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Ochratoxin A in Corn and Wheat: Geographical Association with Endemic Nephropathy

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Aim. To determine the presence and concentration of ochratoxin A in wheat and corn from Slavonski Brod surroundings, the area of endemic nephropathy allegedly caused by ochratoxin.

Methods. Thin-layer chromatography was used to determine ochratoxin A concentrations in 92 wheat and 51 corn samples from the surroundings of Slavonski Brod, Osijek, Hrvatsko Zagorje, Istria, and Celje (Slovenia).

Results. Ochratoxin A was present in 74 of 92 (75.8%) wheat samples and 17 of 51 (33.3%) corn samples, in a concentration range of 0.02-160.00 g/kg in wheat and 0.02-40.00 g/kg in corn. Wheat samples from the Slavonski Brod surroundings contained the highest level of ochratoxin A (38.8 27.2 g/kg), followed by Osijek (8.7 8.3 g/kg). Ochratoxin A levels in the wheat from Hrvatsko Zagorje, Istria, and Celje were considerably lower (2.1 1.5, 1.3 2.6 and 0.2 0.5 g/kg, respectively). Wheat samples from Slavonski Brod significantly differed from all other sample groups (p 0.001), and wheat samples from Osijek differed from those from Hrvatsko Zagorje, Istria, and Celje (p 0.001, p=0.003, p 0.001, respectively). Ochratoxin A level was the highest in the corn samples from the Slavonski Brod surroundings (20.0 14.8 g/kg) and considerably lower in samples from Osijek, Celje, Hrvatsko Zagorje, and Istria (0.8±1.4, 0.7 1.9, 0.4 0.4, and 0.4 0.8 g/kg, respectively). A statistically significant difference was also observed between the Slavonski Brod samples and all other corn samples (p 0.001).

Conclusion. Irrespective of the real association between ochratoxin A and endemic nephropathy, our data clearly demonstrate their geographical overlap.

Key words: *Balkan nephropathy; cereals; Croatia; diet; legislation, food; mycotoxins; ochratoxins; Slovenia*

During vegetation and storage, cereals and cereal products are exposed to the growth and development of a great number of molds that synthesize biologically active substances, mycotoxins (1-3). In addition to some less important groups, there are two large groups of mycotoxins that have come into the focus of scientific interest for their demonstrated or suspected direct and/or indirect harmful effects on human health: aflatoxins (G_1, B_1, and M_1) and ochratoxins (A, B, and C) (1-3). Due to the demonstrated hepatotoxicity of aflatoxins and evidence for their causative relationship with primary hepatic carcinoma (1-3), the allowed amounts of aflatoxins in foodstuffs have been restricted by respective legal provisions in many countries, including Croatia (4). The effects of ochratoxins on human health have not yet been fully investigated and confirmed, in spite of their unquestionable animal toxicity *in vivo* and *in vitro,* nor has an international consensus on the maximal allowed amounts of ochratoxins in foodstuffs been achieved to date (5-11).

Ochratoxins A, B, and C are toxic products of seven molds of the genus *Penicillium* and six molds of the genus *Aspergillus,* parasitizing on cereals. Ochratoxins may persist in foodstuffs even after mold destruction. The amount of the toxin is reduced by thermal food processing (baking) by not more than 20%, whereas boiling has no such effect at all (1-3,12,13). The predisposing factors for their occurrence include mechanical or parasitic damage to the grain, mineral deficiency during grain development, and inappropriate temperature and humidity during the plant growth and ripening (1-3,12-15). Ochratoxin A, a typical nephrotoxin, is exclusively responsible for animal and human toxicity (5-11), its oral LD_{50} for animals being estimated to range between 0.2 for dogs, 1.0 mg/kg for pigs, and 4.7 mg/kg for tronts (16). Ochratoxin A is the etiologic factor for porcine mycotoxic nephropathy (5-10), a disease having much in common with a human disease known as Balkan endemic nephropathy. This is a chronic tubulointerstitial disease of unknown etiology, detected some 50 years ago in Romania, Bulgaria, and countries of the former Yugoslavia (Croatia, Bosnia and Herzegovina, and FR Yugoslavia). The disease occurs in the basins of Danube joining rivers, ie, along the Sava river in Croatia (17-20, Fig. 1). The long-standing, painstaking search for the etiologic cause of the disease has now boiled down to

Figure 1. Map of Balkan peninsula with zone of endemic nephropathy (17-19). Triangles indicate sampling sites in this study and dark grey spots the areas with Balkan endemic nephropathy.

four hypotheses: viral infection, genetic predisposition, water contamination with lignite toxins, and ingesion of ochratoxin with food. The viral hypothesis is supported by successful isolation of coronavirus from kidney biopsies of Balkan endemic nephropathy (21). The detection of "Balkan endemic nephropathy markers" at certain gene locations in affected subjects and their "healthy" relatives, with recently found initial changes characteristic of Balkan endemic nephropathy, speaks in favor of genetic predisposition (22). The existence of lignite deposits in the endemic areas has given rise to a hypothesis on the disease development due to continuous exposure to polycyclic aromatic carbohydrates and other toxic substances from lignite that reach drinking water supplies (17,18,22,23). However, the hypothesis on the ingestion of small doses of ochratoxin A with food over a period of time has become increasingly attractive (24-27).

