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HUMAN AND EXPERIMENTAL FEATURES OF ARISTOLOCHIC ACID NEPHROPATHY (AAN; FORMERLY CHINESE HERBS NEPHROPATHY - CHN): ARE THEY RELEVANT TO BALKAN ENDEMIC NEPHROPATHY (BEN)?

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Summary. *Chinese herbs nephropathy, a rapidly progressive interstitial nephropathy, has been reported in young Belgian women given a slimming regimen including Chinese herbs. It is characterized by early, severe anemia, mild tubular proteinuria, and normal arterial blood pressure. Renal histology shows an extensive, remarkably a- or hypocellular interstitial fibrosis associated with tubular atrophy and global sclerosis of glomeruli with a decreasing cortico-medullary gradient. A 40% to 46% prevalence of upper urothelial malignancy of the upper urinary tract has been reported.*
The nephrotoxic and carcinogenic aristolochic acids (AAs), extracted from the Aristolochia species, have been identified in herbs included in the slimming pills. The hypothesis that these alkaloids were the cause of CHN was substantiated by the identification of pre-mutagenic AA-DNA adducts in the kidney tissue of CHN patients. The causal role of AAs in CHN has eventually been demonstrated by the induction of the salient biologic and morphologic features of CHN in rabbits fed pure AAs without other components of the slimming regimen.
Biological and morphological features of human and experimental AA-induced nephropathy are strikingly similar to those reported in BEN suggesting that AAs play also an etiological role in BEN, a hypothesis considered many years ago and yet to be fully explored. Confirmation of this hypothesis requires evidence that patients with an unequivocal diagnosis of BEN have ingested foods containing AAs and harbour AA-DNA adducts in their renal tissue.

Key words: *Balkan endemic nephropathy, Chinese herbs nephropathy, aristolochic acid nephropathy, interstitial nephropathy, urothelial cancer*

Chinese herbs nephropathy (CHN)

Breakthrough

During the first semester of 1992, JL Vanherweghem et al noticed a significant increase in the number of women who were on hemodialysis in Brussels for interstitial nephritis (1). All patients were women under the age of 50 years who had followed the same slimming regimen at Clinic X in Brussels. This nephropathy is characterized by a subacute course, normal blood pressure, early anemia, normoglycemic glucosuria and tubular proteinuria (2). The regimen included intradermal injections of artichoke extract, euphyllin and pills containing fenfluramine, diethylpropion, cascara powder, acetazolamide, belladonna extract and meprobamate as well as two herbs imported from China, *Magnolia offcinalis* and *Stephania tetrandra*. These herbs had been added from May 1990 onwards, raising the hypothesis that they may be responsible for the interstitial nephropathy.

Pathology

The few kidney biopsies available in the initial report (1) disclosed an interstitial fibrosis. The availability in 1992-1993 of 4 nephroureterectomy specimens removed at the time of renal transplantation in 3 such patients allowed us to provide subsequently a more comprehensive analysis of the nature and topography of the kidney lesions. A unique pathological picture emerged (3) which was confirmed a few months later by others on the basis of renal biopsies (4): extensive hypocellular interstitial sclerosis, tubular atrophy and global glomerular sclerosis decreasing from the outer to the inner cortex, including the columns of Bertin; severe fibromucoid to fibrous intimal thickening of mainly interlobular arteries, normal or collapsed residual glomeruli; constant association with mild to moderate atypia of the collecting ducts epithelium and pelviureteric urothelium.

The development of urothelial malignancy in one of the young women (5) strongly supported the suggestion that the ingested Chinese herbs had a carcinogenic ef-

fect on the urothelium. We thus decided in 1994 to remove both native kidneys and ureters in all patients with CHN at the time of transplantation or shortly thereafter. We discovered a 40% prevalence of urothelial carcinoma in situ (6) and, thus, established the final pathological hallmark of CHN: a constant association of renal subcapsular, hypocellular interstitial fibrosis with urothelial atypia leading to carcinomas in approximately 40% of the cases. This picture has been recently confirmed in a larger series of CHN patients; a similar incidence of urothelial cancer (46%) was found (7).

Etiology

The Chinese Medicinal Material Research Center at the Chinese University of Hong Kong recognized *Aristolochia fangchi*, a nonprescribed aristolochic acid (AA)-containing herb in a sample of the incriminated herbs imported into Belgium under the name of *Stephania tetrandra* (*fangji*) (8). This prompted Vanherweghem et al to look for the presence of AA, the alkaloid found in the plant *Aristolochia,* in the herbs imported in Belgium to prepare the slimming pills. Variable amounts of AA were indeed found in 10/12 of these batches (9). In contrast, no tetrandrine, the alkaloid derived from *Stephania tetrandra*, was found. This raised the suggestion that AA had inadvertently been included in the slimming pills due to a confusion between "*fangji*" and "*fangchi*" and led to the suspicion that this alkaloid was the cause of CHN, a hypothesis disputed by a few others (10-12).

