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URINE OCHRATOXIN A AND SPHINGANINE/ SPHINGOSINE RATIO IN RESIDENTS OF THE ENDEMIC NEPHROPATHY AREA IN CROATIA*

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The most plausible theory of the aetiology of endemic nephropathy links it with exposure to nephrotoxic mycotoxin ochratoxin A (OTA). In this study, the concentration of OTA and sphinganine/sphingosine (Sa/So) ratio, the biomarker of another nephrotoxic mycotoxin fumonisin B, exposure, were analysed in 45 human urine samples collected in the endemic village of Kaniža in Croatia and in 18 samples from control village. Samples were collected twice from the same persons in 2000 and 2005. In both years the frequency of OTA-positive samples was higher in Kaniža (43 % and 18 %, respectively) than in the control village (28 % and 6 %, respectively). OTA concentrations in samples collected in Kaniža were higher in 2000 than in 2005 ($p < 0.005$). Although in both years Sa/So ratio was higher in Kaniža, the difference from the control group was not statistically significant. No control sample contained OTA and had the Sa/So ratio > 1 at the same time, while in Kaniža four such samples were collected in 2000 and one in 2005.

KEY WORDS: *endemic nephropathy, fumonisin B, HPLC, ochratoxin A, sphingolipids*

Endemic nephropathy (EN) is a fatal human kidney disease that occurs in 14 villages in the eastern part of the Croatian Brodsko-posavska county. In the 1970s, the aetiology of the disease was believed to be related to exposure to mycotoxin ochratoxin A (OTA) (1). High incidence of otherwise rare urothelial tumours observed later in the same endemic region was also associated with this toxin (2, 3). Studies on laboratory and domestic animals have shown that OTA is nephrotoxic, carcinogenic, genotoxic, and immunotoxic (4). The International Agency for Research on Cancer (IARC) has classified OTA as Group 2B carcinogens (possible human carcinogen)

(5). OTA was found in various commodities from endemic villages (6), and its concentration was much higher in maize collected in the endemic than in control regions in the 1997 crop (7). The same difference was observed by Puntarić et al. (8) for wheat and maize samples collected in 1999. Although OTA was more frequent in the plasma of the residents of endemic villages, it was also found in the plasma of the residents of control villages (9). Low concentrations of OTA are frequent in the healthy population of Croatia and other countries where EN is not known (10).

Some earlier studies on maize contamination with mycotoxins in the endemic region and the whole of Croatia revealed a high percentage of samples contaminated with fumonisins and to a lesser extent with OTA (7, 11). The most frequent fumonisin in

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maize was fumonisin B₁ (FB₁). In some regions of Africa and China unusually frequent oesophageal cancer and primary liver cancer in humans was associated with high exposure to FB₁. IARC classified FB₁ in the same group of carcinogens as OTA (Group 2B) (12). Animal studies have shown that FB₁ is poorly absorbed and rapidly distributed and eliminated. This makes FB₁ measurement in biological materials useless for evaluation of exposure to this mycotoxin. However, it is established that FB₁ toxicity is the consequence of the inhibition of ceramide synthase, a key enzyme in the biosynthesis of sphingolipids. Animal studies have shown that an elevated ratio of sphingolipids sphinganine and sphingosine (Sa/So) is a reliable biomarker of exposure to fumonisins. This marker had already been evaluated in the serum and urine of subjects living in the EN region (13), and the study showed higher Sa and So concentrations, and higher Sa/So ratio in residents of the endemic region.

The geographically limited occurrence of EN and particular urothelial tumours suggest that some other natural contaminant could add to OTA toxicity and contribute to the development of EN. Since earlier exposure data for the residents of the endemic villages are separate for OTA and fumonisins (Sa/So ratio), the aim of this study was to see whether exposure to these two mycotoxins was in fact combined. The same subjects were tested for these two mycotoxins in 2000 and 2005 to accommodate for high variability of grain contamination from year to year.

MATERIALS AND METHODS

Chemicals

Standards of OTA, sphingoid bases (C₁₈-D-sphingosine and C₁₈-DL-erythro-dihydrosphingosine), *o*-phthalaldehyde (OPA), and 2-mercaptoethanol were purchased from Sigma (St. Louis, MO, USA). Water for HPLC mobile phase and silica gel Si-60 (15 µm to 40 µm) were obtained from Merck (Darmstadt, Germany). Methanol, acetic acid, hydrochloric acid, chloroform, sodium sulphate anhydrous crystal, and formic acid were supplied by Kemika (Zagreb, Croatia). All chemicals were of pro-analysis grade. Water and methanol used for HPLC mobile phase were of HPLC grade.

