Table 4-2 Histopathological findings for male rats administered AA followed by MNU/SDM treatment

Organs	Findings	Groups	0 ppm (control)	20 ppm	40 ppm	80 ppm	0 ppm + SDM	40 ppm + SDM
		No. of animals	23	23	21	23	23	20
Lung								
	Alveolar epithelial hyperplasia		20 (87)	13 (57) *	13 (62)	14 (61) *	9 (39) ***	5 (25) ***
	Adenoma		13 (57)	18 (78)	16 (76)	17 (74)	22 (96) **	17 (85) *
	Adenocarcinoma		6 (26)	12 (52)	10 (48)	14 (61) *	16 (70) **	9 (45)
	Adenoma + Adenocarcinoma		15 (65)	20 (87)	17 (81)	18 (78)	23 (100) **	20 (100) **
Skin								
	Keratoacanthoma		2 (9)	4 (17)	3 (14)	2 (9)	5 (22)	3 (15)
Forestomach								
	Squamous cell hyperplasia		9 (39)	8 (35)	9 (43)	6 (26)	7 (30)	3 (15)
Small intestine								
	Adenocarcinoma		1 (4)	2 (9)	4 (19)	0 (0)	0 (0)	0 (0)
Large intestine								
	Adenocarcinoma		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 5-1 Histopathological findings for female rats administered AA followed by MNU/SDM treatment

Organs	Findings	Groups	0 ppm (control)	20 ppm	40 ppm	80 ppm	0 ppm + SDM	40 ppm + SDM
		No. of animals	22	24	24	22	24	19
Thyroid								
	Focal hyperplasia		0 (0)	0 (0)	1 (4)	0 (0)	14 (58) ***	12 (63) ***
	Follicular adenoma		0 (0)	0 (0)	0 (0)	0 (0)	2 (8)	4 (21) *
	Follicular carcinoma		0 (0)	0 (0)	1 (4)	0 (0)	2 (8)	1 (5)
	Adenoma + Carcinoma		0 (0)	0 (0)	1 (4)	0 (0)	4 (17)	4 (21) *
Mammary gland								
	Fibroadenoma		0 (0)	1 (4)	0 (0)	1 (5)	1 (4)	0 (0)
	Adenocarcinoma		9 (41)	9 (38)	7 (29)	8 (36)	16 (67)	7 (37)
Brain								
	Malignant astrocytoma		0 (0)	1 (4)	0 (0)	0 (0)	0 (0)	1 (5)
Spinal cord								
	Oligodendroglioma		0 (0)	0 (0)	0 (0)	1 (5)	0 (0)	0 (0)
Pituitary								
	Focal hyperplasia		0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (5)
Uterus								
	Cystic endometrial hyperplasia		4 (18)	3 (13)	3 (13)	4 (18)	4 (17)	4 (21)
	Endometrial stromal polyp		1 (5)	3 (13)	1 (4)	1 (5)	4 (17)	1 (5)
	Focal glandular hyperplasia		0 (0)	2 (8)	2 (8)	1 (5)	2 (8)	4 (21) *
	Endometrial adenoma		0 (0)	0 (0)	0 (0)	0 (0)	1 (4)	0 (0)
Clitoral gland								
	Adenoma		2 (9)	1 (4)	0 (0)	0 (0)	2 (8)	0 (0)
	Carcinoma		2 (9)	1 (4)	2 (8)	1 (5)	1 (4)	1 (5)
Oral cavity								
	Squamous cell hyperplasia		0 (0)	1 (4)	4 (17)	2 (9)	2 (8)	2 (11)

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

Table 5-2 Histopathological findings for female rats administered AA followed by MNU/SDM treatment

Organs	Findings	Groups	0 ppm (control)	20 ppm	40 ppm	80 ppm	0 ppm + SDM	40 ppm + SDM
		No. of animals	22	24	24	22	24	19
Lung								
	Alveolar epithelial hyperplasia		12 (55)	8 (33)	7 (29)	11 (50)	13 (54)	11 (58)
	Adenoma		12 (55)	10 (42)	12 (50)	10 (45)	9 (38)	9 (47)
	Adenocarcinoma		0 (0)	3 (13)	2 (8)	1 (5)	4 (17)	3 (16)
	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)

別添4

厚生労働科学研究費補助金・食品の安心・安全性確保推進研究事業
食品中の遺伝毒性を有する有害物質のリスク管理に資する総合研究

平成20年度分担研究報告書

ライフステージを勘案したアクリルアミドの遺伝毒性誘発機構の解析

研究分担者： 本間正充 国立医薬品食品衛生研究所 変異遺伝部第一室 室長
研究協力者： 安井 学 国立医薬品食品衛生研究所 変異遺伝部第一室
小山直己 国立医薬品食品衛生研究所 変異遺伝部第一室

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、スナック菓子等に比較的多く含まれることが報告されて以来、小児への影響が懸念されている。本研究は、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する目的で、3（幼若）, 11(成熟)週齢の各群のトランスジェニック雄ラット(*gpt* delta F344、またはSDラット)にAAを20～80 ppmまたは50～200 ppmを28日間飲水投与し、各種臓器における遺伝毒性試験[突然変異(肝臓、精巣)、小核(末梢血、骨髄、精巣)、コメット(肝臓、精巣)]を実施した。また、甲状腺、乳腺、肝臓、精巣でのDNAアダクトも測定した。成熟、および幼若ラットでは末梢血もしくは骨髄での小核の用量依存的な増加と、肝臓でのコメット試験によるDNA損傷が確認されたが、成熟、および幼若ラットでこれら誘発性に顕著な差は認められなかった。また、肝臓でのgptでの突然変異は成熟、幼若ラット共に誘発は観察されなかった。一方、精巣でのgptの突然変異は幼若ラットのみで観察された。精巣においてはコメット試験、小核試験でも幼若ラットで高い感受性が観察された。DNAアダクトに関しては、肝臓、乳腺、精巣で用量依存的な蓄積が認められ、特に精巣では幼若ラットで成熟ラットの5倍以上ものアダクトの蓄積を認めた。これらのことから、特に精巣に関してアダクトの蓄積量と相関する幼若ラットでの高い遺伝毒性感受性が明らかとなった。本来、AAには精巣で強い染色体異常が観察されることが報告されており、今後、精巣に対するAAの遺伝毒性メカニズムを解明すると同時に、小児に対するAAの遺伝毒性リスクを考察したい。

キーワード；アクリルアミド、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、強い遺伝素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、日常生活に置いて、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 はグルタチオン還元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。

今年度は in vivo での AA の遺伝毒性発現の特徴と、ライフステージにおけるそれら違いを検討するために、3（幼若）, 11(成熟)週齢の gpt デルタトランスジェニックラット(F443)、と SD ラットを用いて多臓器（末梢血、骨髄、肝臓、精巣）マルチエンドポイント（gpt 遺伝子突然変異試験、小核試験、コメット試験）の遺伝毒性試験を行った。また、AA の発がん標的組織と考えられる甲状腺、乳腺、および遺伝毒性試験の対象となる肝臓、精巣での DNA アダクトの定量を行った。

