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Chinese herbs nephropathy-associated slimming regimen induces tumours in the forestomach but no interstitial nephropathy in rats

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Abstract Chinese herbs nephropathy (CHN), a rapidly progressive interstitial fibrosis of the kidney, has been described in approximately 100 young Belgian women who had followed a slimming regimen containing some Chinese herbs. In 4 patients multifocal transitional cell carcinomas (TCC) were observed. Aristolochic acid (AA), suspected as the causal factor of CHN, is a well known carcinogen but its ability to induce fibrosis has never been demonstrated. The objective of this study was to evaluate the latter using doses of AA, durations of intoxication and delays of sacrifice known to yield tumours in rats. We also tested the hypothesis that a possible fibrogenic role of AA was enhanced by the other components of the slimming regimen. Male and female rats were treated orally with 10 mg isolated AA/kg per day for 5 days/week, or with approximately 0.15 mg AA/kg per day 5 days/week contained in the herbal powder together with the other components prescribed in the slimming pills for 3 months. The animals were killed respectively 3 and 11 months later. At sacrifice, animals in both groups had developed the expected tumours but not fibrosis of the renal interstitium. Whether the fibrotic response observed in man is due to species and/or strain related differences in the response to AA or to other factors, remains to be determined. Interestingly, despite the addition of fenfluramine and diethylpropion, two drugs incriminated in the development of valvular heart disease, no cardiac abnormalities were observed.

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Abbreviations *dA-AAI* 7-(deoxyadenosine-N^6-yl)-aristolactam I · *dA-AAII* 7-(deoxyadenosine-N^6-yl)-aristolactam II · *dG-AAI* 7-(deoxyguanosine-N^2-yl)-aristolactam I · *dG-AAII* 7-(deoxyguanosine-N^2-yl)-aristolactam II · Lac I aristolactam I · Lac II aristolactam II

Key words Aristolochic acid · Chinese herbs · Carcinogenicity · Toxicology

Introduction

Chinese herbs nephropathy (CHN) is a new type of subacute interstitial fibrosis of the kidney. It has been observed in approximately 100 patients who had followed a slimming regimen cure including the prolonged use of Chinese herbs (Cosyns et al. 1994a; Depierreux et al. 1994; Reginster et al. 1997; Vanherweghem et al. 1993). One-half of the patients needed renal replacement therapy (Reginster et al. 1994; van Ypersele de Strihou and Vanherweghem 1995). In four cases to date, multifocal transitional cell carcinomas (TCC) of the urinary tract were also observed (Cosyns et al. 1994b; Vanherweghem et al. 1995; Cosyns et al. two unpublished observations).

There was absence in the delivered capsules of one of the prescribed Chinese herbs (*Stephania tetrandra*). Aristolochic acid (AA), the major alkaloid extracted from *Aristolochia* plants, was identified in 11 out of 12 different samples of herb powders imported into Belgium under the name of *S. tetrandra*. Both of these facts led to the suggestion that *S. tetrandra* had been inadvertently replaced by *Aristolochia fangchi*, another Chinese herb (Vanhaelen et al. 1994). This hypothesis is supported by the demonstration of AA DNA adducts in eight kidneys and one ureter from six patients with CHN (Bieler et al. 1997; Schmeiser et al. 1996).

Aristolochic acid (AA), a known nephrotoxin and carcinogen, is a mixture of structurally related nitrophenanthrene carboxylic acids, AAI and AAII being the

major components. Carcinogenicity of AA is most probably due to its ability to form DNA adducts by covalently binding to the exocyclic amino group of purine residues. Using the ^{32}P- postlabelling method, the four structurally known DNA adducts of AA; dA-AAI, dA-AAII, dG-AAI and dG-AAII were actually detected and identified in DNA extracted from rat organs after oral feeding with the natural mixture of AA (Stiborova et al. 1994). These adducts were similar to those reported in CHN (Bieler et al. 1997; Schmeiser et al. 1996). Experimental work on AA has concentrated on its carcinogenic action.

