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ANALYSIS OF OCHRATOXIN A IN SERUM AND URINE OF INHABITANTS FROM AN AREA WITH BALKAN ENDEMIC NEPHROPATHY: A ONE MONTH FOLLOW UP STUDY

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Summary. *In the 1950s a series of publications from Bulgaria, Yugoslavia and Romania described a kidney disease occurring in geographically limited areas of these three Balkan countries. Several studies implicated a mycotoxin etiology, and particularly ochratoxin A. In Bulgaria, the exposure of populations to OTA was supported by a very high prevalence of OTA levels exceeding 2 ng/ml in the blood of affected population. OTA has also been found more often in the urine of people living in BEN-endemic villages than in those in non-endemic villages, and the highest levels were seen in patients with BEN or urinary tract tumors. In the present study we follow up the blood and urine OTA levels of some population from the Bulgarian high incidence area of BEN, not affected by any renal diseases. Samples were thus collected for one month, at the beginning of week one and then at the end of each week. This study strengthens our previous results and demonstrate that the population in the Balkans is more exposed to OTA than the general population in Europe.*

Key words: *Balkan Endemic Nephropathy, ochratoxin A, serum & urine analysis*

Introduction

In the 1950s a series of publications from Bulgaria (1), Yugoslavia (2) and Romania (3) described a kidney disease occurring in geographically limited areas of these three Balkan countries. The progressive, untreatable course and fatal outcome in uraemia shortly after manifestation of symptoms made this disease a major problem of renal pathology. In 1964, a group of World Health Organization (WHO) experts reviewed critically the available data and provided the following description of the disease (4): "…progressive and very gradually developing renal failure with insidious onset. It develops without a nephrotic syndrome and usually without hypertension. There is a marked anemia, mild proteinuria, and trivial urinary deposit. The kidney concentration power is reduced in all cases and out of proportion to the degree of restriction of the glomerular filtration"; "interstitial nephropathy of non-inflammatory origin with heavy damage of the tubular epithelium and only late and secondary destruction of the glomeruli leading eventually to an extreme renal contraction… The last stage shows marked fibrosis, especially in the outer zone of the cortex, where there is a complete absence of the tubules and many fibrotic glomeruli... At this stage chronic and/or acute inflammation is not frequent". The disease was thereafter referred to as Balkan endemic nephropathy (BEN). Later, an association between BEN and tumors of the urinary tract was recognized (5-11), so that the problem of BEN became not only a nephrological but also an oncological one. Although a few cases of BEN have been diagnosed before the age of 50, most occur in the range of 50-60 years and tumors may be detected even later (9). The most affected organs are the renal pelvis and ureter, with combined age-adjusted incidence for these two organs of $74.2/10^5$ and $43.5/10^5$ for females and males, respectively, in Bulgaria (9).

In 1972, on the basis of a series of epidemiological observations, Akhmeteli (12) suggested that fungal toxins were involved in the aetiology of BEN and in the same year Krogh (13), in view of the similarities between BEN and ochratoxin A (OTA)-induced porcine nephropathy, suggested that this toxin may be involved in the aetiology of BEN.

Hult et al. (14) detected OTA in 7% of blood from

humans in the BEN area from Yugoslavia. This was confirmed by Fuchs et al. (1991) (15), Radic et al. (16) and in Croatia by Peraica et al. (17). In Bulgaria, the exposure of population to OTA was supported by a very high prevalence of OTA levels exceeding 2 ng/ml in the blood of affected population (18, 19). OTA has also been found more often in the urine of people living in BEN-endemic villages than in those in non-endemic villages, and the highest levels were seen in patients with BEN or urinary tract tumours (20).

In the present study we follow up the blood and urine OTA levels of some inhibitans from the high incidence area of BEN, not affected by any renal diseases. Samples were collected for one month, at the beginning of week one and then at the end of each week.