B．研究方法

1）In vivo 試験(1)：トランスジェニック F344 ラット

i) 動物

F344 を系統とする gpt デルタトランスジェニックラット（雄）を日本エスエルシー(株)より購入し、馴化後 3 週（幼若）、および 11 週齢(成熟)の動物を使用した。

ii) 投与

gpt ラットを 4 群各 5 匹ずつに分け、それぞれの群に蒸留水で調整した 0、20、40、80ppm 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) 小核試験

投与開始 2 日後、28 日後（最終投与日）に、動物の尾静脈または心臓から血液を約 100 uL 採取し、小核試験を行った。また、投与開始 28 日後（最終投与日）に、精巣を摘出して、小核試験を行った。小核試験は林らの方法に従って行った。

iv) アルカリコメット試験

最終投与日に屠殺後解剖し、肝臓の左側葉の一部及び胃を採取し、アルカリコメット試験を行った。アルカリコメット試験は JaCVAM コメット試験共同研究のプロトコールに従った。

v) 遺伝子突然変異試験

アルカリコメット試験用のサンプル採取後、肝臓及び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。

vi) その他

肝臓、精巣、甲状腺、乳腺の一部から DNA を抽出し、DNA アダクト検出用サンプルとした。また、肝臓、精巣の一部から病理切片標本を作成し、病理学的検討を行った。

2) In vivo 試験(2) : SD ラット

i) 動物

SD ラット（雄）を日本エスエルシー(株)より購入し、馴化後 3 週（幼若）、および 11 週齢(成熟)の動物を使用した。

ii) 投与

SD ラットを 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、50、100、200ppm 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) 小核試験

投与開始 28 日後（最終投与日）に、動物の尾静脈または心臓から血液を約 100 uL 採取し、小核試験を行った。また、投与開始 28 日後 (最終投与日) に、精巣を摘出して、小核試験を行った。小核試験は林らの方法に従って行った。

iv) アルカリコメット試験

最終投与日に屠殺後解剖し、肝臓の左側葉の一部及び胃を採取し、アルカリコメット試験を行った。アルカリコメット試験は JaCVAM コメット試験共同研究のプロトコールに従った。

3) DNA アダクトの定量

AA による主たる DNA アダクトである *N*7-GA-Gua を LC/MS/MS により測定した。*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

1） トランスジェニック F344 ラットでの試験

gpt デルタトランスジェニックラットを用いて多臓器、マルチエンドポイントの遺伝毒性試験を行った。それぞれの群に蒸留水で調整した 0、20、40、80ppm 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。28 日間飲水投与し、肝臓、精巣における遺伝子突然変異、血液、精巣の小核、肝臓のコメット試験を行った。

肝臓ではわずかであるがコメット試験による DNA 損傷が確認されたが、幼若(3W)と成熟（11W）での顕著な差は認められなかった(図 1)。

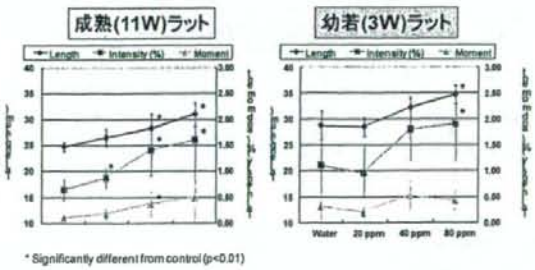

図 1

一方、骨髄での小核の誘発に関しては成熟ラットでは顕著な誘発は認められなかったが、幼若ラットのみにて最高用量で統計的に有意差が認められた(図２)。

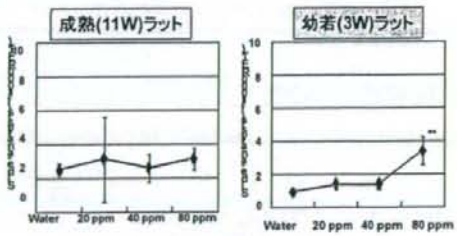

図２

gpt 突然変異に関してはすべての解析はまだ、終了していないが、これまでのところ成熟、幼若ラットにおける AA による肝臓での突然変異の誘発性は認められず、また成熟、幼若の差も認められなかった。精巣に関しては最高用量のみ(80ppm)で幼若ラットで突然変異の誘発に有意差が認められた（表１)。

成熟(11W)ラット

Acrylamide Dose	Number of rat	Total Population	confirmed 6TG Mutants	Mutant Frequency (X 10 $^{-6}$)
solvent	5	5,277,000	10	2.11±1.87
20ppm	5	8,097,000	24	3.23±1.79
40ppm	5	12,864,000	6	0.50±0.20
80ppm	5	7,296,000	22	4.34±2.86

幼若(3W)ラット

Acrylamide Dose	Number of rat	Total Population	confirmed 6TG Mutants	Mutant Frequency (X 10 $^{-6}$)
solvent	4	2,745,000	7	2.38±1.67
20ppm	3	2,670,000	4	2.23±2.68
40ppm	3	4,008,000	6	1.57±0.63
80ppm	4	3,540,000	16	4.85±2.01

表１

AA の主たる DNA アダクトである *N*7-GA-Gua について LC/MS/MS を用いて、肝臓、甲状腺、乳腺、精巣で解析を行った（図３)。

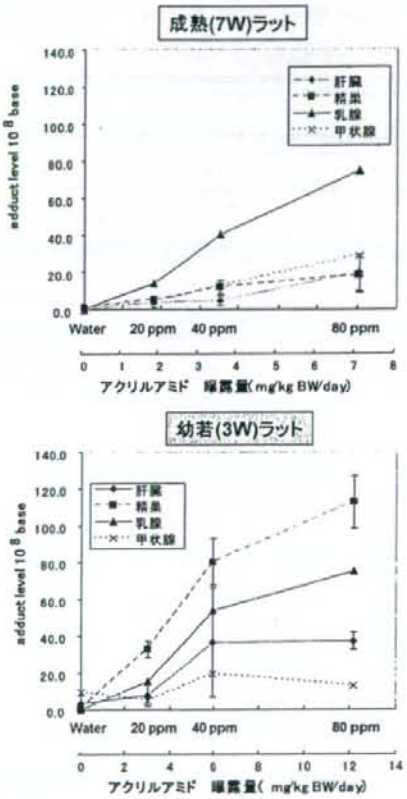

図３

肝臓、乳腺、精巣で用量依存的な増加を観察した。乳腺では成熟、幼若ラット共に同程度の高いアダクトの生成が観察された。肝臓、精巣では成熟、幼若ラットにとも用量依存的な増加を示し、さらに幼若、成熟ラットで差が観察された。特に、精巣においては幼若ラットで５倍以上ものアダクトの蓄積が観察された。

２）SD ラットでの試験

SD ラットのそれぞれの群に蒸留水で

調整した0、50、100、200ppm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。末梢血と精巣での小核試験、肝臓、精巣でのコメット試験を実施した。

末梢血ので小核は最高用量でわずかに増加したが、幼若、成熟ラットでの差は認められなかった。肝臓でのコメットも用量依存的に両群で増加したが、むしろ幼若ラットの方が増加率は低かった。

一方、精巣に関しては小核、コメットともに幼若ラットでのみ用量依存的な反応性と、有意な誘発を認めた。精巣はAAに対する遺伝毒性に対して、特に幼若マウスで感受性が高いことが示された。

