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REVIEW OF THE COOPERATIVE STUDIES OF THE NATIONAL ONCOLOGICAL CENTRE IN BULGARIA ON BALKAN ENDEMIC NEPHROPATHY AND ASSOCIATED URINARY TRACT TUMOURS (1991 – 2001)

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Very few cases of two closely connected diseases in humans have been reported in medical literature so far. One of the most striking examples for such relationship at both population and personal level are the Balkan endemic nephropathy and urinary tract tumours in the endemic regions of former Yugoslavia, Romania and Bulgaria. This strange constellation of impaired health has been studied for over half a century but still its etiology remains obscure despite very hard work done by national and international groups and programs.

In our short review we report the major findings obtained for a period of about 30 years by the collaborative efforts of the authors and their groups in Bulgaria, France, UK and Germany.

Epidemiological studies

The results of the first large population based epidemiological study on the occurrence of Balkan endemic nephropathy (BEN) and Urinary Tract Tumors (UTT) among the population of 27 villages in the endemic area of Vratza district, Bulgaria, for 1965-1974, were published in 1977 by Chernozemsky et al (1). One of the most striking characteristics of BEN was a very high incidence of UTT found among the population at high BEN risk. It was found that the UTT age-related incidence rates per 100 000 inhabitants were 88.8 for men and 104.5 for women in villages with high incidence of BEN and 11.2 and 9.8 for men and women respectively in villages without BEN from the same region.

A second biostatistical study of UTT in the area with BEN in Vratza district, Bulgaria during the period 1975-1991 was completed by Nikolov et al. (2,3). We confirmed the positive correlation between the incidence of UTT and BEN demonstrated in our 1977 study. A UTT incidence of 98.9 per 100 000 men and 74.7 per 100 000 women was found in villages most affected by BEN when compared with 11.0 and 6.7 per 100 000 for men and women, respectively, in non-endemic villages. The relative risk (RR) of developing endemic UTT strongly

Table 1. Age-Standardized Incidence Rates of UTT by Sex and BEN Group Villages (per 100 000 Population)

Groups of villages according to BEN incidence	Sex	Period	
		1965-1974	1975-1991
A – High	M	88.8	98.9
	F	104.5	74.7
B – Moderate	M	45.3	38.0
	F	36.6	44.0
C – Very low	M	9.6	17.1
	F	14.3	19.6
D – Control nonBEN villages	M	11.2	11.0
	F	9.8	6.8

Table 2. Relative Risk (RR) of developing UTT in relation to BEN and sex

Groups of villages according to BEN incidence	Kidney pelvis and ureter		Urinary bladder		All UTT	
	M	F	M	F	M	F
A – High	29 18-48	35 21-56	4 2-5	11 7-17	7 5-9	14 11-19
B – Moderate	8 4-17	27 16-44	2 1-3	6 3-10	3 2-5	10 7-13
C – Very low	6 4-10	6 4-10	2 1-2	3 2-5	2 2-5	4 3-5
D – Control nonBEN villages	1	1	1	1	1	1

depended on the person's residence (in or out of BEN villages) and was higher in women –35 against 29 – in men. One of the most striking finding of the second study was change in the pattern of distribution of UTT on the village base: at that time new villages moved to the top of the list of high incidence endemic UTT villages.

Analytical studies

As the most plausible hypothesis for the etiology of BEN and UTT which has been recently put forward involves mycotoxins; a survey on the possible involvement of the nephrotoxic and carcinogenic mycotoxins Ochratoxin A (OTA) in staple food from endemic and non-endemic areas in Bulgaria was carried out.

OTA was found in samples of home-produced and home-stored beans and maize from both endemic and non-endemic areas, but the samples from affected families were contaminated at higer percentage and at higher levels, than those from unaffected households.

Table 3. OTA contamination of beans and maize samples from different harvests (1984-1990) collected from BEN and control villages in Vratza District, Bulgaria

Cereals	Households	Number of samples	OTA contaminated	
			%	Range (ug/kg)
Beans	BEN	147	36.6-88.2	0.5-264.0
	Control	113	5.0-15.0	0.2-220.0
Maiz	BEN	147	43.7-97.2	5.0-1417.0
	Control	113	5.0-20.0	0.7-235.0

The survey was extended to screen also for the presence of other nephrotoxic mycotoxin – Citrinin. It was found that more than 40% of analyzed beans and maize samples from BEN area were contaminated with Citrinin in the range of 50-1500 ug/kg.