Material and methods

Selection of study subjects

Two villages [Gorno Peshtene (GP) and Beli Izvor (BI)], located in Vratza District (a high risk area for BEN), north-west Bulgaria were selected by the staff of the National Center of Oncology from Sofia. Sixteen healthy volunteers - 5 from GP and 11 from BI participated in the investigation. These people were selected in the age range 20 to 30 years to avoid subjects with impaired renal function. All subjects signed a written consent form and had clinically normal blood biochemistry, haematology and urinalysis values. Pre-study females were not lactating and shown not to be pregnant by a negative pregnancy test. Participants had a negative result to hepatitis B surface antibodies. Subjects with a history of hepatic, renal or metabolic disorders, cardiovascular or gastrointestinal disease were excluded. Subjects who had undergone surgical operation (s) on the digestive tract (other than appendectomy) or had a history of alcohol or drug abuse were also excluded. Participants declared that they would consume their normal diet for the duration (1 month) of the study.

Blood and urine collection and preparation

All the preparation of samples has been performed by the staff from the National Center of Oncology in Sofia.

Blood samples were collected (in tube containing the aqueous citrate sodium anticoagulant) from each subject on days 0, 7, 14, 21 and 28 of the investigated month. Sera (5 ml) were prepared by centrifugation of blood at 400 *g* and heated at 56°C for 30 min to discard any possibility of HIV contamination. The separated sera were frozen and sent to IARC-Lyon for ochratoxin A analysis where they were stored at -80°C until assayed.

Urine samples were collected during twenty-four hours and the volumes recorded. Aliquots (50 ml) of these 24 hour-urine samples were taken from each subject on days - 1, 6, 13, 20 and 27 of the same month. They were frozen and sent to ENSA-Toulouse for ochratoxin A analysis where they were stored at -80°C until assayed.

Chemicals

All reagents and solvents for the extraction were of analytical grade quality. For HPLC analysis, solvents were of HPLC grade. The sodium acetate solution contained 0.005 M in water.

Ochraprep® immunoaffinity columns (IAC) were purchased from Rhône Diagnostic Technologies (Lyon, France).

Millex-GVS filters: 4 mm, 0.22 µm (Millipore, Molsheim, France)

Analysis of OTA in serum and urine

Blood samples: A solution containing 1 volume of 0.1 M hydrochloric acid and 1 volume of 0.2 M magnesium chloride was prepared. Forty mL of this solution were added to a sample of 2 mL serum in a centrifuge tube and gently mixed for 1 min. After addition of 10 mL of chloroform and intensively mixing for 10 min, the mixture was centrifuged at 3000 *g* for 30 min. The clear organic phase (chloroform) at the bottom of the tube was carefully withdrawn by a Pasteur pipette and transferred to a pear-shaped flask. The extraction was repeated with another 10 mL of chloroform. The combined extracts were evaporated to dryness under a stream of nitrogen at 60 °C. The residue was re-suspended in 1 mL of methanol and diluted with 50 mL TRIS/HCl pH 7.5 (12.11 g of TRIS in 500 mL distilled water was acidified by HCl to pH 7.5). The total volume was loaded onto the IAC. The IAC was then washed with 20 mL of distilled water. The bound ochratoxin A was slowly eluted from the column with 5 mL of acidified methanol (methanol/acetic acid - 98/2) allowing this to pass through the column by gravity and collected in a sample vial. The air was passed through the column to collect the last drops of eluate. Then the eluate was evaporated to dryness at 50°C under a stream of nitrogen and the residue was re-suspended in 1 mL of mobile phase and filtered on Millex filters. An aliquot (50 µL) of this solution was injected onto an HPLC system.

