 40 ppm males. Taken together with previous reports, juvenile rats are not necessarily more susceptible to AA-induced toxicity as compared with young adults. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: acrylamide; juvenile rats; histopathology

INTRODUCTION

Acrylamide (AA), a vinyl monomer that improves the aqueous solubility, adhesion and cross-linking of polymers, is used as a mobility control agent for oil recovery, a flocculant for wastewater treatment and soil stabilization and a reagent for scientific research (IARC, 1994). There are many reports demonstrating various toxic effects of AA in experimental animals, including neurotoxicity (Burek *et al.*, 1980; LoPachin *et al.*, 2003; Tyl *et al.*, 2000a), testicular toxicity (Burek *et al.*, 1980; Yang *et al.*, 2005), reproductive toxicity (Tyl *et al.*, 2000a, b) and genotoxicity (Maniere *et al.*, 2005; Paulsson *et al.*, 2003; Yang *et al.*, 2005). In addition, AA increased the incidence of lung and skin tumors in mice and consistently induced mesotheliomas, thyroid follicular cell and mammary tumors in two carcinogenicity studies in rats (Bull *et al.*, 1984a, b; Friedman *et al.*, 1995; Johnson *et al.*, 1986). The International Agency for Research on Cancer (IARC) has determined that AA is probably carcinogenic to humans (group 2A; IARC, 1994).

There have been several peripheral neuropathy cases as a result of AA intoxication by potential occupational exposure in man (Garland and Patterson, 1967; IARC, 1994; Spencer and Schaumburg, 1974, 1975), but environmental exposure under natural conditions, except for cigarette smoking, was previously thought to be excluded and controllable in the general population (Bergmark, 1997; IARC, 1994; Spencer and Schaumburg, 1975). However, in 2002, new analytical data from Swedish scientists showed spontaneous AA formation in fried and baked foods at various concentrations, e.g. moderate levels of AA (5–50 μg kg^{-1}) were detectable in heated protein-rich foods and higher contents (150–4000 μg kg^{-1}) were detectable in carbohydrate-rich foods, while AA could not be measured in unheated control or boiled foods (<5 μg kg^{-1}) (Tareke *et al.*, 2002). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) concluded that the average AA intake for the general population and high consumers was approximately 1 and 4 μg kg^{-1} body weight per day, respectively, with margin of exposure (MOE) values of 200 and 50 for morphological changes, such as demyelination and/or degeneration of axons in nerves in rats, 310 and 78 for induction of mammary tumors in rats and 180 and 45 for induction of Harderian gland tumors in mice. These MOE values are relatively low compared with other genotoxic contaminants in foods (JECFA, 2010). Furthermore, human exposure levels to AA from cooked foods during childhood are estimated to be higher than in adults

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(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 ± SD), 0.82 ± 0.50, 0.89 ± 0.42 and $0.51 \pm 0.38\%$, respectively, in males and 0.16 ± 0.19, 0.29 ± 0.11, 0.33 ± 0.21 and $0.22 \pm 0.10\%$, 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.

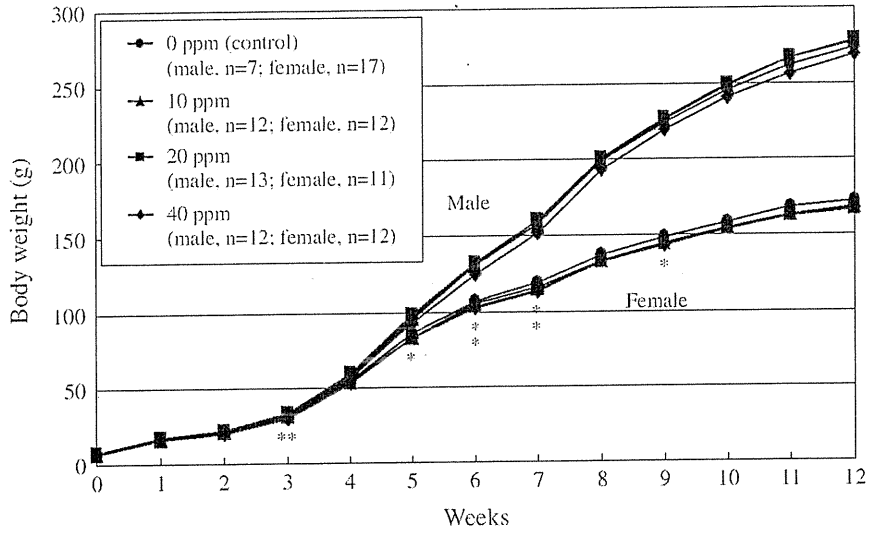

Figure 1. Experiment 1 – body weight curves for rats given drinking water containing acrylamide for 12 weeks. *, **Significantly different from the controls at $P<0.05$ and 0.01, respectively.

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

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.

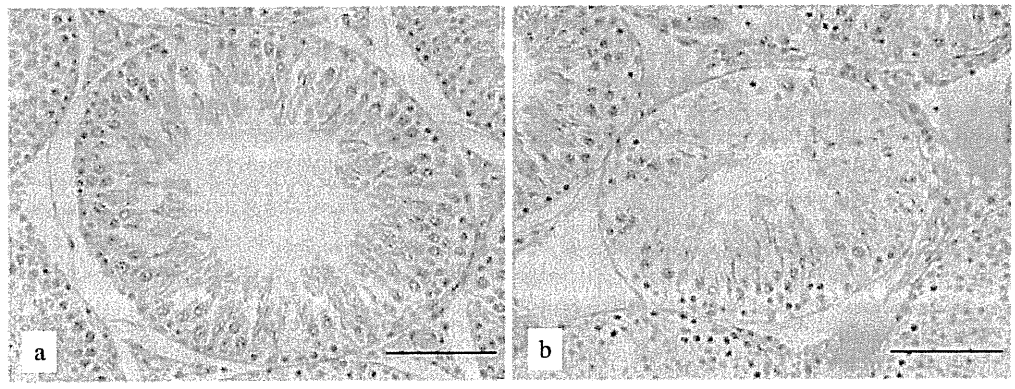

Figure 2. Experiment 1 – testis. (a) Control, a male. The seminiferous tubules of the testis are normal. The tubule in the central zone is in stage XIII. (b) The 40 ppm group, a male. Note the degeneration and necrosis of seminiferous tubules. The tubule in the central zone is in stages XII–XIII. Bar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

