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OCHRATOXIN A IN BLOOD OF HEALTHY POPULATION IN ZAGREB

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Healthy blood donors from the city of Zagreb were checked for the presence of a nephrotoxic mycotoxin ochratoxin A (OTA) in the plasma. Samples of blood were collected in June, September, and December 1997, and March 1998, totalling 200 or 50 in each round. The concentrations of OTA were measured using high pressure liquid chromatography (HPLC) method (detection limit 0.2 ng OTA/ml of plasma). The frequency of OTA-positive samples (>0.2 ng/ml of plasma) showed significant seasonal variation ($P<0.001$). The frequency of OTA-positive samples was the highest in March (65%) and it gradually decreased towards December (12%). The high frequency of positive samples coincided with seasons favouring growth of moulds and production of toxins. The daily intake of OTA by healthy persons in Zagreb was estimated from the mean concentration of OTA in samples collected during the whole year (0.19 ng OTA/ml plasma). The estimated daily intake was 0.26 ng/kg b.w., that is, substantially below the tolerable daily intake proposed by World Health Organization (16.0 ng/kg b.w.).

Key words:
blood donors, endemic nephropathy, HPLC analysis, mycotoxins, ochratoxin A intake

Ochratoxins are the mycotoxins produced by storage moulds of *Aspergillus* and *Penicillium* strains which contaminate cereals, meat, and various other commodities (1). The most important ochratoxin is the nephrotoxic ochratoxin A (OTA), present in cereals, meat, coffee, wine, and beer. It is supposed to play a role in the etiology of endemic nephropathy (2, 3). Its presence was first detected in the blood of inhabitants from an endemic region of Croatia (4). Consequently, it had systematically been measured in the blood of inhabitants in endemic villages for several years (5, 6). As a matter of fact, OTA was found in the plasma of inhabitants from endemic and from control villages, but the concentration of OTA was higher in endemic villages (5–7).

The high frequency of samples with low concentrations of OTA was found in other countries where the endemic nephropathy was not detected (8–11). Countries like France, Switzerland, and Sweden showed regional differences in mean concentrations of OTA (8, 11, 12) probably as the consequence of different dietary habits in those regions.

The aim of our investigation was to check the frequency and the concentration of OTA in healthy inhabitants of Zagreb and to compare them with healthy inhabitants of other countries. The mean concentration of OTA in plasma makes it possible to estimate the daily intake of this toxin and the comparison with recommended limits.

MATERIALS AND METHODS

Chemicals

Crystalline OTA was obtained from Sigma (St. Louis, MO, USA). Acetic acid, hydrochloric acid, chloroform, magnesium chloride, and TRIS buffer were obtained from Kemika (Zagreb, Croatia), and acetonitrile and water, both HPLC-grade, came from MERCK (Darmstadt, Germany).

Standards

Standard samples with OTA were prepared by adding known amounts of stock solution (0.1 mg OTA/ml of TRIS, pH 7.4) to portions of pooled plasma.

Plasma samples

Blood samples of healthy volunteers were collected at the Croatian Institute for Transfusion Medicine in Zagreb in the first week of June, September, and December 1997, and March 1998. Each time 50 samples of about 5 ml of blood were taken. Samples were centrifuged and the plasma was stored at -80 °C until analysed. The project was approved by the Ethical Committee of the Croatian Institute for Transfusion Medicine and the volunteers were informed about its purpose.

Extraction and purification of plasma samples

Plasma samples were extracted and purified using the method described by *Beker and Radić* (13), with 87% recovery of OTA from standards. The evaporated residue was kept at -20 °C until analysed.

The detection limit of the method was 0.2 ng of OTA/ml plasma. The limit was calculated as the amount of toxin that gave a peak height three times higher than the mean noise signal.

High pressure liquid chromatography

Equipment consisted of isocratic pump (Gilson 305), manometer (Gilson 805), injector (Rheodyne 7125) with loop (50 μl), fluorescent detector (Thermo Separation Products – Spectra System FL 2000) printer (Gilson N1), column, and guard column filled

with C_{18} reverse phase (Licrospher, Merck), with dimensions 4.0 x 125.0 mm and 4.0 x 4.0 mm, respectively. The particles of the stationary phase were 5 μm.

Chromatographic conditions

The ratio of acetonitrile:water:acetic acid in the mobile phase was 500:500:5, respectively at pH 3.16. At room temperature the flow rate was 0.5 ml/min and the pressure 22 bars. The wave length of the fluorescent detector was 336 nmλ_{ex} and 464 nm λ_{em}. The retention time was about nine minutes and the injected volume was 50 μl.