Urine samples: A 25 mL test portion of urine was acidified to pH 2.5 with HCl in a centrifuge tube. After addition of 25 mL of chloroform and gently mixing for 10 min, the mixture was centrifuged at 3000 *g* for 30 min. The clear organic phase (chloroform) at the bottom of the tube was carefully withdrawn by Pasteur pipette and transferred to a pear-shaped flask. The extract was evaporated to dryness under a stream of nitrogen at 60°C. The residue was re-suspended in 1 mL of methanol and diluted with 50 mL TRIS/HCl pH 7.5 (12.11 g of TRIS in 500 mL distilled water was acidified with HCl to pH 7.5). The total volume was loaded onto the IAC. The next steps of immunoaffinity clean-up were as described above for the blood samples. The dried eluate was then taken in 200 µL of solvent and the volume injected onto the HPLC system was 20 µL.

High performance liquid chromatography

The HPLC analysis of all blood samples was carried out using a Thermo Separation Products HPLC liquid chromatographic system (Spectra Series P200) coupled to an automatic injector (Spectra system AS 3000) on a 250 mm × 4.6 mm, 5 µm Kromasil C18 column, Fluorescence (Perkin Elmer LS40) was monitored at an excitation of 335 nm and an emission of 465 nm. The system was run isocratically with a mobile phase containing: methanol/acetonitrile/0.005 M sodium acetate solution /acetic acid (300/300/400/14) at a flow rate of 1 mL/min. Ochratoxin A standard solutions were used every day for calibration and prepared daily by dilution of the stock standard ochratoxin A solution with 10% acetonitrile. Typical injection volumes were 50 µL.

The HPLC analysis of all urine samples was carried out using a Gilson 811B dynamic chromatography pump, a Spectra Physic 2000 fluorescence spectrophotometer and ICS auto sampler. A spherisorb column (5 µm C18, 0.46 × 25 cm) from ICS was used. The mobile phase and other analytical conditions were as above.

Statistical analysis

The data were analyzed for the comparison of the medians from both villages by the method of Mc Gill et al. (21). In addition, the weekly averages for all individuals in both villages were analyzed by the ANOVA test.

Results

Characterization of the methods of OTA analysis

The calibration curve was established in the range 0.01 ng to 6 ng/mL (r = 0.9818). Limit of quantification was equivalent to 0.1 ng/mL and 4 ng/mL for blood and urine respectively. In the sample of blood enriched with OTA at the levels of 1 ng/mL, the recoveries were about 85%. In the samples of urine (25 mL) enriched with 10 ng OTA, the recovery were about 70%. Examples of chromatogram are given figures 1 to 3 for blood and urine. Elution time of OTA was between 7 and 8 min depending on the system used. The results were corrected for recovery.

OTA contamination in blood and urine

The results from the analysis of OTA in the serum and urine samples from GP and BI are respectively presented in Tables 1 and 2. In addition, we have calculated from the volume of excreted urine, the amount of OTA excreted during the day.

All samples of blood are contaminated by OTA above the limit of detection. The average concentration of OTA in blood are: 2.01 ± 0.54 (BI); 0.67 ± 0.24 (GP) and 1.59 ± 0.44 (both villages). The highest concentrations of OTA in blood are found in BI village. For participants BI 07 and BI 08, high levels of OTA (>5 µg/L) in serum are continuously detected over the month. For two others (GP 04 and BI 03) medium levels (1-2.5 µg/L) of OTA are detected with little variation over the month. For the other participants lower levels, generally below 1 µg/L, are detected with again little variability during the month. In both villages, the medians with confidence intervals at 95% are 0.4 ± 0.22 and 0.7 ± 0.15 respectively for GP and BI. These are not statistically different. In GP village, all people except GP 04, have an average blood OTA level of about 0.5 µg/L. GP 04 is statistically different with an average weekly level of 1.46 µg/l. For BI village, the average weekly levels are more widely distributed ranging from 0.26 to 8.36 µg/l. Three individuals (BI 03, BI 07, BI 08) are statistically different from the others.