The aim of the study was to determine whether cereals from the Slavonski Brod surroundings, an area of endemic nephropathy, contained ochratoxin A and if so, in what amounts, compared with other areas with rare or no occurrence of the disease. The study was also done as a support effort towards legal control of ochratoxin A maximal allowed amounts in human food.

Material and Methods

The sampling of cereals was carried out during 1999 and the beginning of 2000 in the surroundings of Slavonski Brod, Osijek, Hrvatsko Zagorje, Istria, and Celje (Republic of Slovenia) surroundings. The sites of sampling were randomly chosen from the list of individual producers, grain elevators, desiccators, and mills. A kilogram sample was obtained from each sampling site. The samples were scaled, encoded, and transported to the Department of Food Control, Zagreb Public Health Institute. The total number of samples from Hrvatsko Zagorje and Istria was lower because cereals are not extensively grown in these areas. Thus, the number of potential sampling sites was low and hardly adequate for the study purpose. A total of 92 wheat samples and 51 corn samples were obtained (Tables 1 and 2).

Specific ochratoxin A extraction from samples was made by means of organic solvents. After that, extract purification was done on SiOH column, and continued by column conditioning with Na_2SO_4, hexane, and dichloromethane. The samples were passaged through the column, the column was washed with dichloromethane, hexane, and toluene, and the preparation phase was finished with sample elution and evaporation. Identification and quantification of the samples were performed with thin-layer chromatography (TLC; Merck, Darmstadt, Germany), whereas high-pressure liquid chromatography (HPLC; Hewlett Packard, Waldbron, Germany) was used as a confirmation method (28-31).

The limit of detection was 0.02 g/kg.

Statistical analysis was performed by use of non-parametric Mann-Whitney U-test (32). Statistical differences between samples were estimated at alpha level p 0.01.

Results

By far the highest proportion of samples containing ochratoxin A levels above the limit of detection (ie, all 33 wheat samples and 9 of 11 or 81.8% of corn samples) and the highest mean values of ochratoxin A (38.8 27.2 and 20.0 14.8 g/kg for wheat and corn, respectively) were recorded in the group of samples from Slavonski Brod surroundings (Tables 1 and 2). This was confirmed by statistically significant differences in the amount of ochratoxin A in wheat and corn samples between these and all other groups of samples ($p<0.001$). A statistically

Table 1. Ochratoxin A (g/kg; mean±SD, range) in wheat samples from different geographic locations

Sample origin	No. of samples	Ochratoxin A	Statistical analysis[a]				
			Slavonski Brod	Osijek	Hrvatsko Zagorje	Istria	Celje (Slovenia)
Slavonski Brod	33	38.78±27.15 (2.00-160.00)	median 40.00	U=131.50 p<0.001	U=6.00 p<0.001	U=1.00 p<0.001	U=0.00 p<0.001
Osijek	27	8.71±8.28 (0.019-32.00)		median 8.00	U=90.50 p<0.001	U=21.00 p=0.003	U=16.0, p<0.001
Hrvatsko Zagorje	17	2.07±1.47 (0.019-4.80)			median 1.60	U=30.00 p=0.155	U=19.00 p=0.001
Istria	6	1.33±2.56 (0.019-6.40)				median 0.019	U=20.50 p=0.45
Celje (Slovenia)	9	0.18±0.53 (0.019-1.60)					median 0.019

[a]Mann-Whitney U-test.

significant difference (p<0.001, p=0.003, p<0.001) was also found between wheat samples from Osijek surroundings (24 of 27 or 88.9% of positive samples, with a mean ochratoxin A level of 8.7 8.3 g/kg) and the remaining three groups of samples. The samples from Celje surroundings showed by far the lowest proportion of positive findings (1 of 9 or 11.1% for wheat, and 2 of 16 or 12.5% for corn).

Discussion

Results of our study support the hypothesis on the possible relationship between ochratoxin A and Balkan endemic nephropathy because Slavonski Brod area is endemic for this disease and our wheat and corn samples from that area had extremly high concentrations of ochratoxin A (Tables 1 and 2) and also significantly higher than those from any other tested area. It is interesting that this was true for the samples of cereals from Slavonski Brod area that were collected in villages (Gornja Bebrina, Rastušje, Podcrkavlje, Glogovnica, Donji Slatnik) in which Balkan endemic nephropathy has not been reported. Moreover, a significantly higher level of ochratoxin A was also found in wheat samples from the Osijek area, which is not endemic for Balkan endemic nephropathy, but where sporadic cases of the disease have been recorded. Thus, although not associated with specific local foci of the disease, increased contamination of wheat and corn with ochratoxin A was clearly associated with affected areas in this study.