Statistics

Standard curves were calculated from the results of analysis of the spiked plasma samples using the least-square method. The results of plasma sample analysis were divided in two groups: below and above the detection limit of 0.2 ng OTA/ml plasma. The statistical significance of the seasonal variations of OTA-positive samples was calculated using the χ^2-test, and was confirmed through the Fisher's exact test.

Estimated daily intake of ochratoxin A

The daily intake of OTA was estimated from the mean concentrations of OTA in all samples collected in one season, using the equation given by *Breitholtz and coworkers* (12):

$$k_o = 1.34 \times Cp$$

where k_o is the constant of daily intake (ng/kg b.w.) and Cp is the concentration of OTA in plasma.

RESULTS

Table 1 shows the results of the OTA analysis of plasma samples of healthy blood donors. OTA-positive samples were found in plasma collected in all seasons. The analysis of OTA-positive samples was not even throughout the year and the differences in the frequency of positive samples were significant ($P<0.001$). Under the null hypothesis of equal proportions, the frequency of positive samples was lower than expected in March and higher than expected in December. Only two plasma samples contained over 1.0 ng/ml OTA, one collected in March (1.2 ng/ml) and the other in June (1.3 ng/ml).

Table 1 *Seasonal variations in the frequency of ochratoxin A-positive samples in Zagreb (P>0.001, χ^2-test)*

		Concentration of OTA (ng/ml)		Number of samples
		<0.2	>0.2	
March	N samples	17	31	48
	%	35.4	64.6	
	expected	26.667	21.333	
	χ^2	3.504	4.3802	
June	N samples	21	29	50
	%	42.0	58.0	
	expected	27.778	22.222	
	χ^2	1.654	2.067	
September	N samples	28	22	50
	%	56.0	44.0	
	expected	27.778	22.222	
	χ^2	0.002	0.002	
December	N samples	44	6	50
	%	88.0	12.0	
	expected	27.778	22.222	
	χ^2	9.474	11.842	
Total		110	88	198

Table 2 shows mean concentrations of OTA in all samples from each collection round. The mean concentration reached the peak in March, after which it kept falling. The mean concentration in all collected samples is low and so is the calculated daily intake of OTA for the donors.

Table 2 *Seasonal variations in the mean concentration of ochratoxin A in plasma and the estimated daily intake in Zagreb*

	Mean concentration of OTA (ng/ml)	Daily intake of OTA (ng/kg b.w.)
March	0.27	0.36
June	0.26	0.35
September	0.16	0.21
December	0.08	0.11
Average	0.19	0.26

DISCUSSION

The similarity of morphological changes in the kidney of patients with endemic nephropathy and in the kidney of pigs exposed to ochratoxin A in feed in Scandinavia suggests the possibility that OTA plays a role in the etiology of endemic nephropathy (2). Inhabitants of endemic villages showed significantly higher blood OTA concentrations than did controls from nonendemic villages (6, 7). However, OTA was often found in small concentrations in a number of European countries (Table 3). The

Table 3 *The frequency of ochratoxin A-positive samples of plasma in inhabitants of European countries. The mean concentration was calculated in all samples analysed*

Country	Collecting period	OTA positive/ analysed (%)		Mean concentration (ng/ml)	Range (ng/ml)	Reference (no.)
Bulgaria	1984 – 1990	9/125	(7)	0.2		(7)
Czechoslovakia	1990	35/143	(24)	0.14	0.1 – 1.3	(14)
Czech Republic	1994	734/809	(91)	0.23	0.1 – 13.7	(15)
	1995	404/413	(98)	0.24	0.1 – 1.9	(15)
Denmark	1986 – 1988	78/144	(54)	1.8	0.1 – 13.2	(9)
France	1991 – 1992					(8)
Alsace		97/500	(19)		0.1 – 11.8	
Aquitaine		385/2055	(19)		0.1 – 16.0	
Rhone-Alpe		75/515	(15)		0.1 – 4.3	
Germany	1977	84/164	(51)	0.4	0.1 – 14.4	(16)
	1985	89/141	(63)	0.3	0.1 – 1.8	(16)
	1988	142/208	(68)	0.75	0.1 – 8.0	(17)
Hungary	1994	52/100	(52)	0.48		(18)
	1995	291/355	(82)		0.2 – 10.0	(19)
	1997	213/277	(77)		0.1 – 1.4	(20)
Italy	1992	65/65	(100)	0.53	0.1 – 2.0	(10)
	1997	87/100	(87)	0.45		(21)
Poland	1983 – 1984	25/397	(6)	0.2	1.0 – 13.0	(22)
	1984 – 1985	52/668	(8)	0.3	1.0 – 40.0	(22)
Spain	1996 – 1998	40/75	(53)	0.71	0.5 – 4.0	(23)
Sweden	1989					(12)
Visby		29/99	(29)	0.26	0.3 – 7.0	
Uppsala		3/99	(3)	0.02	0.3 – 0.8	
Ostersund		6/99	(6)	0.03	0.3 – 0.8	
Switzerland	1989					(11)
north of the Alps		251/252	(100)		0.06 – 2.14	
south of the Alps		116/116	(100)		0.11 – 6.02	