The objective of the present study was to evaluate fibrogenicity using doses of AA, duration of intoxication and delays before sacrifice known to yield tumours in rodents (Mengs 1988; Mengs and Stotzem 1993; Mengs et al. 1982). Male and female rats were thus given 10 mg isolated AA/kg per day 5 days/week for 3 months and killed 3 months later. We also tested the hypothesis that a possible fibrogenic role of AA was enhanced by the other components of the slimming regimen. Another group of rats was thus given a sample of *S. tetrandra* powder contaminated with AA together with the other components prescribed in the slimming pills for 3 months and killed 11 months later.

Materials and methods

Animals and environment

Eight-week-old male and female Wistar rats (Iffa Credo, Brussels, Belgium) weighing 230 ± 10 g and 170 ± 10 g respectively, were used after a 14-day acclimatisation period. Rats were kept in groups of four males and eight females in polycarbonate cages in fully air-conditioned animal rooms with an artificial light/dark cycle. The temperature was maintained at 20 ± 1 °C with a relative humidity of 50 ± 5%. Standardised food pellets (Rats et Souris Elevage 'A03', UAR, Epinay-sur-Orge, France) and tap water from polycarbonate bottles with steel nipples were available ad libitum.

Identification of AAI and AAII in AA and Chinese herbs

The natural mixture AA was purchased as its sodium salt from Sigma Aldrich Co, Milwaukee, Wisconsin, USA and analysed by high performance liquid chromatography (HPLC) as described (Stiborova et al. 1994). Separation was performed on an Ultrasphere ODS RP-18 5 μm (250.0 × 4.6 mm) column (Beckman) under isocratic conditions with 65% methanol, 34% water and 1% acetic acid (v/v). Pills containing Chinese herbs were prepared in order to provide all the ingredients described by Vanherweghem et al. (1993). Three batches of herb powders imported in Belgium under the name of *S. tetrandra* and one batch under the name of *Magnolia officinalis* similar to those used in 1990 were analysed.

A different chromatography system was used for the analysis of the so-called *S. tetrandra*, which provided a better separation of AAI and AAII as well as their corresponding reduced forms, aristolactam I (LacI) and II (LacII). The so-called *S. tetrandra* herbal powder was extracted by treatment with 5 ml methanol in order to identify AA, and thus confirm the substitution of *S. tetrandra* for *A. fangchi*. The methanolic extract was concentrated to 2.5 ml and aliquots (50–100 μl) were analysed by HPLC. Separation was performed on a reversed phase column (Beckman Ultrasphere ODS RP 18, 5 μm) using a linear gradient from 5 to 50% acetonitrile in 0.1 M triethylammonium acetate pH 7.0, within 45 min at a flow rate of 1 ml/min. UV monitoring was at 260 nm and fluorescence monitoring at excitation 360 nm, emission 490 nm.

Group 1

Rats treated with pure AA

The dose of 10 mg AA/kg, for 5 days a week during 3 months followed by sacrifice 3 months later was selected on the basis of previous experiments showing a multisystemic tumorigenicity under such conditions (Mengs et al. 1982).

The natural mixture of AA dispersed in 0.5 ml olive oil was administered through a gastric tube to eight males (group 1a) and eight females (group 1b). Six male and six female control rats received the vehicle only (groups 1c and 1c*).

Group 2

Rats treated with slimming regimen pills containing AA

In an effort to reproduce the conditions leading to CHN, we treated another group of eight males (group 2a) and eight females (group 2b) with the slimming regimen (Vanherweghem et al. 1993). In addition to intradermal injections of artichoke extract and euphyllin, rats were given slimming pills containing *S. tetrandra*, *M. officinalis*, fenfluramine, diethylpropion, cascara powder, acetazolamide, belladonna extract and meprobamate as described subsequently (Table 1).

1. The contents of the pills were dispersed in 0.5 ml olive oil and administered through a gastric tube for 5 days a week.

2. A sample of *S. tetrandra* powder, which has shown to contain a methanol extractable amount of 2.2 mg AA/g, was used to feed the rats. The daily amount of *S. tetranda* powder expressed per kg body weight (and *M. officinalis*, though this latter did not contain AA) delivered to the rats were 10 times higher than the estimated doses administered to the patients, who received on average 7 mg *S. tetrandra* powder/kg per day for an average person of 65 kg (Vanherweghem et al. 1993). On the basis of the weekly recorded body weight, a daily amount of 70 mg *S. tetrandra* powder/kg was thus delivered to the rats who received 0.15 mg AA/kg per day together with the other components prescribed in the slimming pills.