Higher mean levels of ochratoxin A were found in wheat than in corn samples, which is consistent with literature data (33) that the highest ochratoxin A levels are found in wheat, as compared with other cereals, irrespective of their ripening season (33). These findings obviously call for additional investigations.

The incontestable relationship between the ochratoxin A and porcine mycotoxic nephropathy, described some 20 years ago, and strong similarities between this disease and Balkan endemic nephropathy in humans have stimulated studies on the etiologic relationship between ochratoxin A and Balkan endemic nephropathy (5-11). The proposed association has been supported by a number of epidemiological features of Balkan endemic nephropathy. The disease never occurs in children,

Table 2. Ochratoxin A (g/kg, mean±SD, range) in corn samples from different geographic locations

Sample origin	No. of Samples	Ochratoxin A	Statistical analysis[a]				
			Slavonski Brod	Osijek	Hrvatsko Zagorje	Istria	Celje (Slovenia)
Slavonski Brod	11	20.00±14.83 (0.019-40.00)	median 20.00				
Osijek	15	0.81±1.35 (0.019-4.00)	U=19.00 p<0.001	median 0.019			
Hrvatsko Zagorje	5	0.42±0.41 (0.019-2.00)	U=6.00 p=0.01	U=34.00 p=0.80	median 0.019		
Istria	4	0.40±0.79 (0.019-1.60)	U=5.00 p=0.026	U=27.50 p=0.81	U=10.00 p=1.09	median 0.019	
Celje (Slovenia)	16	0.70±1.93 (0.019-6.40)	U=18.00 p<0.001	U=107.00 p=0.63	U=38.00 p=0.90	U=29.00 p=0.82	median 0.019

[a]Mann-Whitney U-test.

which points to a prolonged period of incubation and/or etiologic agent exposure. This suggestion is further corroborated by the fact that settlers are equally affected as the native population, provided they have lived in the endemic area for more than 10 years. Exclusively rural population is involved; there is a pattern of familial clustering that cannot be fully explained by the genetic rules of inheritance, as well as a strong susceptibility to the development of otherwise very rare urinary tract tumors (17-20,34-36). Over the last two decades, the incidence of the disease has been on a decrease, while its clinical course appears to become longer and less severe. For example, the incidence reached the highest level during the 1970-79 period (mean rank 2.75/10,000), whereas in the 1995-97 period, it declined to 0.96/10,000 (17-19). This has been ascribed to the improvements gradually implemented in the storage and preservation of foods (refrigerators, cooling chambers) as well as in the pattern of nutrition (ready-made flour products) (18,34). Differences in the level of ochratoxin A between foodstuffs from the endemic area and those from non-endemic areas have been reported in a number of studies (33,37-41). Also, higher serum levels of ochratoxin A were generally, but not uniformly, found in both Balkan endemic nephropathy affected and non-affected subjects from the endemic area, compared with other population groups (42-44). The hypothesis seems to be definitely confirmed by the discovery of a Balkan endemic nephropathy-like disease in Tunisia, where increased levels of ochratoxin A were detected in the foodstuffs taken by affected individuals, as well as in their serum samples (45,46).

However, the ochratoxin theory suffers from a number of shortcomings. Studies have shown that ochratoxin A can be found in the serum of healthy individuals not only in the countries with Balkan endemic nephropathy-involved areas (47) but practically all over Europe (13,17,48,49). Food contamination with ochratoxin A has been recorded in many developed countries, as well as in some African countries, where the disease has never been described (8,12,13,17,37,48-51). The increased serum levels of ochratoxin A in affected subjects might simply result from its accumulation in the body due to renal function impairment by some other etiologic agent (17,48).

Irrespective of how likely the association of Balkan endemic nephropathy and ochratoxin A may or may not seem, it is beyond belief that there are no legal provisions, national or international, to regulate the maximal allowed amount of ochratoxin A in human foods. In Croatia, there are only regulations on the maximal allowed concentration of ochratoxin A in stock feed (52). There are recommendations on the tolerable daily intake of ochratoxin A, ranging from 1.5 to 5.7 and even up to 16 ng/kg body weight (13,47,48,53). However, these restrictions are not mandatory for manufacturers. We belive that the presence of ochratoxins in foodstuffs should be strictly regulated by amendments to the Act on Food Safety and respective by-laws, especially in the light of recent concepts on their highly probable carcinogenicity (12,27,48,53,54), teratogenicity (11,48,54), genotoxicity (35,53,54) and immunotoxicity (48), in addition to their already proven nephrotoxicity. It should be mentioned that, besides cereals and cereal products, ochratoxin A can also be found in other foods, such as beans (25). Moreover, residual ochratoxin A was detected in pork and pork products contaminated with animal feed (7,8,10) and, as a rarity, in chicken eggs (25). The growing interest in the issue is also indicated by the fact that coordination of the standards for ochratoxin A in foodstuffs, based on the principle of reasonable minimum, is under way in the European Union countries (3).

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