frequency of OTA-positive samples depends on the sensitivity of the method used. In Switzerland, OTA was found in all samples of human plasma. The investigation used a very sensitive HPLC method with immunoaffinity column cleanup (detection limit 0.06 ng OTA/ml plasma) (11). A study in Poland using a spectrofluorimetric method (detection limit 1.0 ng OTA/ml plasma), however, revealed only 6–8% of OTA-positive samples (22). As it is difficult to compare the frequency of positive samples, it seems that the mean concentration in all samples would be a better parameter for the comparison. In our investigation, the mean OTA concentration in all samples regardless of the collection period was 0.19 ng of OTA/ml of plasma. This concentration is not high and corresponds to the mean concentration of OTA in blood of healthy inhabitants in other European countries with continental climate (Czech Republic, Bulgaria, and Poland).

Most similar investigations in European countries do not state the season in which the samples were collected. Our investigation showed significant seasonal variations in the frequency of OTA-positive samples in Zagreb; OTA-positive samples were the most frequent in March, decreasing gradually towards December. The period with the highest frequency of OTA-positive samples (and the highest mean concentration of OTA) corresponds to the season favouring the growth of moulds and consequent production of toxin.

The frequency of OTA-positive samples (>0.2 ng/ml plasma) found in Zagreb did not differ from the frequency in Split, Rijeka, and Varaždin from our previous investigation carried out in June (24). However, all samples from Osijek were OTA-positive, and the mean concentration was 0.68 ng of OTA/ml of plasma. An investigation in France, Switzerland, and Sweden revealed significant regional variations in the frequency of OTA-positive samples or in the mean concentration of OTA (8, 11, 12). The authors believe that these variations are the consequence of specific dietary habits in these regions. The same is probably true for the inhabitants of Osijek who »excel« in consumption and preference of fresh and dried pork over fruit and vegetables with respect to other inhabitants of Croatia (25).

The daily intake of OTA could be estimated from the OTA concentration in food or from the OTA concentration in human plasma. Both calculations include some approximations. The first calculation poses the problem of food sampling. The determination of OTA in various commodities requires different methods of analysis. This method of estimation should rely on the analysis of a large number of food samples. The advantage of the latter method of estimation (from the mean OTA concentration in population) is that it only needs a proper method of blood analysis. Positive blood OTA confirms the exposure to this toxin, but it does not indicate the origin of exposure. On the other side, toxicokinetics of OTA in humans is not established and this calculation should include the approximations from toxicokinetics in monkey. Both calculations were compared and gave very similar results (26).

Seasonal variations of daily intake of OTA in Zagreb range from 0.11 to 0.36 ng/kg b.w. The mean daily intake of 0.26 ng OTA/kg b.w. is lower than the mean European daily intake estimated from the mean blood concentration (0.9 ng/kg b.w.) found in a large investigation which included 13 European countries (27). The estimated daily intake of OTA in Zagreb is much below the limit of 16.0 ng/kg b.w. recommended by World Health Organisation (28).

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Sažetak

NALAZ OKRATOKSINA A U PLAZMI ZDRAVIH LJUDI U GRADU ZAGREBU

Prisutnost okratoksina A (OTA) mjerena je u plazmi zdravog stanovništva u Zagrebu. U ožujku, lipnju, rujnu i prosincu skupljeno je po 50 uzoraka krvi. Koncentracija OTA određena je visokotlačnom tekućinskom kromatografijom uz granicu detekcije od 0.2 ng/ml plazme. Učestalost uzoraka iznad detekcijske granice bila je različita u različitim godišnjim dobima (χ^2-test, P<0,001). Učestalost uzoraka s koncentracijom OTA iznad granice detekcije bila je najveća u ožujku (65%) te je postepeno opadala do prosinca (12%). Najveći postotak uzoraka s OTA iznad detekcijske granice uzorkovan je u onim mjesecima koji pogoduju rastu plijesni i sintezi mikotoksina. Dnevni unos OTA u zdravih stanovnika grada Zagreba procijenjen je iz srednje koncentracije u svim uzorcima plazme (0,19 ng/ml). Procijenjeni dnevni unos OTA u gradu Zagrebu je 0,26 ng/kg tj. t., odnosno mnogo je manji od granične vrijednosti od 16,0 ng/kg tj. t. što ga je predložila Svjetska zdravstvena organizacija.

Ključne riječi:
endemska nefropatija, mikotoksini, visokotlačna tekućinska kromatografija, zdravi stanovnici

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