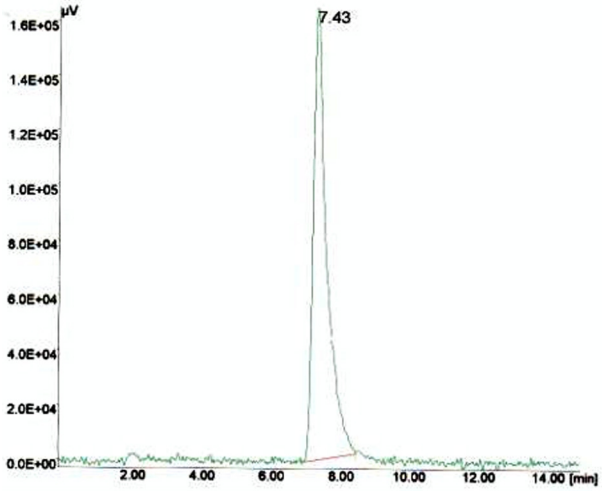

Fig. 1. Chromatogram of a blood sample contaminated by OTA

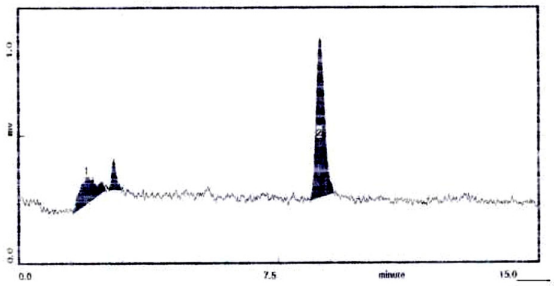

Fig. 2. Chromatogram of a urine sample contaminated by OTA

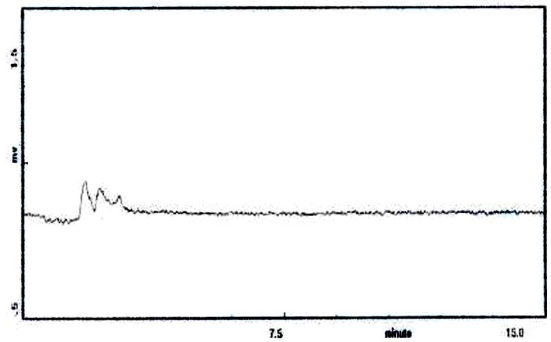

Fig. 3. Chromatogram of a urine sample not contaminated by OTA

Table 1. Summary of analytical results for ochratoxin A in serum and urine of subjects from Gorno Peshtene (GP), Vratza District, Bulgaria

Subject N° - week	OTA level in serum (µg/L)	OTA level in urine (ng/L)	OTA excreted in urine (ng/day)
GP 01 0	0.8	30	25.47
GP 01 1	0.3	10	5.90
GP 01 2	0.4	10	4.28
GP 01 3	0.4	N.D	N.D
GP 01 4	0.4	60	23.51
GP 01 Average ± SD	0.46 ± 0.19	22 ± 23.87	11.83 ± 11.77
GP 02 0	0.4	20	8.49
GP 02 1	0.4	20	13.73
GP 02 2	0.4	20	11.89
GP 02 3	0.3	20	12.88
GP 02 4	0.7	N.D	N.D
GP 02 Average ± SD	0.44 ± 0.15	16 ± 8.94	9.42 ± 5.63
GP 03 0	0.3	50	28.31
GP 03 1	0.4	90	26.94
GP 03 2	0.2	60	23.09
GP 03 3	0.7	40	24.86
GP 03 4	1.1	30	11.73
GP 03 Average ± SD	0.54 ± 0.36	54 ± 23.02	22.99 ± 6.6
GP 04 0	1.6	90	53.15
GP 04 1	1.3	40	30.97
GP 04 2	1.6	60	51.14
GP 04 3	1.2	20	12.45
GP 04 4	1.6	110	47.28
GP 04 Average ± SD	1.46 ± 0.19	64 ± 36.47	39 ± 17.21
GP 09 0	0.5	50	44.53
GP 09 1	0.2	40	41.97
GP 09 2	0.2	40	35.32
GP 09 3	0.4	30	30.72
GP 09 4	0.9	330	211.68
GP 09 Average ± SD	0.44 ± 0.29	98 ± 129.88	72.64 ± 77.89
Total average	0.67 ± 0.24	50.8 ± 44.44	31.17 ± 23.82
Median	0.4 ± 0.22	40 ± 12.6	24.86 ± 7.36