3. The weekly intradermal injections of purified extract of fresh leaves of *Cynara scolymus* (Chophytol) and of aminophyllinum (Euphyllin) were inadvertently increased to 1 mg/kg and 9.6 mg/kg, respectively.

4. Duration of treatment lasted 3 months followed by sacrifice 11 months later according to previous experiments yielding tu-

Table 1 Modified Chinese herbs nephropathy-associated slimming regimen (Vanherweghem et al. 1993) used in Wistar rats

	Doses (mg/kg)
Injection[a]:	
Purified extract of fresh leaves of *Cynara scolymus*	1.00
Aminophyllinum	9.60
Capsules A + B (gastric tube):	
Fenfluramine	0.97
Diethylpropion	0.97
Meprobamate	1.15
Cascara powder	3.92
Acetazolamide	1.61
Belladonna extract	0.07
Stephania tetrandra	70.00
Magnolia officinalis	70.00

[a] Intradermal injection once a week

mours with such a daily dose of pure AA (Mengs et al. 1982). Eight male and eight female control rats (groups 2c and 2c*) were given the vehicle only and injected with saline only.

Sacrifice and post-mortem examinations

All rats surviving 6 months after the start of treatment with AA or 14 months after the start of treatment with the slimming regimen were killed by intracardiac punction during ether anaesthesia. After external inspection, each animal was autopsied. Samples of lungs, heart, liver, pancreas, spleen, stomach, small and large intestine, kidneys, adrenals, urinary bladder, testes, seminal vesicles, prostate, uterus, salivary glands, tongue, trachea, oesophagus, brain, skin, skeletal muscle, as well as any tissue with abnormal appearance were excised and fixed in formalin and/or Duboscq-Brasil for histological examination. Paraffin sections were stained with haematoxylin and eosin (HE). Renal tubulointerstitial fibrosis was defined as an increased interstitial matrix with or without tubular atrophy and/or obsolescent glomeruli.

Blood and urinary chemistry

In order to determine serum creatinine levels, blood was obtained from all rats at sacrifice by intracardiac punction.

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) and Fisher's exact test with a Statview program. Results are expressed as mean ± SD.

Results

Detection of AAI and AAII in the natural mixture of AA and in *S. tetrandra* powder

The nature mixture AA purchased from Sigma consisted of 44% AAI and of 56% AAII. Besides several unknown UV-active substances, AAI and AAII were identified in two out of three samples of so-called *S. tetrandra* herbal powder after methanol extraction according to their retention times (AAI 30 min; AAII 28 min) with aristolochic acid purchased from Sigma. Likewise fluorescence monitoring showed the presence of the corresponding aristolactams, LacI (retention time, 44 min) and LacII (retention time, 42 min) in trace amounts. Quantitation by peak integration revealed that the sample of *S. tetrandra* powder used to feed the rats contained methanol-extractable AA of approximately 2 mg AAI/g (91%) and 0.2 mg AAII/g (9%). The daily amount of *S. tetrandra* powder delivered to the rats (70 mg/kg) thus corresponded to a daily dose of approximately 0.15 mg AA/kg.

Group 1

Rats treated with pure AA

Serum creatinine values, available at sacrifice in three male and four female treated rats only, were within the normal range. Compared with control rats, body weights of male but not of female treated rats were lower during the whole length of the experiment: 427 ± 55 g vs 491 ± 33 g at the time of sacrifice ($P = 0.024$). Post-mortem examination revealed no differences in organ weights of treated vs control rats.

Four treated rats (two males and two females) from this group died accidentally before sacrifice. At sacrifice, 3 months after the end of the treatment (6 months into the study), 6/8 treated females, 6/8 treated males, 6/6 male and 6/6 female controls were available. No parenchymal fibrosis was found in either subgroup. One or more cardiac valve leaflets and/or chordae were visible by light microscopy on HE stained sections in 7/12 treated and in 8/12 control rats. No abnormality was observed except, in 1 treated male rat, a well differentiated myocardial fibrosarcoma. Group 1 tumours consisted of 10/12 squamous cell papillomas and 3/12 squamous cell carcinomas of the forestomach; 7/12 leiomyosarcomas, 4/12 angiosarcomas and 1/12 osteosarcoma of the small intestine; 4/12 adenomas and 2/12 malignant tumours of unclear histogenesis of the kidney; 3/12 atypical hyperplasia and 1/12 papillary TCC of the bladder; 1/6 adenocarcinoma of the mammary gland. Control rats showed 6/6 benign as well as malignant prostatic hyperplasia.