Urine samples

For OTA and sphingolipid analysis, spot human urine samples were collected from 63 subjects in the spring of 2000 and 2005, and kept frozen at -80 °C until analysis. Forty-five samples were from the endemic village of Kaniža and 18 from a control village where EN is not known. Urine was sampled according to the Croatian law, with the previous approval of the Ethics Committee of the Institute for Medical Research and Occupational Health in Zagreb.

HPLC system

The high-performance liquid chromatograph used in the experiment consisted of a gradient pump (INERT 9012, Varian, Walnut-Creek, CA, USA), manual injector (Rheodyne 7125, Cotati, CA, USA) with a 50 µL loop, and a fluorescent detector (9075, Varian, Walnut-Creek, CA, USA). The guard column and analytical column were LiChrospher RP-18 (Merck, Darmstadt, Germany) with 5 µm particles and their size was 4 mm x 4 mm and 125 mm x 4 mm, respectively. Chromatographic data were collected and processed using Star Chromatography Workstation software (Ver. 5.0, Varian, Walnut-Creek, CA, USA). The analysis was performed at room temperature.

Determination of OTA

To determine OTA concentrations in the urine we adopted the method of Pascale and Visconti (14). This method uses immunoaffinity columns (OchraTest™, Vicam, Watertown, MA, USA) for sample cleanup procedure. Urine samples (10.0 mL) were diluted with 5 % NaHCO₃ (10.0 mL), mixed, and filtered through Whatman No.1 filter paper. Ten mL of filtered sample were transferred to OchraTest™ immunoaffinity columns. Immunoaffinity columns were washed twice with distilled water (5.0 mL), and OTA was eluted with 2 mL methanol. The eluted extract was evaporated under a stream of nitrogen in a water bath at 60 °C. The residues were kept at +4 °C until analysis. Just before HPLC analysis, the residues were dissolved in 300 µL of the mobile phase.

For OTA analysis, the mobile phase consisted of methanol, water, and acetic acid (70:30:2), and the flow-rate was 0.5 mL min⁻¹. The excitation wavelength of the fluorescence detector was set at 336 nm, and the emission wavelength was 464 nm. Validation of the method showed linear standard curve (r²=0.998). OTA detection limit of the method was 0.005 ng mL⁻¹ and reproducibility (day to day precision), expressed

as relative standard deviation (RSD), was 3.5 %. The presence of OTA in some OTA-positive samples was confirmed by adding OTA standard to the sample.

Determination of sphingolipids

Free So and Sa concentrations in the urine samples were determined using the method of Solfrizzo et al. (15). Urine (2.0 mL) was diluted with methanol (1.9 mL), alkalised with 1.2 mL NH_4OH (0.35 mol L^{-1}), and extracted with chloroform (4.0 mL). After centrifugation, the chloroform extract was cleaned up through a silica gel mini-column consisting of 5.0 g of sodium sulphate anhydrous crystal packed on top of 0.2 g silica gel 60. The mini-column was conditioned with chloroform and then loaded with the sample. So and Sa were eluted from the mini-column with 4 mL of $\text{CHCl}_3:\text{MeOH}:\text{NH}_4\text{OH}$ (50:50:2). The collected eluate was evaporated to dryness in a water bath under a stream of nitrogen at 60 °C. Before injection, the sample was redissolved in 250 μL of methanol, and derivatised in reaction with 50 μL of o-phthalaldehyde (OPA).

The mobile phase consisted of methanol and water (90:10). The flow rate was 1.0 mL min^{-1} . Wavelengths of the detector were set at λ_{em} 334 nm and λ_{ex} 440 nm. Validation of the method showed linear standard curves for So and Sa (with $r^2=0.997$ and $r^2=0.996$, respectively). The detection limit for sphingolipids was 0.1 ng mL^{-1} and RSD below 10 %.

Statistics

Differences in the means of two independent samples between the groups were evaluated with the Student's *t*-test using Statistica 5.0. Probability values of $p<0.05$ were considered statistically significant.