D．考　察

これまでの我々in vitroの研究では、AAとGAの遺伝毒性について比較したところ、AAはほとんどin vitroでは毒性を示さなかったのに対して、GAの遺伝毒性は強く、主として点突然変異を主体とするDNA損傷作用を持つことを報告した。このことから、AAの毒性の本体はその代謝物であるGAであると結論した。AAはCYP2E1により、エポキシ環をもつGAに変換され、強い遺伝毒性物質に変わるが予想される。

Manjanathaはトランスジェニック BigBlueマウスを用いてAAとGAの突然変異の検討を行った。雄雌ラットそれぞれに100、500ppmの飲水で4週間日間投与し、肝臓での突然変異を見たところ、両化合物とも有意に上昇し、むしろAAでその誘発率が高いと報告している。また、突然変異スペクトルを比較したところ、AAもGAもGC>TAのトランスバージョンを主体とする点突然変異であることが示された。

我々の今回の実験はこのManjanathaらの報告に基づいて行った。しかしながら、100、500ppmの飲水投与は非常に毒性が強く、特に幼若ラットでの生存性に問題があると判断したため、20-200ppmの低濃度領域で試験を行った。尚、100ppmの飲水投与でのAA摂取量は10mg/kg/dayに相当し、これはこれまでのAAでの毒性試験の1/10程度の量に相当する。全ての試験動物について生育に伴う顕著な体重の抑制、飲水量の変化は認められなかった。また、28日後の剖検においても顕著な病理的異常は認められなかった。

骨髄、末梢血に関しては、予想通り顕著な小核誘発は認められなかった。Manjanathaらの報告でも、100ppmでは骨髄小核の誘発は認められていない。我々のgptラットを用いた試験では統計的には幼若ラットでのみ末梢血、骨髄で有意差が認められた。また、SDラットでは両者で有意差が認められた。従って、造血系に対してAAは弱い遺伝毒性を示すが、幼若ラットで特に強い影響があるとは考えにくい。

一方、肝臓ではコメット、gpt突然変異が試験された。コメットは2回の実験で用量依存的な陽性反応を示した。一般にコメット試験は処理直後に試験を実施しないと、DNA修復が働くため、DNA損傷の検出が困難であると言われているが、今回の実験で、飲水投与のような長期暴露によるDNA損傷も検出できることがわかった。gpt突然変異に関しては全ての用量で無処理群と比較して突然変異の増加は観察されなかった。Manjanathaの報告でも100ppmでは突然変異の誘発は顕著でなく、我々の80ppmでは

突然変異の誘発がないことはこのことを支持するものである。

精巣に関しては、SDラットの実験で、成熟、幼若ラットと共に用量依存性の増加が観察された。また、この増加は幼若ラットで顕著であった。gptラットでの突然変異でも80ppmの最高用量において両者で突然変異の増加が観察された。また、この増加は幼若ラットのみで有意であった。これらの結果は、少なくとも精巣に関しては幼若ラットがAAの遺伝毒性に強い感受性を持つことを示している。

DNAアダクトの結果はこのことを裏付けるものである。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が、N7-G-GAが全体の90%以上を占めるため、今回、このアダクトにのみ注目して測定した。精巣でのN7-G—GAは幼若、成熟ラットともに用量依存的に増加し、特に幼若ラットでは80ppmで120/10^8bpの増加量であった。これはバックグランドレベルの20倍で成熟ラットの場合と比較しても6倍以上高い。

これまで、精巣にはAAのアダクトが蓄積しやすく、その原因としてプロタミンとの結合が考えられている。また、その約5%はDNAともアダクトを形成する。AAは精巣細胞に強い遺伝毒性を示し、転座型の染色体異常を示すこと、低い濃度でも優性致死試験で陽性を示すことが知られている。このようにAAは特に生殖細胞に遺伝毒性を示し、それがアダクトの生成と相関するものと考えられる。さらに今回、我々の実験ではこの傾向が幼若ラットで顕著に現れることが示された。この原因については不明であり、今後の解明が待たれるが、少なくともAAの生殖細胞に対する遺伝毒性感受性には年齢が関係しており、幼年期でのAAの過剰摂取に関しては、注意が必要であると考えられる。

また、乳腺でも高いDNAアダクトが観察された。乳腺はAAの発がん標的組織の一つであり、今後雌ラットなどでの検証も必要である。

E. 結　論

低用量（20-100ppm）のアクリルアミドを飲水で成熟、および幼若ラットに投与し、各種遺伝毒性と、DNAアダクト形成を比較し、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。多くの組織で、ライフステージの違いよる差は認められなかったのに対して、精巣ではDNA損傷の蓄積、小核の誘発、遺伝子突然変異の誘発が幼若ラットで顕著であった。また、それに対応したDNAアダクト量の増加も観察された。特にアダクト量は、幼若ラットで成熟ラットの5倍以上もの蓄積を認めた。本来、AAには生殖細胞に対する強い遺伝毒性が報告されている。今後、ライフステージを勘案した生殖細胞に対するAAの遺伝毒性メカニズムを解明すると同時に、低年齢層に対するAAの遺伝毒性リスクを考察すべきである。

F. 研究発表

1．論文発表

Yatagai, F., Umebayashi, Y., Honma, M., Sugasawa, K., Takayama, Y., and Hanaoka, F. Mutagenic radioadaptation in a human

lymphoblastoid cell line. Mutat. Res., 638, 48-55 (2008)

Yasui, M., Suenaga, E., Koyama, N., Masutani.C., Hanaoka, F., Gruz, P., Shibutani, S., Nohmi T., Hayashi, M., and Honma, M. Miscoding properties of 2'-deoxyinosine, a nitric oxide-derived DNA Adduct, during translesion synthesis catalyzed by human DNA polymerases. J Mol Biol, 377, 1015-1023 (2008)

Yatagai, F., Suzuki, M., Ishioka, N., Ohmori, H., Honma, M. Repair of I-SceI Induced DSB at a specific site of chromosome in human cells: influence of low-dose, low-dose-rate gamma-rays. Radiat Environ Biophys 47, 439-44 (2008)

Nhomi, T., Toyoda-Hokaiwado, N., Yamada, M., Masumura, K., Honma, M., and Fukushima, S. International Symposium on Genotoxic and Carcinogenic Thresholds. Genes and Environment, 30, 101-107 (2008)

本間正充；遺伝毒性物質に閾値はあるのか？ファルマシア 45, 143-148 (2009)

2．学会発表

本間正充: 医薬品に不純物として含まれる遺伝毒性物質の分類と許容量 第 35 回日本トキシコロジー学会学術年会(2008.6)

本間正充: ICH における新しい遺伝毒性試験ガイドライン（S2R1）と試験実施タイミング 第 35 回日本トキシコロジー学会学術年会(2008.6)

Honma, M. Ultimate Threshold and Genetic Consequence of A Single Double Strand Break in Human Cells. International Symposium on Genotoxic and Carcinogenic Thresholds (2008.7)

Honma, M. Genome Mapping of Damaged Chromosome Regions Induced by Ionizing Irradiation Using DNA Microarray Analysis. 38th European Environmental Mutagen Society (2008.9)