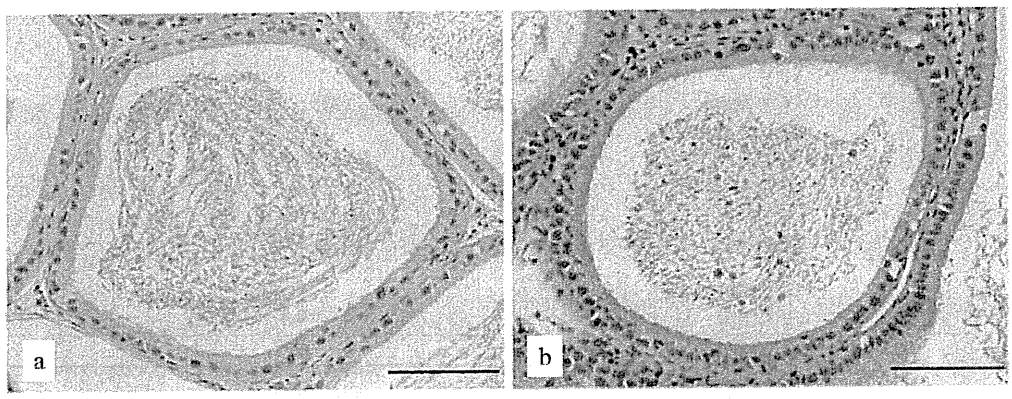

Figure 3. Experiment 1 – epididymis. (a) Control, a male. The epididymal ducts of the epididymis are normal. (b) The 40 ppm group, a male. Note desquamated epithelial cells in the epididymal ducts of the epididymis. Bar = 100 μm. HE staining.

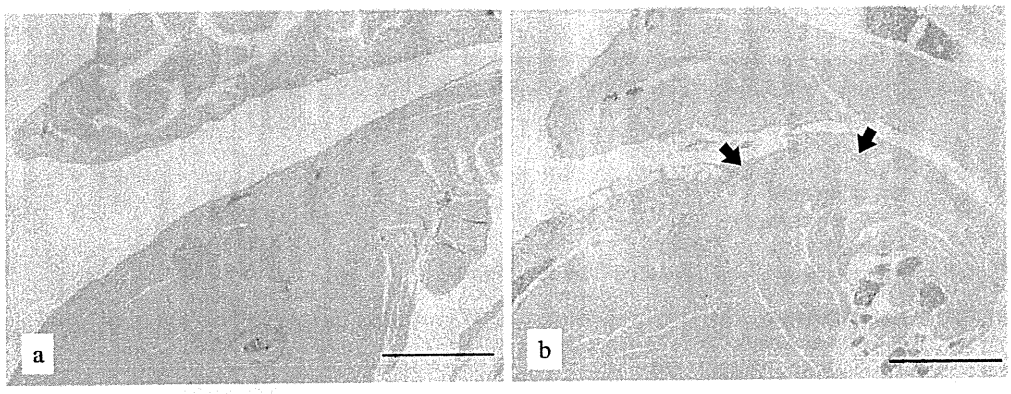

Figure 4. Experiment 1 – heart. (a) Control, a male. The side of the right ventricle of the heart is normal. (b) The 40 ppm group, a male. Note moderate infiltration of lymphocytes and macrophages with degeneration of myofibers (arrows) in the heart. Bar = 500 μm. HE staining.

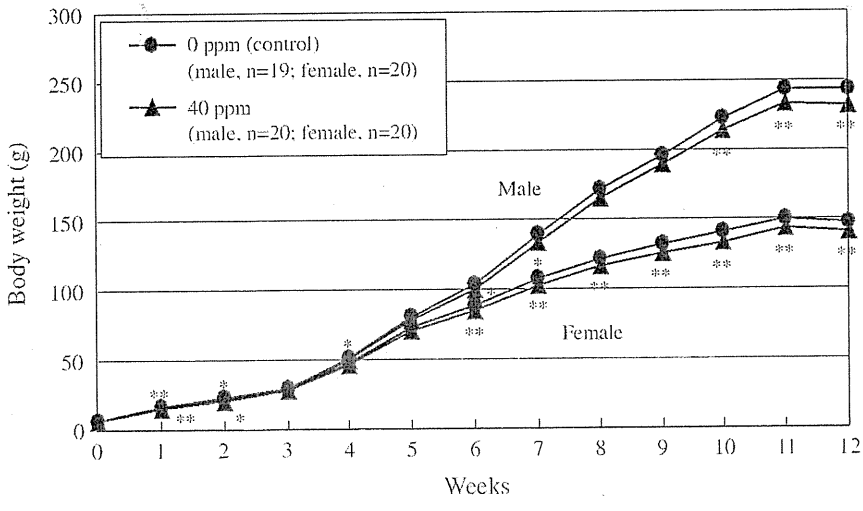

Figure 5. Experiment 2 – body weight curves for rats given drinking water containing acrylamide for 12 weeks. *, **Significantly different from the controls at $P < 0.05$, 0.01, respectively.

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

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.

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