N.D.: Below the limit of detection ; - : Sample not available

As compared to OTA blood level, the urinary OTA excretion shows more fluctuation. Except for the participants BI 07 and BI 08, who have by far the highest urinary excretion clearly related to the high levels of OTA in blood, the levels of OTA excreted are not correlated to the OTA blood level. The medians with confidence intervals at 95% of OTA urine level are 40 ± 12.6 and 70 ± 19 ng/L, respectively for GP and BI village. These are not statistically different. The amount of OTA excreted per day is more relevant than the urinary concentration. The average excretion levels of OTA in GP village varies from 9.4 to 72.84 ng/day, and in BI village from 19.9 to 1272.43 ng/day or 70.8 when excluding BI 07 and BI 08. When the latter are excluded there is no statistical difference between both villages.

Table 2. Summary of analytical results for ochratoxin A in serum and urine of subjects from Beli Izvor (BI), Vratza District, Bulgaria

Subject N° - week	OTA level in serum (µg/L)	OTA level in urine (ng/L)	OTA excreted in urine (ng/day)
BI 01 0	0.5	80	104.73
BI 01 1	0.5	-	-
BI 01 2	1	140	187.40
BI 01 3	0.8	70	121.40
BI 01 4	0.9	80	115.34
BI 01 average	0.74 ± 0.23	92.5 ± 31.02	132.22 ± 37.43
BI 02 0	0.1	10	15.59
BI 02 1	0.3	50	52.35
BI 02 2	0.5	30	48.56
BI 02 3	0.2	100	93.48
BI 02 4	0.2	80	70.23
BI 02 average	0.26 ± 0.15	54 ± 36.47	56.04 ± 28.76
BI 03 0	1.4	170	67.58
BI 03 1	1.2	80	95.66
BI 03 2	1.2	70	77.22
BI 03 3	2.2	70	54.19
BI 03 4	2.5	70	36.15
BI 03 average	1.7 ± 0.61	92 ± 43.82	66.16 ± 22.57
BI 04 0	0.4	-	-
BI 04 1	0.7	60	52.53
BI 04 2	0.5	30	26.87
BI 04 3	0.5	N.D	N.D
BI 04 4	0.5	60	39.97
BI 04 average	0.52 ± 0.11	37.5 ± 28.72	29.84 ± 22.48
BI 05 0	0.9	-	-
BI 05 1	0.3	40	30.42
BI 05 2	0.3	40	32.53
BI 05 3	0.4	60	39.84
BI 05 4	0.9	10	6.75
BI 05 average	0.56 ± 0.31	37.5 ± 20.61	27.38 ± 14.34
BI 06 0	0.6	100	69.80
BI 06 1	0.6	40	32.34
BI 06 2	0.9	10	17.35
BI 06 3	1	140	98.29
BI 06 4	0.5	80	57.52
BI 06 average	0.72 ± 0.22	74 ± 50.79	55.06 ± 31.74
BI 07 0	9.3	520	833.96
BI 07 1	6.3	220	378.17
BI 07 2	7.9	610	920.38
BI 07 3	7.9	1910	2672.15
BI 07 4	10.4	1040	1557.47
BI 07 average	8.36 ± 1.56	860 ± 656.24	1272.43 ± 888.31
BI 08 0	5.7	570	805.26
BI 08 1	9	100	163.32
BI 08 2	10.9	-	-
BI 08 3	6.1	560	731.92
BI 08 4	6.8	590	773.5
BI 08 average	7.7 ± 2.2	455 ± 236.99	1266.43 ± 888.31
BI 09 0	0.5	30	26.53
BI 09 1	0.5	20	23.09
BI 09 2	0.5	10	6.37
BI 09 3	0.7	90	83.80
BI 09 4	0.5	30	26.67
BI 09 average	0.54 ± 0.09	36 ± 31.31	32.29 ± 29.45
BI 11 0	0.7	-	-
BI 11 1	1	150	131.57
BI 11 2	0.8	30	40.30
BI 11 3	0.6	130	166.88
BI 11 4	0.4	20	15.15
BI 11 average	0.7 ± 0.22	82.5 ± 67.02	88.47 ± 72.35
BI 12 0	0.2	30	23.44
BI 12 1	0.7	N.D	N.D
BI 12 2	0.4	40	20.71
BI 12 3	0.3	60	23.62
BI 12 4	0.2	40	31.70
BI 12 average	0.36 ± 0.21	34 ± 21.91	88.47 ± 72.35
Total average	2.01 ± 0.54	168.64 ± 111.45	277.04 ± 202.87
Median	0.7 ± 0.15	70 ± 19	52.53 ± 17.51