In short, it was found that in Bulgarian endemic areas, BEN and/or UTT affected families are much more frequently and at higher level exposed to OTA and Citrinin (4).

The presence of OTA in food samples from areas of Bulgaria with BEN and a high incidence of UTT shows that the populations of such areas are likely to consume this mycotoxin and it should be detectable in biological fluids from exposed populations. Thus a survey was conducted to determine the occurrence of OTA in blood and urine from people living in the endemic area who were either affected or unaffected by the two diseases as well as in blood and urine from people living in control regions where these diseases do not occur. OTA was found more frequently and at higher levels in blood serums and urines from patients with BEN and/or UTT in comparison with the unaffected people from endemic and control areas (5,6).

Table 4. OTA in blood samples from people in BEN and nonBEN areas in Bulgaria (1984-1990)

Group of persons	Number of subjects	Samples containing OTA	
		%	Range (ng/g)
BEN/UTT patients	105	23.3 – 29.2	20.0 –27.2
Healthy persons from BEN families	111	13.3 – 18.2	12.5 – 15.0
Healthy persons from nonBEN families in BEN villages	116	10.8 – 14.0	10.0 – 12.0
Healthy persons from nonBEN villages in BEN area	129	10.0 – 11.7	10.0 – 18.0
Healthy persons from nonBEN area	125	6.6 – 7.7	8.0 – 10.0

Table 5. OTA in urine samples from people in BEN and non BEN areas in Bulgaria (1984-1990)

Group of persons	Number of subjects	Samples containing OTA	
		%	Range (ng/l)
BEN/UTT patients	36	38.9	5.0 – 604
Healthy persons from BEN families	25	48.0	5.0 – 33.0
Healthy persons from nonBEN families in BEN villages	32	44.0	5.0 – 43.0
Healthy persons from nonBEN villages in BEN area	31	12.9	17.0 – 41.0
Healthy persons from nonBEN area	3	0	0

Assessment of human exposure to OTA (pilot study)

Using duplicate diet method involving healthy young volunteers from two villages heavily affected by BEN and UTT and consuming a free choice of diet, we studied the relationship between OTA levels in urine and serum with that of dietary intake of OTA. The aim was to investigate the possible validity of both serum and urine as markers of OTA exposure. Composite samples of each volunteer's weekly and monthly diet were analyzed together with composite serum and urine samples taken over the same period.

The results of this pilot study showed that ***about 60% of duplicate diets contained OTA.***

The intake of OTA by some persons ranged from 110 ng to 14152 ng.during defferent weeks. OTA was found also in all serum samples, in the range 0.1 to 11.4 ng/ml and in 85% of the urine samples, in the range 0.01 to 0.33 ng/*ml* *(Nikolov, Petkova-Bocharova, Vrabcheva, Pfohl-Leszkowicz, Dragacci, Castegnaro; unpublished data). Further work to define better the relationship between OTA intake and serum and urine OTA levels should be undertaken.*

Table 6. Mean weekly level of OTA in diet, blood serum and urine in young healthy persons from two villages heavily affected by BEN and UTT in Vratza District, Bulgaria

Village	Subject No	OTA level in diet (ng/g)	OTA consumed weekly (ng)	OTA level in blood serum (ng/ml)	OTA level in urine (ng/ml)
Beli Izvor	2	0.29	1968	0.26	0.04
	3	0.24	1220	1.70	0.09
	5	0.09	321	0.56	0.04
	6	0.10	85	0.72	0.07
	7	1.36	6373	8.40	0.86
	8	2.60	6489	6.85	0.59
	9	0.11	442	0.54	0.04
	11	0.12	305	0.70	0.08
Gorno Peshtene	1	0.15	224	0.46	0.02
	2	0.11	521	0.98	0.02
	3	0.09	198	0.59	0.05
	4	0.11	582	1.46	0.06
	5	0.25	387	1.00	0.09
	6	0.07	98	0.42	0.01
	9	0.16	309	0.44	0.10

We analysed also the diets of the same young and healthy volunteers for the occurrence of another nephrotoxic mycotoxin, namely Citrinin, which was found in about 58% of the samples. The weekly intake of Citrinin by the persons was found in the range of 4000 ng to 10000 ng (7).