鈴木孝昌、小木美恵子、小原有弘、本間正充、田邊思帆里、山口照英：SNP および CGH マイクロアレイを用いた c-myc 遺伝子増幅に関する詳細解析 第 67 回日本癌学会総会(2008.10)

谷田貝文夫、菅澤薫、榎本秀一、本間正充：DSB 修復効率からの適応応答の追求 日本放射線影響学会第 51 回大会(2008.11)

本間正充：DNA 二本鎖切断修復と遺伝的不安定性 日本環境変異原学会第 37 回大会(2008.12)

安井 学、本間正充：ヒトリンパ球細胞のゲノム内に導入させた 8-オキソグアニン 1 分子の突然変異誘発能 日本環境変異原学会第 37 回大会(2008.12)

小山直己、木村葵、安井学、高見成昭、高橋美和、今井俊夫、山本歩、汲田和歌子、増村健一、増田修一、木苗直秀、松田知成、能美健彦、本間正充：ライフステージを考慮したアクリルアミドの多臓器遺伝毒性評価 日本環境変異原学会第 37 回大会

(2008.12)

斉藤美香、松藤寛、千野誠、林真、本間正充、山形一雄:過酸化水素によって誘導されたヒトリンパ芽球細胞 TK6 の細胞増殖と遺伝毒性に対する天然抗酸化物質の保護効果　日本環境変異原学会第 37 回大会(2008.12)

木村葵、坂本浩子、西郷和彦、洲加本孝幸、本間正充:*In vitro* コメットアッセイプロトコールの検証　日本環境変異原学会第 37 回大会(2008.12)

Wang, J., Sawyer, J., Honma, M., Chen, T., and Moore, M. The Mouse Lymphoma Assay detects recombination, deletion, and aneuploidy. 日本環境変異原学会第 37 回大会(2008.12)

谷田貝文夫、高橋昭久、本間正充、鈴木ひろみ、大森克徳、関真也、橋爪藤子、島津徹、榎本秀一、大西武雄、石岡憲昭:宇宙実験:放射線影響の LOH 検出系による解析　日本環境変異原学会第 37 回大会(2008.12)

鈴木孝昌、小泉朋子、本間正充、中嶋圓、濱田修一、渡辺貴志、降旗千恵:トキシコゲノミクスに関する JEMS/MMS 共同研究 II:遺伝子傷害性発癌物質の迅速スクリーニング系としての TaqMan Low Density Array の評価　日本環境変異原学会第 37 回大会(2008.12)

本間正充、櫻庭真弓、汲田和歌子、林　真:DNA マイクロアレイによる放射線損傷領域のゲノムマッピング　日本環境変異原学会第 37 回大会(2008.12)

中嶋圓、鈴木雅也、田中仁、本間正充、林真:*In vitro* コメットアッセイ国際バリデーションデータ解析に関する一考察　日本環境変異原学会第 37 回大会(2008.12)

安井学、Suzuki, N.、本間正充、Shibutani, S.:一酸化窒素によって形成する DNA 付加体の誤塩基対形成メカニズム　第 31 回分子生物学会(2008.12)

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
Takahashi, M., Shibutani, M., et al.	Pathological assessment of the nervous and male reproductive systems of rat offspring exposed maternally to acrylamide during the gestation and lactation periods - a preliminary study.	J. Toxicol. Sci.	33, 1	11-24	2008
Takahashi, M., Shibutani, M., et al.	Limited lactational transfer of acrylamide to rat offspring on maternal oral administration during the gestation and lactation periods.	Arch. Toxicol.		In press	2009

The Journal of Toxicological Sciences (J. Toxicol. Sci.)
Vol.33, No.1, 11-24, 2008

Original Article

Pathological assessment of the nervous and male reproductive systems of rat offspring exposed maternally to acrylamide during the gestation and lactation periods – a preliminary study

Miwa Takahashi[1], Makoto Shibutani[1,2], Kaoru Inoue[1], Hitoshi Fujimoto[1], Masao Hirose[1,3] and Akiyoshi Nishikawa[1]

[1]Division of Pathology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
[2]Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu City, Tokyo 183-8509, Japan
[3]Food Safety Commission, 2-13-10 Prudential Tower 6th Floor, Nagata-cho, Chiyoda-ku, Tokyo 100-8989, Japan

(Received September 10, 2007; Accepted October 16, 2007)

ABSTRACT — To evaluate the developmental effects of exposure to acrylamide (ACR) on the nervous and male reproductive systems, pregnant Sprague-Dawley rats were given ACR at 0, 50, 100 or 200 ppm in the drinking water from gestational day 10 to postnatal day 21 and histopathological assessment of offspring was performed at weaning and postnatal week 11. Neurotoxicity was quantitatively assessed with reference to nerve fiber density, percentages of degenerated and small caliber axons in the sciatic nerves, and numbers of aberrant dot-like structures immunoreactive for synaptophysin in the cerebellar molecular layer. Although maternal neurotoxicity was evident from 100 ppm, no changes suggestive of neurotoxicity or testicular toxicity were observed in offspring. However, lowering of body weights was dose-dependently observed from birth at the dose levels of ≥50 ppm in males and ≥100 ppm in females. Maternal malnutrition was apparent at ≥100 ppm during the lactation period. Therefore, poor lactational ACR-exposure due to maternal toxicity might account for the lack of ACR-induced offspring toxicity other than retarded body growth.

Key words: Acrylamide; Neurotoxicity; Testicular toxicity; Developmental exposure; Rat

INTRODUCTION

Acrylamide (ACR), a water-soluble vinyl monomer, is widely used to synthesize polymers for industrial applications such as soil conditioning, wastewater treatment, and cosmetic, paper and textile industries (Friedman, 2003). Exposure to monometric ACR results in peripheral neuropathy characterized by ataxia, skeletal muscle weakness, and weight loss, and multifocal neurofilamentous swelling and eventual degeneration beginning from the distal ends of peripheral nerve axons (Spencer and Schaumburg, 1974; Le Quesne, 1985; LoPachin and Lehning, 1994). The initial target appears to be nerve terminals, in both the central and peripheral nervous systems, and the result is autonomic, behavioral, sensory, and motor disturbance (LoPachin *et al.*, 2003), although the underlying mechanisms remain controversial. Additionally, ACR has carcinogenic potential (Dearfield *et al.*, 1988; IARC, 1994), and exerts adverse effects on male reproduction, including dominant lethality, degeneration of testicular epithelial tissue, and impaired fertilization (Sakamoto *et al.*, 1988; Adler *et al.*, 2000; Tyl and Friedman, 2003). It has been demonstrated that reproductive toxicity is not only induced by ACR, but also by glycidamide (GA), an oxidized metabolite of ACR generated by CYP2E1, which exerts clastogenic effects on spermatids (Adler *et al.*, 2000; Costa

Correspondence: Makoto Shibutani (E-mail: shibutan@nihs.go.jp)

et al., 1992; Ghanayem *et al.*, 2005).