AA is the active principle extracted from *Aristolochia* plants (13). AA is a mixture of structurally related nitrophenanthrene carboxylic acids, AAI and AAII being the major components. Noteworthy, both components were found in 2/3 samples of the so-called *Stephania tetrandra* herbal powder used to prepare the slimming pills (14, 15).

AAs have a strong carcinogenic effect in rodents. The mutagenicity of AA has been established in several short-term tests (16-19).Time- and dose-dependent multisystemic tumors have been subsequently documented in rats and mice after chronic oral intake of AA (20, 21). After daily oral intake of 10 mg/kg, all rats developed tumors mainly in the forestomach and bladder within 3 months.

Mutagenic and carcinogenic effects were found to be associated with the formation of pre-mutagenic AA-DNA adducts (22).

In order to substantiate the suspected etiologic role of AAs in CHN, we looked in collaboration with the Heidelberg group for the presence of AA-DNA adducts in 8 kidneys and 1 ureter from 6 CHN patients. Using the ^{32}P postlabelling method (23) and cochromatographic analyses (TLC and HPLC) with authentic markers, we identified 1 major DNA adduct as the 7-(deoxyadenosin-N6-yl)-aristolactam I (dA-AAI), and 2 minor DNA adducts as the 7-(deoxyguanosin-N2-yl)-aristolactam I (dGAAI) and the 7-(deoxyadenosin-N6-yl)-aristolactam II (dA-AAII), respectively, in all the kidney samples and in the ureter removed from these 6 patients up to 44 months after the end of the slimming regimen (14, 24). The specificity of these DNA adducts was established by their absence in end-stage kidneys taken from 6 control patients with various renal diseases different from CHN. These results have been recently confirmed (7). They conclusively demonstrate that CHN is associated with the intake of AAs and that the amount of AAs was sufficient to alter cellular DNA. AA's premutagenic activity provides a pathophysiological clue as to the cause of the constant urothelial atypia found in patients with CHN as well as for the multifocal TCC observed in 40%-46% of them (6, 7).

In contrast with the well known carcinogenicity of AA in rodents and with its acute nephrotoxicity characterized by acute tubular necrosis in several species (25-27), little is known about its potential chronic nephrotoxicity.

In 1970, Ivic reported the presence of renal interstitial fibrosis but not of urothelial atypia or carcinomas in NZW rabbits after eleven months of oral intake of *Aristolochia* seeds (28). We wondered whether chronic administration of the alkaloid AA given in isolation, i.e., without inclusion of the other substances of the slimming regimen, could induce urothelial malignancies, renal interstitial fibrosis and the biologic features characterizing CHN. In female NZW rabbits given an AAs dose equivalent to 10 times that reported in CHN patients for 17 and 21 months, we found extensive hypocellular interstitial fibrosis with tubular atrophy predominantly in medullary rays and superficial cortex (29). It was associated with extensive, mainly proximal, tubular epithelial cell flattening, interstitial edema and scanty lymphocytic infiltration. Moreover, urothelial atypia and a flat high-grade TCC invading the lamina propria were found in all treated animals and in one 21 months AA-treated rabbit, respectively. AA-treated animals had glucosuria from 4 months onwards, tubular proteinuria and a raised serum creatinine level. They became anemic but remained normotensive. Classification of AA-treated rabbits according to the severity of interstitial fibrosis in the outer cortical labyrinth (OCL) allowed us to classify the animals into three patterns, all of which include an extensive involvement of medullary rays (MR). In pattern I, interstitial fibrosis is almost restricted to MR and corresponding outer medulla (OM). This suggests that the initial insult occurs in the straight part (S3 segment) of the proximal tubules of the the superficial nephrons. In pattern II, fibrosis extends to the outer cortical labyrinth (OCL) suggesting subsequent involvement of the convoluted parts (S1 and S2 segments) of the proximal tubules of the superficial nephrons. In pattern III, additional involvement of the inner cortical labyrinth (ICL) and the corresponding OM suggests an extension of the injury to the proximal tubules of deep nephrons. This progression of interstitial fibrosis provides an explanation for its decreasing intensity from the OCL to the ICL in these animals. Finally, the kidneys of AA-treated rabbits contained the same

AA-DNA adducts as those found in the renal tissue of CHN patients (29).

In conclusion, we have demonstrated in this model that in addition to their well known carcinogenic activity, AAs cause renal interstitial fibrosis with several morphological and salient biological features of CHN. Moreover, induction of these changes by AAs alone without association with the other compounds prescribed in the slimming regimen indicate that AAs play likely a significant role in the pathogenesis of CHN.