Discussion

These results confirm those from the previous studies performed in Bulgaria some 10 years ago (18, 19, 20).

The blood concentration of OTA is in the same range as those reported previously in this region with highest levels around 10 µg/L. Since we have used a more sensitive method (Limit of detection of 0.1 ng/mL) we found 100% of positive samples while in the previous studies we reported only 23.3-29% of samples above 1 µg/L. In this study 4/16 (25%) of participants continuously present concentrations of OTA greater than 1 µg/L. The level of OTA in blood found in the other participants ranged between 0.11 and 1 µg/L. In the previous studies in the same area, 10 to 12% of the population have OTA levels in blood above 2 µg/L (18, 19), while in this study we found 12.5% above 2 µg/L. These results also corroborate the results from Yugoslavia and Croatia (16, 17) in which up to 100% of samples were above the limit of detection and the highest concentrations of OTA were found in the town of Osijek (Slavonski-Brod) which present the highest incidence of BEN. In all the previous studies, spot tests were performed on samples collected at random. This study adds that over a period of a month, the blood contamination of the participants does not vary widely. Thus these people having continuously circulating OTA are more exposed to the noxious effects of this toxin.

When comparing our results to those from healthy persons in other European countries using similar analytical methods, Table 3 demonstrates than in the BEN area the population is more frequently exposed to OTA and to higher amounts. The highest average blood concentrations in Denmark was 1.8 µg/L but only half of the population was affected. When most of the population was affected (Czech Republic, Germany, Italy, Switzerland, Sweden, U.K.), the average concentrations are significantly lower (0.17–1.09 µg/L) and in some case the limit of detection was much lower.

For the urine samples, 98% of the samples contain OTA in the range 10 – 1910 ng/L confirming also our previous results from Bulgaria (20) in which OTA was detected in about 33% of the samples in the range 5 – 604 ng/L. The apparent discrepancy in the incidence of positive samples may be due to the improved recovery by using IAC purification.

To our knowledge, there is only one study in Europe referring to OTA levels in urine. MacDonald et al. (34) found 10–58 ng/L in healthy individuals from U.K. except in 4 samples. In the present study one third of these individual results are above 60 ng/L.

Since in our study we collected the 24h-urines, we could calculate the amount of OTA excreted on that day. This modulates the absolute OTA concentration level in urine. When comparing the OTA level in serum and those excreted in the urine no proportionality is observed. For example GP 04 and BI 03 having an average blood OTA level of 1.4 and 1.7 µg/L respectively, did not excrete more OTA in urine than all other GPs' or BIs' participants (except BI 07 and BI 08).