Recently, exposure to ACR in foodstuffs has become a worldwide concern because of its generation in a variety of fried and oven-baked foods during cooking through Maillard reactions of sugars with asparagine residues (Mottram *et al.*, 2002; Friedman, 2003). The 64th Joint FAO/WHO Expert Committee on Food Additives concluded that an intake of 1 μg/kg body weight/day of ACR could be taken to represent the average for the general population (WHO/IPCS, 2006). However, infants and small children may be more highly exposed to ACR due to their lower bodyweights and high consumption of snacks, so that their relative ACR intake is estimated to be 2 to 3-fold higher than in adults (WHO/IPCS, 2006). Because toxicity studies of ACR have hitherto mainly been performed using adult animals to mimic occupational exposure, the data for effects during fetal, infantile and pubertal periods are rather limited. Therefore, it is important for risk assessment of ACR exposure in human to evaluate toxicity taking into account physiological differences between adults and fetuses/infants that might influence sensitivity.

Regarding age-related susceptibility to ACR-induced toxicity, data from animal studies are insufficient and the findings are inconsistent (Kaplan and Murphy, 1972; Ko *et al.*, 1999). Placental transfer of ACR has been reported in experimental animals and humans, and the fetal internal level of ACR on a weight-adjusted basis was estimated to be at least equal to that of human mothers (Ikeda *et al.*, 1983; Schettgen *et al.*, 2004). The potential for exposure to ACR through human milk has been demonstrated in a study conducted with two women (Sörgel *et al.*, 2002). It is evident that ACR is a developmental toxicant in rodents, because suppression of offspring body weight results from maternal exposure (Zenick *et al.*, 1986; Field *et al.*, 1990; Wise *et al.*, 1995; Garey *et al.*, 2005). Furthermore, it has been reported that ACR can produce developmental neurotoxicity, such as decreased motor activity and auditory startle responses in Sprague-Dawley (SD) rat offspring, at a maternal gavage dose of 15 mg/kg body weight/day, a daily dose level that can cause neurotoxicity to maternal animals (Wise *et al.*, 1995).

Many previous studies on ACR-induced toxicity during the developmental stages were conducted with the focus on behavioral endpoints, but detailed assessment of changes in target organs/tissues has been scarce. In the present study, we thereby performed a histopathological assessment of the nervous and male reproductive systems of rat offspring exposed maternally to ACR during the gestation and lactation periods. In humans, since exposure to ACR occurs through ingestion of food in general population, it is reasonable to examine toxic effects of such chemicals by oral administration through food or drinking water to experimental animals. In the present study, ACR was administered to maternal rats through drinking water with the dose levels in inducing histologically apparent neurotoxicity and testicular toxicity after 4 weeks administration in adult male SD rats in our previous studies (Lee *et al.*, 2005; Woo *et al.*, 2007).

MATERIALS AND METHODS

Chemicals and animals

ACR was purchased from Sigma (St. Louis, MO, USA; CAS #79-06-1) as a white powder with a purity of >98%. Pregnant CD® (SD) IGS rats were obtained from Charles River Japan Inc. (Kanagawa, Japan) at gestational day (GD) 3 (the day when a vaginal plug was observed was designated as GD 0) and housed individually in polycarbonate cages with wood chip bedding, maintained in an air-conditioned animal room (temperature: 24 ± 1°C, relative humidity: 55 ± 5%) with a 12-hr light/dark cycle. They received powdered basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water *ad libitum* during the 1 week acclimatization period.

Experimental design

On GD 10, dams were randomly divided into 4 groups of 3 dams and given ACR at 0, 50, 100 or 200 ppm in the drinking water from GD 10 to postnatal day (PND) 21. The highest dose was chosen since it is known to induce neurotoxicity within 4 weeks in adult male rats of the same strain used in the present study (Lee *et al.*, 2005). Litters were culled randomly to preserve 8 pups, mostly 4 per sex per litter on PND 3. On PND 21, the offspring were weaned, and all dams and half of the pups were killed by exsanguination from the abdominal aorta under deep anesthesia with ether. The remaining offspring were housed up to 4 littermates of each sex per cage, and maintained until postnatal week (PNW) 11.

Daily observation for clinical signs and mortality was conducted throughout the experimental period. In addition, to assess ACR-induced neurological deficits, animals were scored with respect to the appearance of gait abnormalities (Moser, 1991; Shell *et al.*, 1992; Lee *et al.*, 2005) by placing individual animals free in Plexiglas boxes (90 × 90 × 20 cm) for 3 min. Degrees of abnormality were classified into four categories: grade 1 as normal gait; grade 2 as slightly abnormal gait with slight degrees of ataxia, hopping gait, and foot splay; grade 3 as moderately abnormal gait with moderate degrees of ataxia, foot splay and limb abduction; grade 4 as severely affected gait, including inability to support the body weight as well as foot splay.

Body weights of dams and pups were measured twice a week before weaning, and then recorded every week. Maternal food and water consumption was also recorded twice a week during ACR exposure.

The animal protocol was reviewed and approved by the Animal Care and Use Committee of the National Institute of Health Sciences, Japan.

Histopathological assessment

At necropsy, the brain, liver, spleen, kidneys, testes and epididymides were removed and weighed. The trigeminal nerve, spinal cord and skeletal muscle were also removed. In dams, numbers of implantations were also recorded. The brains were fixed in methacam solution at 4°C overnight, and the testes in Bouin's solution at room temperature overnight. The other organs were fixed in 10% buffered formalin. All were then routinely processed for paraffin embedding, sectioning at 3 μm, and staining with hematoxylin and eosin (HE). For histopathological assessment of axons in the peripheral nerves, the sciatic nerves 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. The portion located at the ankle position was carefully excised and further fixed with fresh fixative overnight, postfixed in 1% osmium tetroxide in the same buffer for 2 hr at 4°C, and embedded in epoxy resin (TAAB Laboratories Equipment Ltd., Berkshire, UK). Semithin sections, 1 μm in thickness, were stained with toluidine blue for light microscopic assessment.

Immunohistochemistry for synaptophisin (SYP) in the cerebellum and skeletal muscle

To evaluate aberrant SYP-immunoreactivity in the cerebellar molecular layer, sections obtained from methacarn-fixed brain slices including the cerebellum were subjected to immunohistochemistry for SYP. Rabbit polyclonal antibody Ab-4 (1: 200, Lab Vision Corp., Fremont, CA, USA) was used as the primary antibody and immunodetection was conducted with the horseradish peroxidase-avidin-biotin complex method utilizing a VECTASTAIN® Elite ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) with 3,3'-diaminobenzidine/H_2O_2 as the chromogen, as described previously (Woo *et al.*, 2007). In addition, SYP-immunoreactivity was also examined in skeletal muscle using formalin-fixed sections to evaluate the distribution of neuromuscular junctions. For antigen retrieval, the sections of skeletal muscle were heated by autoclaving in 10 mM citrate buffer for 5 min before incubation with the primary antibody.