Group 2

Rats treated with the slimming regimen containing AA

Serum creatinine values available at sacrifice in three male and four female treated rats were within the normal range. Compared with control rats, total body weights were not significantly different at sacrifice in both female and male rats treated by the slimming regimen. Post-mortem examination revealed no differences in organ weights of treated vs control rats. Eight treated (four males and four females) and four control rats (one male and three females) from this group were accidentally lost before sacrifice. At sacrifice, 11 months after treatment (14 months into the study), 4/8 treated males, 4/8 treated females, 7/8 male controls and 5/8 female controls were available.

Multifocal areas of tubulointerstitial fibrosis were observed in the kidneys of 2/4 male treated vs 1/7 male control rats (not significant, NS). No other evidence of parenchymal fibrosis was present in the kidneys or in the other organs. One or more cardiac valve leaflets and/or chordae were visible by light microscopy on HE stained sections in 4/8 treated and 10/12 control rats. No abnormality was observed except, in one male treated rat which displayed focal chordal calcifications. The prevalence of forestomach squamous cell papillomas was higher than in controls but the difference did not reach statistical significance (318 vs 2112). By contrast, malignant well differentiated squamous cell carcinomas invading the submucosa of the forestomach were observed in 214 treated males. This group showed no other benign or

malignant tumours other than benign as well as malignant prostatic hyperplasia (in all male rats; see Table 2).

Discussion

This is the first time that HPLC was used to demonstrate the presence of AA in a batch of so-called *S. tetrandra* and the absence of this drug in a batch of *M. officinalis* similar to those used during the slimming cure (in Vanherweghem et al. 1993). Thus, our results confirm those previously reported by others using thin-layer chromatography (But 1993; Vanhaelen et al. 1994).

As expected, the administration of AA whether alone or together with the other components included in the slimming pills led to the development of multisystemic tumours and forestomach epidermoid carcinomas. The results are similar to those reported by Mengs (1988), Mengs and Stotzem (1993) and Mengs et al. (1982). However, we found no evidence of kidney fibrosis. The failure to observe renal fibrosis after AA ingestion cannot be ascribed to the absence of an enhancing compound given to patients. Indeed the administration of AA together with the other substances prescribed in the regimen also failed to induce fibrosis.

We have previously shown that in humans the carcinogenic and the fibrogenic properties of the slimming cure were closely associated: all patients had evidence of both extensive renal fibrosis as well as pelviureteral atypia with, in four of them, the subsequent development of multifocal TCC (Cosyns et al. 1994b; Vanherweghem et al. 1995; Cosyns et al. two unpublished observations). In the rat, in contrast both properties appear dissociated as only tumours and no fibrosis were induced. Such a dissociation is unlikely to be related to differences in the metabolic pathways for AA: both rats and humans share a nitroreductase activity (Schmeiser et al. 1986) responsible for the formation of specific DNA adducts, which were indeed found in tissues from both humans suffering from CHN and from rats after oral administration of the natural mixture of AA. These promutagenic adducts might well be implicated in the tumours observed both in rats given AA and in CHN patients (Bieler et al. 1997; Schmeiser et al. 1996; Stiborova et al. 1994).

The dissociation of the fibrotic and carcinogenic processes in the rat remains a matter of speculation. The induction of sclerosis might be controlled by genetic factors, which differ between rat and man and which are unrelated to the initiation of cancer. The existence of a genetic predisposition to fibrosis is supported by the

Table 2 Nature and frequency of tumoral lesions in Wistar rats 3 and 11 months after 5 days/week for 3 months tube-feeding respectively with 10 mg aristolochic acid/kg per day and 0.15 mg aristolochic acid/kg per day as included in the Chinese herbs nephropathy-associated slimming regimen