Conclusions

This study strengthens our previous results obtained in Bulgaria and demonstrates that the population in the Balkans is more exposed to OTA than the general population in Europe. More studies of this type related to the OTA intake should allow to determine the best marker of exposure to OTA. Indeed if it can be confirmed that measurement of OTA in urine is a good marker of exposure as hypothesized by Gilbert et al. (36), it will be less invasive than blood for follow up of population exposure. In addition, the data of this study demonstrate that twenty years after no improvement has occurred in the food sanitary status of this population, which will soon become a member of the European Union.

Finally when related to the analysis of DNA adducts in urinary tract tissues from exposed patients, the role of OTA in the induction of BEN and / or other nephropathies of unknown aetiology and urinary tract tumors in Europe could be investigated more easily.

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Table 3. Data from other European countries from healthy persons, using conventional HPLC techniques

Country	% positive samples	Range (Mean) µg/L	Reference
Denmark	54.2	0.1 – 13.2 (1.8)	Hald (1991), 22
France (Alsace)	19.4	0.1 – 11.8	Creppy et al. (1993), 23
France (Rhône Alpes)	14.6	0.1 – 4.3	Creppy et al. (1993), 23
Germany (1977-1985)	56.5	0.1 – 14.4 (0.6)	Bauer & Gareis (1987), 24
Germany (1988)	68	0.1 – 8.4 (0.75)	Hadlok (1993), 25
Germany (1999)	98.1	0.06 – 2.03(0.27)	Rosner et al. (2000), 26
Italy (1992)	100	0.1 – 2.0 (0.53)	Breitholtz-Emanuelsson et al. (1994), 27
Italy (1994)	97	0.12 – 57.2 (0.56)	Palli et al. (1999), 28
Poland (1983-1985)	7.2	1 – 40 (0.28)	Golinski (1987), 29
Spain	53	0.5 – 4.0 (0.71)	Jimenez et al. (1998), 30
Switzerland	100	0.06 – 6.02	Zimmerli & Dick (1995), 31
Sweden (1989)	12.8	0.3 – 7.0 (0.20)	Breitholtz et al. (1991), 32
Sweden (1990-1991)	100	0.09 – 0.94(0.17)	Breitholtz-Emanuelsson et al. (1993), 33
U. K.	100	0.4 – 3.11(1.09)	MacDonald et al. (2000), 34
Czech Republic	93.1	0.1 – 13.7(0.24)	Malir et al. (1998), 35

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ANALIZA OCHRATOXIN-a U SERUMU I URINU STANOVNIŠTVA IZ OBLASTI SA BALKANSKOM ENDEMSKOM NEFROPATIJOM: JEDNOMESEČNA STUDIJA

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Kratak sadržaj: *U toku 1950-ih serija publikacija iz Bugarske, Jugoslavije i Rumunije opisala je bolest bubrega koja se javlja u geografski definisanim regijama ove tri balkanske zemlje. Nekoliko studija upućuje na etiološku povezanost sa mycotoxin-om i delimično sa ochratoxin-om A. Izloženost populacije OTA u Bugarskoj je praćena visokom prevalencijom nivoa OTA koji prevazilazi 2 ng/ml u krvi pogođene populacije. OTA je takođe nađen češće u urinu ljudi koji žive u BEN-endemskim u poređenju sa neendemskim naseljima, a najviše vrednosti se beleže kod obolelih od BEN ili tumora urotrakta. U ovoj studiji mi pratimo nivo OTA u krvi i urinu populacije sa visokom incidencom BEN u Bugarskoj bez znakova bolesti bubrega. Uzorci su sakupljani u toku jednog meseca početkom i krajem nedelje. Ispitivanje je potvrdilo naše prethodne rezultate koji pokazuju da je populacija na Balkanu izloženija delovanju OTA no što je to slučaj sa populacijom u Evropi.*

Ključne reči: *Balkanska endemska nefropatija, ochratoxin A, analize urina i seruma*