Morphometric assessment

To quantify axonal degeneration and atrophy in the sciatic nerves and SYP-immunoreactive aberrant dot-like structures in the cerebellar molecular layer, digital photomicrographs were taken with a Vanox-S microscope (Olympus Corp., Tokyo, Japan) equipped Fujix Digital Camera System (Fujifilm Corp., Tokyo, Japan). Measurement was then performed using a MacSCOPE image analysis software package (Version 3.61, Mitani Corp. Tokyo, Japan). The total number of axons/unit area and the number of degenerated axons were counted in one cross sectional area at 400× magnification of toluidine blue-stained specimens from each animal, and the density and the percentage of degenerated axons were calculated. For the evaluation of sciatic nerve atrophy, numbers of myelinated axons sized >3 μm and <3 μm in diameter were also differentially counted, based on an earlier study that showed a shift in the distribution of axon diameters with a peak frequency around 3 μm on ACR treatment (Saita *et al.*, 1996). 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 20× magnification and the number of SYP-immunoreactive dots/unit length of the cortex was calculated.

Statistical analysis

In dams, data were collected for up to 3 animals per group. Therefore, maternal body and organ weights, food consumption, reproductive parameters and values for morphometric assessment in the sciatic nerves and cerebellar molecular layer were analyzed by the Student's *t*-test when the variance was proven to be homogenous among the groups using a test for equal variance. If a significant difference in variance was observed, Welch's *t*-test was performed. In pups, variance in data for body and organ weights, and values from morphometric assessment in the sciatic nerves and cerebellar molecular layer 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. With histopathological changes, incidences were compared using the Fisher's exact probability test and severity data were analyzed with the Mann-Whitney's *U*-test.

RESULTS

Maternal toxicity

One dam at 200 ppm was euthanized on PND 2 because of non-delivery and worsening general condition, and therefore, statistical analysis could not be applied in this group because of the small number (n=2). Maternal water consumption was decreased at 200 ppm throughout the gestation and lactation periods, and suppressed water consumption at 100 ppm became evident after delivery (Fig. 1A). Also, food consumption was reduced in a dose-related manner during the lactation period (Fig. 1B). Body weight gain of dams was suppressed at 100 and 200 ppm from the gestation period, and the suppression became more prominent in the later stages of the dosing period (Fig. 2A). Mean daily intake of ACR by dams during the gestation and lactation periods was 9.9 ± 0.5, 16.7 ± 2.1 and 22.2 mg/kg body weight/day at 50, 100, and 200 ppm, respectively. At 200 ppm, a slightly abnormal gait appeared from GD 20 (Fig. 2B). Symptoms advanced with time, and the gait abnormality score of dams receiving 200 ppm reached grade 4 at PND 14. Dams in the 100 ppm group exhibited gait abnormality from PND 14 and progressed to score 3 or 4 at the end of the experiment. No animals in the 0 and 50 ppm groups exhibited any gait abnormality. ACR did not affect the gestation period, number of implantations, live birth ratio and male pup ratio. However, pup body weights at PND 2 were significantly decreased from 50 ppm in males and from 100 ppm in females (Table 1). At the necropsy at PND 21, body weights in the 100 and 200 ppm groups were decreased by 12% (284.3 ± 26.4 g) and 27% (235.5 g), respectively, as compared with the 0 ppm group value (323.6 ± 14.8 g).

Histopathologically, central chromatolysis of ganglion cells in the trigeminal nerves was observed in maternal rats from 50 ppm (Table 2, Fig. 3A and B). Morphometric assessment of the sciatic nerves showed dose-related increases of degenerated axons and myelinated nerves of < 3 μm in diameter from 100 ppm (Table 2, Fig. 3C and D). In the cerebellar molecular layer, increase of dot-like SYP-immunoreactive structures was observed from 100 ppm as reported previously (Fig. 3E and F; Lee *et al.*, 2005; Woo *et al.*, 2007). However, the distribution of SYP-positive neuromuscular junctions in skeletal muscle did not differ among the groups. No treatment-related histopathologic changes were observed in the liver, spleen, kidneys and spinal cord.

Offspring toxicity until weaning

In both male and female pups, no apparent abnormalities were found on clinical observation for neurological deficit before weaning. Significant depression of body weight was however observed from PND 2 through weaning from 50 or 100 ppm in males and from 100 ppm in females (Fig. 4A and B). At necropsy at weaning, body weights of pups in both sexes were significantly decreased from 100 ppm as compared with those of the 0 ppm group, and pups in the 100 and 200 ppm groups showed little milk content in their stomach.

On histopathological analysis of pups at weaning, increase of remaining external granular cells in the cerebellum was found in ACR-exposed groups with statistical significance for the severity at 200 ppm in both sexes (Table 3). The degree of extramedullary hematopoiesis in the liver and spleen was decreased in a dose-dependent manner, with statistical significance from 100 ppm in males and 200 ppm in females. Though not significant, loss of glycogen in cytoplasm of hepatocytes was observed in the liver at 200 ppm in both sexes. In the testes of the 0 ppm-controls, the seminiferous tubules retained 3 to 4 layers of spermatocytes, although spermatogenesis had not started yet at weaning. In contrast, germinal epithelium of the testes in ACR-exposed groups at 100 and 200 ppm featured 1 to 2 layers of spermatocytes or Sertoli cells only, suggesting retardation of spermatogenesis (Fig. 5A and B). There were no treatment-related histopathological changes in the trigeminal nerves, kidneys and epididymides.

Morphometrically, axonal caliber of pups at weaning was totally smaller than that of dams, and a tendency for decrease of the axonal size was found in association with ACR treatment. In the sciatic nerves, increases of axonal density and the percentage of myelinated nerves of < 3 μm in diameter were observed (Table 4). However, percentages of degenerated axons did not differ between the controls and any of the ACR-exposed groups (Fig. 5C and D). There were also no differences in the numbers of dot-like SYP-immunoreactive structures in the cerebellar molecular layer, and the distribution of SYP-positive neuromuscular junctions was unchanged.

Offspring toxicity after weaning

No animal exhibited any gait abnormality on clinical observation after weaning. One female offspring in the 200-ppm group died at PNW 4, but the cause of death was unclear. After weaning, pups in every group showed a constant increase in body weight gain. In male pups, body weights were significantly lowered as compared to the control from 100 ppm in a dose-dependent manner (Fig. 6A). At 50 ppm, statistically significant suppression was also detected on PNW 4 in males. Similarly, statistically significant lowering of body weights was observed in