	Group 1 Pure aristolochic acid				Group 2 Slimming regimen			
	a: Male rats	b: Female rats	c: Male controls	c*: Female controls	a: Male rats	b: Female rats	c: Male controls	c*: Female controls
Serum Creatinine (mg/dl):	$n^x = 3$	$n = 4$	$n = 6$	$n = 6$	$n = 3$	$n = 4$	$n = 2$	$n = 4$
Mean	0.94	0.63	0.67	0.78	0.75	0.63	0.85	0.67
Standard deviation	0.52	0.06	0.06	0.06	0.06	0.06	0.02	0.06
Lesions:	$N^y = 6$	$N = 6$	$N = 6$	$N = 6$	$N = 4$	$N = 4$	$N = 7$	$N = 5$
Forestomach								
Squamous cell papilloma	5	5	0	0	2	1	0	2
Squamous cell carcinoma	3	0	0	0	2	0	0	0
Small intestine								
Leiomyosarcoma	5	2	0	0	0	0	0	0
Angiosarcoma	3	1	0	0	0	0	0	0
Osteosarcoma	1	0	0	0	0	0	0	0
Kidney								
Adenoma	4	0	0	0	0	0	0	0
Malignant tumour of unclear histogenesis	0	2	0	0	0	0	0	0
Bladder								
Atypical hyperplasia	1	2	0	0	0	0	0	0
Transitional cell carcinoma	1	0	0	0	0	0	0	0
Heart								
Fibrosarcoma	1	0	0	0	0	0	0	0
Prostate								
Benign hyperplasia	6	–	6	–	4	–	7	–
Malignant hyperplasia	6	–	6	–	4	–	7	–
Mammary gland								
Adenocarcinoma	–	1	–	0	–	0	–	0

[x] *n*, Number of available samples
[y] *N*, Number of surviving animals

strain-dependent variability in the response observed in different models of fibrosis : bleomycin-(Schrier et al. 1983), radiation-(Johnston et al. 1995) or ozone-induced (Ohtsuka et al. 1995) lung fibrosis and cyclosporine-induced kidney fibrosis. For instance, Sprague-Dawley rats injected intraperitoneally with 25 mg/kg per day of cyclosporine in olive oil during 28 days (Gillum et al. 1988) developed tubulointerstital fibrosis whereas the same dose given during 2 weeks to Fisher rats failed to produce kidney fibrosis (Farthing et al. 1981). In humans, in contrast, there is no evidence for a genetic predisposition to renal fibrosis. Of note however, is the limited number of patients who developed kidney fibrosis after the ingestion of the slimming pills containing Chinese herbs (van Ypersele de Strihou and Vanherweghem 1995). It remains to be seen whether this interindividual variability is due to some genetic predisposition or to other factors (interindividual variability in the metabolism of AA, batch-to-batch differences of AA contents in the capsules, compliance to the slimming regimen, additional unacknowledged drugs, etc.).

Valvular heart diseases has been reported in up to 42% of the CHN patients. This damage was absent in a control group of patients with other types of interstitial nephropathy (Reginster et al. 1997). Recently, Connolly reported that fenfluramine-phentermine therapy was associated with pulmonary hypertension and valvular heart disease characterized by macroscopic thickening and microscopic fibrotic changes of the cardiac valve leaflets and chordae indistinguishable from the features of carcinoid and ergotamine-induced valve disease (Connolly et al. 1997). Since the CHN patients also took fenfluramine, it now appears likely that anorectic agents and not the Chinese herbs were the cause of the valvular disease (Vanherweghem 1997; van Ypersele de Strihou 1998). No experimental data are available on the relation between anorectic drugs and valvular lesions. Our results are therefore of great interest: despite a duration of 3–11 months oral administration of 0.97 mg fenfluramine/kg per day, a dosage within the range commonly advised for this medication (Connolly et al. 1997), no valve injury had been noted on macroscopic and microscopic examination of the heart. These findings are again compatible with the existence of a species related susceptibility to fibrotic agents.

Altogether, the 3 months administration of a high dose of pure AA as well as of a low dose of AA as in the CHN-associated slimming pills, induced the expected array of tumours but no fibrosis in Wistar rats. Thus, our data demonstrate a dissociation of the carcinogenic and fibrotic properties of the slimming cure in rodents contrary to humans. Whether the fibrotic response observed in man is due to species, or strain related differences in the response to AA or to other factors, remains to be determined.

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