RESULTS

Tables 1 and 2 show urine OTA concentrations and Sa/So ratio measured in samples collected from 45 residents of the endemic village of Kaniža and 18 control subjects in 2000 and 2005. In both years the mean OTA concentration in positive samples was higher in Kaniža than in control samples. Mean OTA concentration in all samples collected in Kaniža in 2000 was higher than in 2005 ($p<0.005$).

In both years the frequency of OTA-positive samples was also higher in Kaniža residents than controls. In addition, it was higher in 2000 than in 2005 in both groups of subjects.

Mean Sa/So ratio was higher in the urine collected from Kaniža residents than from controls in both years (Table 2). No control urine sample had the Sa/So ratio >1.0 . The number of samples from Kaniža with Sa/So ratio >1.0 was higher in 2000 than in 2005. Four samples collected from Kaniža in 2000 and one in 2005 contained both detectable amounts of OTA and Sa/So ratio >1 .

DISCUSSION

Mycotoxins are still the most plausible cause of EN development. EN's geographically limited occurrence in Croatia and other countries suggests that its aetiology should involve a local natural toxin. The most common suspect is OTA, which was found more frequently in the plasma of endemic region residents than in controls (9). As OTA is excreted via urine, positive urine finding is used to confirm human exposure to this mycotoxin (14, 16, 17).

In our study, OTA-positive samples were found in both subject groups and in both years of sampling. This result is not surprising because we had already found OTA in the plasma of healthy Croatian population who did not live in the endemic area (10). However, the frequency of positive samples was higher in the residents of the endemic village of Kaniža than in the control village, which corroborates the study by Radić et al. (9). This could be the consequence of higher OTA contamination of cereals in the endemic than in other areas (7, 8).

It is interesting to note that the higher mean concentration of OTA and higher percentage of OTA-positive urine samples was found in 2000 than in 2005 regardless of the sampling location. The urine samples were collected in the spring, when the subjects still consumed last year's crop. According to the data of the Meteorological and Hydrological Service of Croatia, the years preceding urine sampling were climatically very different. In 1999, the temperature and rainfall in the EN area significantly exceeded the long term average, while in 2004, both parameters were only slightly above the normal (18). In the endemic area of Croatia, significant year-to-year variability of OTA concentration has already been observed for maize (7).

In our study of maize collected in 14 counties of Croatia, FB_1 concentration was above the detection limit in all samples (11). A similar very high frequency of FB_1 -positive samples was found in maize collected

Table 1 Mean OTA concentrations in urine samples and the frequency of positive samples in 2000 and 2005

Year	Samples	N	n	All samples	Positive samples		Frequency of positive samples /%
				Mass concentration of OTA / ng mL ⁻¹			
				Mean ± SD	Range		
2000	Controls	18	5	0.003±0.005	0.01±0.006	0.005 to 0.02	28
	Kaniža	45	19	0.007±0.014	0.017±0.018	0.005 to 0.086	43
2005	Controls	18	1	0.005±0.024	0.01	0.01	6
	Kaniža	45	8	0.001±0.003**	0.007±0.003	0.005 to 0.015	18

N – number of samples

n – number of positive samples

** different from concentration in Kaniža in 2000 ($p < 0.005$)

Table 2 Mean sphingosine (Sa) and sphinganine (So) concentrations, Sa/So ratios, and the frequency of samples with Sa/So ratio below and above 1.0 in 2000 and 2005

Year	Samples	N	Sphingosine	Sphinganine	Sa/So ratio	Sa/So < 1		Sa/So > 1	
			Mass concentration / ng mL ⁻¹			n	Frequency of samples / %	n	Frequency of samples / %
			Mean ± SD						
2000	Controls	18	3.72±2.83	0.79±0.75	0.19±0.09	18	100	-	-
	Kaniža	45	2.54±2.95	2.47±2.90	1.79±1.98	33	73	12	27
2005	Controls	18	5.84±8.05	1.78±2.86	0.33±0.27	18	100	-	-
	Kaniža	45	0.93±0.46	2.38±9.34	1.45±4.44	43	96	2	4

N – number of samples

n – number of positive samples

both from the endemic and non-endemic areas (7). In the only Croatian study of urine Sa/So ratio performed by now, this ratio was significantly higher in persons with suspected EN and in healthy subjects from the endemic area than in controls, regardless of the sex (13). In our study, mean urine Sa/So ratio was also higher in Kaniža residents than in controls, but due to the high variability of data, this difference is not statistically significant (Table 2). Although the endemic area is not known for high consumption of maize, mean Sa/So ratio in the urine collected in 2000 and 2005 in Kaniža was similar or higher (1.79 and 1.45, respectively) than in the urine of persons living in the areas of North Argentina and South Brazil known for high consumption of maize (0.69 and 1.57, respectively) (19). Control values for Sa/So in our study in 2000 and 2005 were 0.19 and 0.33, respectively, which is in accordance with the Sa/So ratios from earlier studies ranging from 0.12 to 0.43 (13, 19-21).