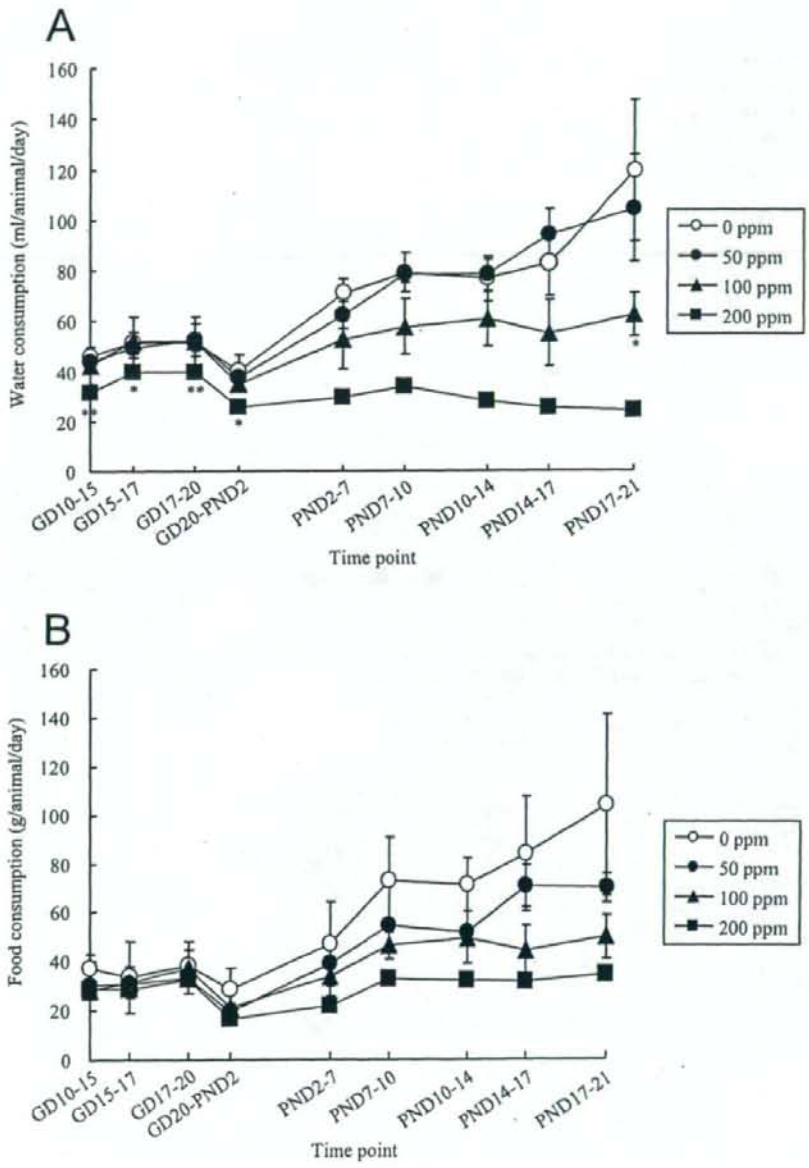

Fig. 1. Time course of change in water and food consumption by dams given ACR in the drinking water for the gestation and lactation periods. *A*. Water consumption. *B*. Food consumption. Data are mean ± SD. n=3 (0, 50 and 100 ppm group), n=3 or 2 (200 ppm group). *, **: $p<0.05$, $p<0.01$ vs. 0 ppm group. Abbreviation: GD, gestation day; PND, postnatal day.

females from 100 ppm at PNWs 4 and 5, and at 200 ppm thereafter (Fig. 6B).

At PNW 11, although some incidental findings were observed, no treatment-related histopathological changes were noted in the examined organs in either sex.

Morphometrically, no differences between the control and ACR-exposed groups were noted in parameters examined in the sciatic nerves and the cerebellum at PNW 11 (Table 4). The distribution of SYP-positive neuromuscular junctions was unchanged between the control and ACR-exposed groups at PNW 11.

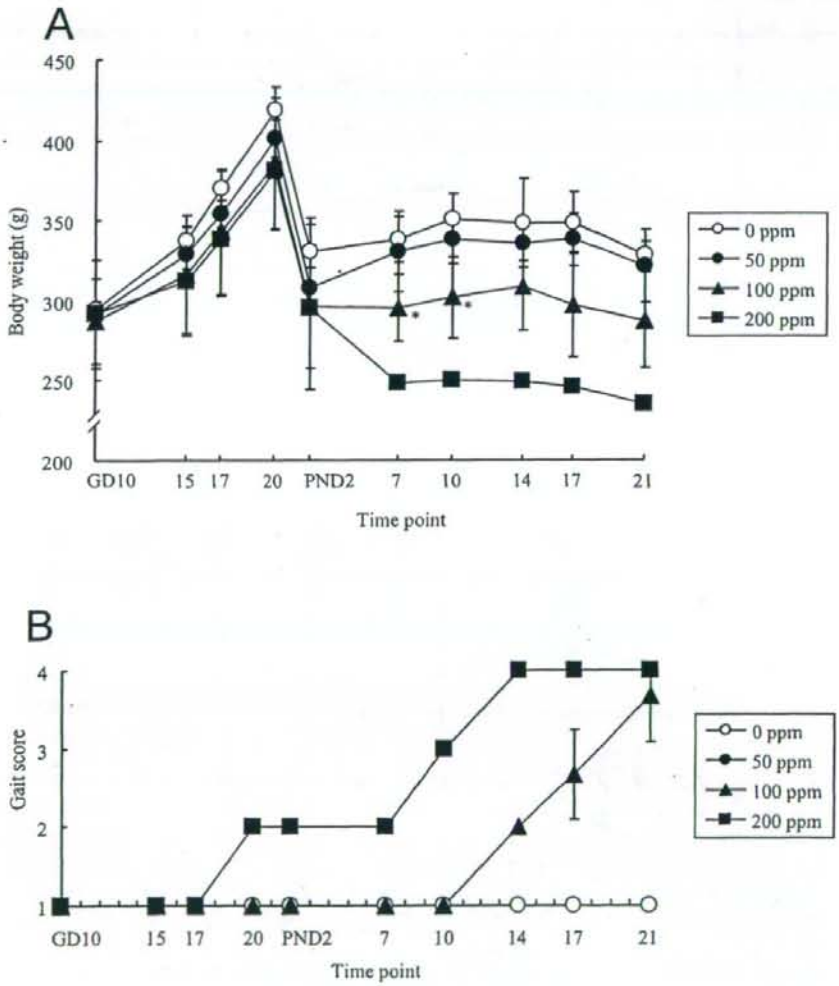

Fig. 2. Body weight changes and scores for gait abnormalities of dams given ACR in the drinking water for the gestation and lactation periods. *A*. Body weight. *B*. Scores for gait abnormalities. Data are mean ± SD. n=3 (0, 50 and 100 ppm group), n=3 or 2 (200 ppm group). *: $p<0.05$ vs. 0 ppm group. Abbreviation: GD, gestation day; PND, postnatal day.

DISCUSSION

In the present study, ACR-induced neurotoxicity in maternal animals was evident from 100 ppm on morphometric assessment of the sciatic nerves and cerebellar molecular layer, as previously reported (Lee *et al.*, 2005; Woo *et al.*, 2007). Dams given ACR at concentrations of ≥100 ppm exhibited depression of body weight and food and water consumption in a dose-dependent fashion. Maternal body weights at weaning were decreased by 12% and 27% relative to the control dams in the 100 and 200 ppm groups, respectively. In a previous study of the effects of feed restriction during the gestation and lactation periods in rats, body weights of dams were decreased by 1-5%, 10-20% and 17-32% in the 10%, 30% and 50% food restriction groups, respectively (Carney *et al.*, 2004), and the body weight curves were similar to those for our ACR-treated dams. Since reduced body weight gain concomitant with reduced food and water consumption was apparent here, particularly in the later stages of the experimental period, it can be considered that the advanced neurotoxicity due to ACR led to maternal malnutrition. ACR did not affect the gestation period, number of implantations, live birth ratio and male pup ratio, as in the report by Zenick *et al.* (Zenick *et al.*, 1986). Although inability to deliver was observed in one dam at 200 ppm, it was unclear whether

Table 1. Reproductive data.