Aristolochic acid nephropathy (AAN): Time to abandon the term CHN.

Several clues suggested that CHN was not restricted to the young women who followed the suspected slimming phytotherapy. We recently reported a Chinese female who presented with clinical features of subacute interstitial nephritis after consumption of pills bought in Shangai (30). Renal biopsy disclosed mainly subcapsular hypocellular interstitial fibrosis with severe tubular atrophy. Glomeruli were either globally sclerosed or normal by light microscopy. Subsequently, AAs were detected in the pills and the major AA-DNA adduct, dA-AAI, was identified in a fragment of the frozen kidney biopsy. This observation of CHN features in a patient who had been exposed to AAs without having ingested the other substances contained in the slimming regimen together with the recent demonstration that AAs given to rabbits as a single drug induced similar features, removes any doubt about the causal role of AAs in the so-called CHN. We have thus proposed to designate the interstitial nephritis in which the unequivocal role of AAs has been fully documented by the name of AA nephropathy (AAN). Accordingly, Belgian epidemy (1-7, 14, 24, 32) and outside patients (30-31, 33) with so-called CHN may be now better called AAN.

Are human and experimental features of AAN relevant to BEN ?

The association of interstitial fibrosis with urothelial atypia and malignancy characterizing AAN in rabbits and humans is reminiscent of another interstitial fibrosing nephropathy, the Balkan endemic nephropathy (BEN). BEN is a chronic tubulointerstitial disease characterized by both a familial (34, 35) and an environmental clustering (35). Diagnosis is made in the third to the fifth decade of life with a slight predominance of females in families farming in some villages of Rumania, Croatia, Bosnia, Serbia and Bulgaria in an area of about 400 to 500 km^2 along the tributaries of the Danube (35-36).

Several findings are common to BEN patients and to either AAN patients or to AAs fed rabbits. Arterial blood pressure remains normal whereas serum creatinine increases, mild tubular proteinuria, normoglycemic glucosuria and anemia develop. Tumors develop in all settings. The main target for malignancy is the urothelium of the upper urinary tract in the animal model as well as in humans. Moreover, the incidence of urothelial malignancy in BEN and AAN patients is identical (40%) (6, 35) and associated with urothelial atypia, a constant finfing in AAN patients and rabbits. On morphological grounds, mainly subcapsular regenerative tubular epithelia of mainly proximal tubules, hypocellular interstitial sclerosis and tubular atrophy with normal or sclerosed glomeruli are found in renal biopsies and end stage kidneys from AAN (3, 4) and BEN patients (35-42). Arterial and glomerular lesions are absent in the rabbit model indicating that the renal vasculature is not a primary target of AAs. It is of interest to note that arteries are normal also in the early stages of AAN (4) and that arteriolar hyalinosis and fibroelastosis of the interlobular arteries are seen in all patients with end-stage AAN (3) as well as in 35% of biopsies from patients with more or less advanced BEN (41). This suggests that vascular lesions observed in AAN and BEN patients are secondary to the progressive kidney destruction.

However, some differences between AAN and BEN patients, and rabbits with AAN should be pointed out. Involvement of the columns of Bertin in BEN autopsied patients appears less prominent than in AAN patients (3, 42-43). On clinical grounds, four findings in AAN patients differ from BEN: firstly, the sex-ratio in BEN is approximately 1/1, in contrast to the female preponderance in human AAN; this reflects the almost exclusive female attendance of the X clinic. Secondly, AAN patients progress to end-stage renal failure within a few months or years, in contrast with the slow evolution of BEN and of AAN in rabbits over time. This could reflect a higher level of toxic exposure in AAN than in BEN patients and interspecies differences in the susceptibility to the toxic agent as suggested by the differences in the metabolic transformation of AAs between humans and animal species (44). Alternatively, it may be hypothesized that toxicity of AAs is potentiated in humans by its admixture with other compounds contained in the herbal preparation. Finally, the development of urothelial malignancy requires a much longer exposure time in BEN patients and in rabbits with AAN. Induction times of 20 and 27 years have been calculated for renal pelvic and bladder carcinomas associated with BEN, respectively (45). Urothelial malignancy has been detected in rabbits 21 months after the start of AA exposure. In contrast, in AAN patients, the delay between the end of AA intake and the diagnosis of urothelial malignancy was approximately 2 to 6 years (6).

The final demonstration that AAs play a role in BEN requires evidence that patients with an unequivocal diagnosis of BEN have ingested foods containing AAs, present the typical biological and morphological characteristics of AAN and harbour AA-DNA adducts in their renal tissue.

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