It is known that FB₁ significantly increases Sa and to a lesser extent So concentration in experimental animals. This discrepancy reflects in changed Sa/So ratio. Studies of humans consuming home-grown maize contaminated with fumonisins showed no increase in the Sa/So ratio (19). It seems that change in the Sa/So ratio is not sensitive enough to be a biomarker of fumonisin exposure in humans, probably because humans are exposed to a much lower level of fumonisins than experimental animals. The arbitrary Sa/So ratio cut off of above 1.0 shows that Sa is higher than So, which in turn suggests exposure to fumonisins. When we set the cut off for Sa/So ratio to 1.0 (see 19), we found that all samples >1.0 originated from Kaniža (Table 2).

Although the co-occurrence of OTA and FB₁ in maize seems to be quite frequent in Croatia (7, 11), only five samples in our study, all of them collected in Kaniža, indicated the exposure to both mycotoxins. This is probably because these mycotoxins are

produced by different moulds (mostly *Penicillium* spp. and *Fusarium* spp., respectively). Human exposure to these two mycotoxins is particularly interesting not only because of additive and even synergistic effect on cultured cells (22, 23) but also because of the synergistic increase in DNA lesions seen in the kidney of experimental animals treated with both mycotoxins (24). Despite the well-known nephrotoxicity and carcinogenicity of OTA and FB₁ *in vitro* and in animal studies, nothing is known about their effect in humans. This is the first report on combined human exposure to these two mycotoxins. Our results have confirmed that human exposure to OTA and FB₁ varies from year to year. Higher frequency of OTA-positive samples and the higher number of samples with Sa/So ratio >1.0 in the endemic than in the control village call for a continued study of this issue.

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Sažetak

OKRATOKSIN A I OMJER SFINGANINA I SFINGOZINA U URINU STANOVNIKA S PODRUČJA ENDEMSKE NEFROPATIJE U HRVATSKOJ

Najprihvatljivija teorija o etiologiji endemske nefropatije povezuje njezin nastanak s izloženosti nefrotoksičnim mikotoksinima. Dok se izloženost mikotoksinu okratoksinu A (OTA) može dokazati njegovim nalazom u biološkim uzorcima kao što su krv i urin, vrlo kratko zadržavanje fumonizina B₁ (FB₁) u organizmu to onemogućava. Na pokusnim je životinjama nađeno da je porast omjera koncentracija sfingolipida sfinganina i sfingozina (Sa/So) biološki pokazatelj izloženosti tom mikotoksinu. U ovom istraživanju mjerena je koncentracija OTA i omjer koncentracija Sa/So u urinu 45 stanovnika u endemskom selu Kaniža i 18 stanovnika u kontrolnom selu. Uzorci urina skupljeni su od istih osoba 2000. i 2005. godine. U obje godine učestalost uzoraka koji su sadržavali OTA bila je veća u Kaniži (43 % i 18 %) negoli u kontrolnom selu (28 % i 6 %). Koncentracija OTA također je bila viša u urinima skupljenim u Kaniži negoli u kontrolnom selu. Koncentracija OTA u uzorcima skupljenim u Kaniži 2000. bila je viša nego u uzorcima iz 2005. ($p < 0.005$). Iako je u urinima iz obje godine omjer koncentracija Sa/So bio viši u Kaniži negoli u kontrolnom selu, razlika nije bila statistički značajna. Nije nađen nijedan uzorak skupljen u kontrolnom selu koji bi istodobno sadržavao mjerljivu koncentraciju OTA i omjer Sa/So veći od jedan. Za razliku od uzoraka iz kontrolnog sela, četiri uzorka skupljena u Kaniži u 2000. godini i jedan uzorak u 2005. godini upućivali su na istodobnu izloženost ovim mikotoksinima.

KLJUČNE RIJEČI: *endemska nefropatija, fumonizin B₁, okratoksin A, sfingolipidi*

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