	Acrylamide in the drinking water (ppm)			
	0	50	100	200
No. of animals	3	3	3	3
Gestational length (days)	22.0 ± 0.0[a]	22.0 ± 0.0	21.3 ± 0.6	21.7 ± 0.6
No. of implantations/dam	13.3 ± 0.6	13.7 ± 1.2	13.7 ± 2.1	13.3 ± 2.1
Live birth ratio (%)[b]	100	100	100	94
No. of live pups/litter[c]	12.7 ± 1.2	12.3 ± 2.5	11.3 ± 1.2	11.3 ± 3.8
No. of male pups[c]	8.7 ± 2.5	6.0 ± 4.6	5.0 ± 1.0	5.3 ± 2.3
No. of female pups[c]	4.0 ± 1.7	6.3 ± 3.2	6.3 ± 2.1	6.0 ± 3.6
Male pup ratio (%)	67.9 ± 15.6	46.7 ± 28.5	45.0 ± 13.6	49.1 ± 17.9
Male pup weight[c] (g)	8.27 ± 0.45	7.58 ± 0.66*	7.27 ± 0.65*	6.01 ± 0.39*
Female pup weight[c] (g)	7.97 ± 0.35	7.52 ± 0.43	6.67 ± 0.74*	5.59 ± 0.47*

[a]: Mean ± SD.
[b]: Live birth (%) = number of live pups delivered/total number of pups delivered ×100.
[c]: Measured at PND 2.
*: $p<0.01$ vs. 0 ppm group.

Table 2. Data for histopathology and morphometry of lesions developing in the nervous system of dams.

		Acrylamide in the drinking water (ppm)			
		0	50	100	200
No. of animals		3	3	3	3
Trigeminal nerve					
Central chromatolysis of ganglion cells (+/++)[a]		0	3 (3/0)	3 (0/3)	3 (0/3)
Sciatic nerve (distal portion)					
Density	(/100 μm²)	1.89 ± 0.31[b]	2.10 ± 0.11	1.96 ± 0.07	1.59[c]
Degenerated axons	(%)	1.04 ± 1.56	0.94 ± 0.20	2.43 ± 0.50*	5.78[c]
Myelinated axons, <3 μm in diameter	(%)	7.82 ± 1.57	10.45 ± 2.80	13.02 ± 1.59*	14.55[c]
Cerebellar cortex, molecular layer					
SYP-immunoreactive aberrant dots	(/mm cortex)	0.44 ± 0.11	0.75 ± 0.27	3.18 ± 0.45**	4.57[c]

[a]: Grade of change: +; mild, ++; moderate.
[b]: Mean ± SD.
[c]: One dam was killed due to inability to deliver.
*, **: $p<0.05$, $p<0.01$ vs. 0 ppm group.
Abbreviation: SYP, synaptophysin.

this was induced by ACR.

In the present study, pup body weights were significantly depressed at PND 2 from 50 ppm in males, and from 100 ppm in females, and decreased body weights persisted through PNW 11. A decrease of pup body weight has been previously reported in rats and mice with gestational exposure to ACR (Zenick *et al.*, 1986; Field *et al.*, 1990; Wise *et al.*, 1995), and such weight decrease has been proven to be a sensitive indicator of developmental ACR-toxicity, because it is apparent at dose levels lower than that producing maternal neurotoxicity (Wise *et al.*, 1995). This latter finding was also the case in the present study, suggesting that the decrease of pup body weight shortly after birth was caused by developmental effects of ACR administered during gestation. However, it has been reported that pup body weights were significantly decreased due to maternal malnutrition along with reduction of maternal body weight, food and water consumption in Wistar rats gavaged with ACR 25 mg/kg body weight/day throughout lactation (Friedman *et al.*, 1999). In addition, maternal food restriction during gestation and lactation suppresses pup body weight (Carney *et al.*, 2004).

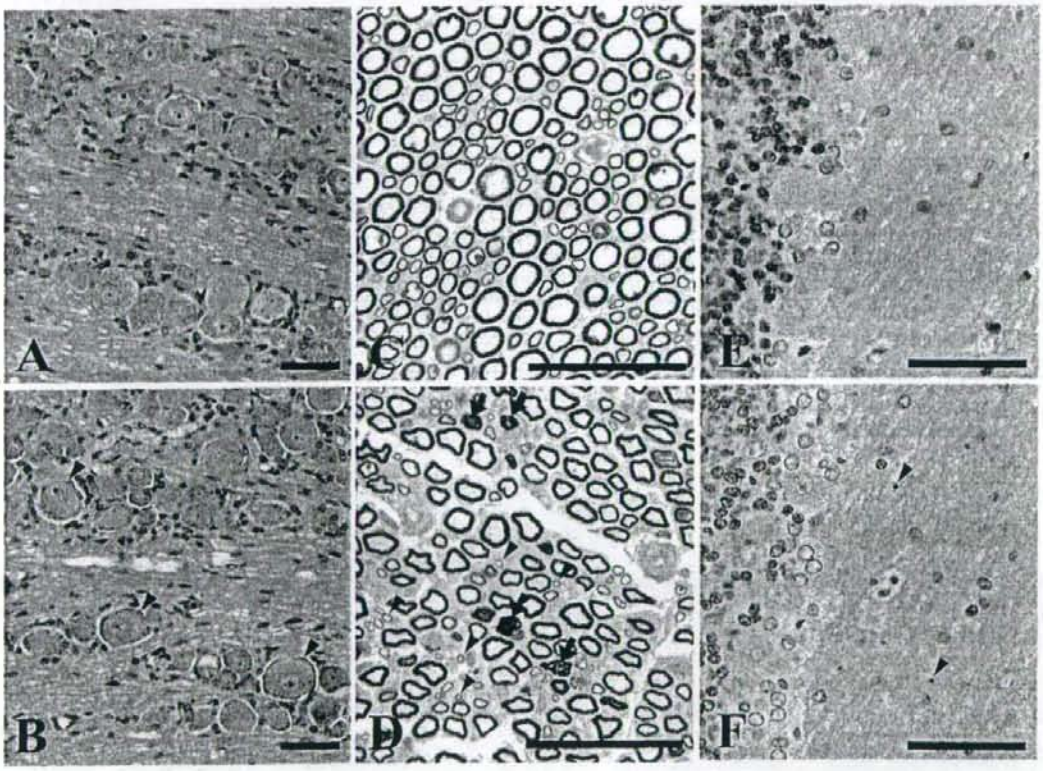

Fig. 3. Histopathology of the nervous system of dams given ACR at 0 (control) or 200 ppm in the drinking water for the gestation and lactation periods. (A) The trigeminal nerve of a dam at 0 ppm. (B) Central chromatolysis of ganglion cells (arrowheads) in the trigeminal nerve of a dam at 200 ppm. (C) The sciatic nerve of a dam at 0 ppm. (D) Increases of degenerated axons (arrows) and atrophic fibers (arrowheads) in the sciatic nerve of a dam at 200 ppm. (E) The cerebellar molecular layer of a dam at 0 ppm. (F) Increase of dot-like SYP-immunoreactive structures (arrowheads) in the cerebellar molecular layer at 200 ppm. A, B: hematoxylin and eosin. C, D: resin-embedded semithin sections stained with toluidine blue. E, F: immunohistochemical staining for SYP. Bar=50 μm.