Biological effects of OTA and studies of the mechanism of action

OTA-related DNA adducts in urinary tract tumours of Bulgarian subjects

In order to establish a possible implication of OTA in urinary tract tumour pathogenesis, we have analyzed TC tumours from three kidneys and five bladders from Bulgarian patients undergoing surgery for cancer and from three non-malignant kidneys collected from French subjects. Since the first step in the tumour formation is the alteration of DNA, we have analyzed the samples for DNA adducts formation. Several adducts with the same RF values as those obtained from mouse kidney after treatment with OTA were detected, mainly in kidney but also in bladder tissues from Bulgarian patients. No adducts were detected in French kidney tissues (8).

OTA-related DNA adducts in progeny through transplacental treatment with a single dose of OTA to pregnant hamsters and mice

We investigated some early signs of genetic impairment including the presence of DNA adducts in target tissues from fetus and progeny of mice and hamsters after administration of a single dose of OTA during the last part of the pregnancy. Several characteristic DNA adducts with the same RF values were detected in kidney and liver of both OTA-treated mice and hamsters and their progeny – the fetuses and the offspring.

Four of the adducts were the same as those found in human kidney tumours, namely adducts No 7,9,12 and 18. No adduct was found in tissues from control animals. High levels of DNA adducts were detected in the kidneys of male progeny, whereas in the female progeny and the mothers they were detected almost exclusively in the liver. Our results provide evidence for the direct genotoxic action of OTA in the progeny through transplacental contamination, which constitutes a new serious health hazard of exposure to this toxin (9,10).

Chromosomal alteration in lymphocytes of BEN patients and of healthy individual after incubation *in vitro* with OTA

Lymphocyte cultures from patients with BEN and from individuals from a nonendemic region were examined and compared with cultures from healthy people which had been incubated *in vitro* with noncytotoxic doses of OTA. The lymphocytes of patients with BEN and/or UTT contain significantly more numerical and structural autosomal aberrations than lymphocytes from controls. The treatment of normal lymphocytes *in vitro* with OTA induced similar anomalies. These results strongly support the idea that OTA is involved in the induction of various chromosomal aberrations and that mycotoxins may play a significant role in the etiopathogenesis of BEN and UTT.

No consistent chromosomal aberration was found in the peripheral lymphocytes of analyzed BEN patients. This investigation therefore excludes the existence of specific chromosomal markers in patients with this disease (11).

Genetic predisposition to Balkan endemic nephropathy

In this line of study we examine the association between efficiency of oxidative metabolism and risk for developing BEN and/or UTT, using Debrisoquine as test substance and urinary samples from subjects with BEN and/or UTT and from healthy subjects from area with BEN and without BEN. The highest urinary recovery of debrisoquine was found among controls from areas with no BEN; recovery in BEN patients was only 50% of the controls. The most interesting result is that BEN patients did not have impaired debrisoquine metabolism: subjects who metabolized <25% of the drug represented only 2.9% of BEN patients, 12.4% of controls from BEN villages and 12.7% of controls from outside the BEN area. The very poor metabolizers represented 1.0% of BEN patients and 4.8-5.8% of controls. The percentages of extensive metabolizers in the same groups were 86.3, 64.5 and 67.4%, respectively. The mean metabolic ratio rose progressively from BEN patients < suspected BEN patients < controls from BEN villages < controls from non-BEN villages; the maximum metabolic ratios were 40, 51 and 87, respectively. The cumulative distribution of the 8-h urinary debriso-

quine metabolic ratios, presented as a normal probability plot, formed a discrete population with values over 10. The distribution among patients with BEN/UTT indicates a predominance of extensive debrisoquine hydroxylation and a lack of poor metabolizers. These results are consistent with the hypothesis that efficiency of oxidative metabolism is greater in BEN patients and that it may be one of the key host factors determining predisposition to these diseases (12).

The Future

Epidemiology

The war in former Yugoslavia (Serbia, Croatia) is a giant health and social experiment on BEN and UTT. A population based epidemiological study will possibly give better understanding of the role of the environmental factors and of the genetic predisposition because of the movement and mixing of big population groups in and out of BEN areas and especially if application of new molecular epidemiological methods will be used.

Mechanisms

Further studies on DNA adduct formation with different xenobiotics.

Application of DNA chip technology.

Application of new markers for individual susceptibility to xenobiotics.

Healthcare

UTT screening in BEN areas with new tumour markers.

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