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Determination of seasonal variations in serum ochratoxin A levels in healthy population living in some regions of Turkey by enzyme-linked immunosorbent assay

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ABSTRACT

This study has been undertaken to investigate the regional and seasonal variability in ochratoxin A (OTA) exposure of healthy population living in Black Sea and Mediterranean regions of Turkey by measuring serum OTA concentrations. The mean serum concentrations of OTA were determined to be 0.137 ng/mL (0.0306–0.887 ng/mL) and 0.312 ng/mL (0.028–1.496 ng/mL) in all samples for winter and summer, respectively by enzyme-linked immunosorbent assay (ELISA). The differences between mean values of OTA in all serum samples collected in summer and winter were statistically significant. The highest OTA concentration was determined in the children living in Black Sea Region in summer. The mean daily intake levels of OTA in all samples were estimated as 0.182 ng/kg b.w./day and 0.408 ng/kg b.w./day in winter and summer, respectively. The results showed that the mean serum concentrations of OTA in healthy population in both regions were found not to be exceeded 1 ng/mL in agreement with the distribution reported in most European countries and that the daily intake levels of OTA were calculated below the tolerable daily intake levels given by regulatory authorities. However, overall results suggest that Turkish population living in these regions is continuously exposed to OTA and that the exposure levels are also elevated in summer period compared to winter.

1. Introduction

Ochratoxins are a group of secondary metabolites produced by a number of *Aspergillus* and *Penicillium* species. The most important of these toxins is Ochratoxin A (OTA). OTA is considered to be the main causal agent of Balkan Endemic Nephropathy (BEN), a disease characterized by progressive renal fibrosis in humans mainly occurring in some areas of South-Eastern Europe. Furthermore, in West African countries, the number of end stage chronic interstitial nephropathy is reported to increase continuously these last decades and could be related to exposure to strong nephrotoxin like OTA (Creppy, 2002; Fogazzi et al., 2003; Fink-Gremmels, 1999; Walker, 2002; Bayman and Baker, 2006.). In addition, OTA disturbs blood coagulation and carbohydrate metabolism and it has been reported to be carcinogenic, teratogenic and immunosuppresive in several animal species (Fink-Gremmels, 1999; Pfohl-Leszkowicz and Manderville, 2007; Beardall and Miller, 1994; Kuiper-Goodman and Scott, 1989; Álvarez et al., 2004; Al-Anati and Petzinger, 2006). OTA is classified as a possible carcinogen to human (Group 2B) by the International Agency for Research on Cancer (IARC, 1993). High concentrations of OTA have been described to provoke inhibition of protein synthesis, alteration of mitochondrial respiration and lipid peroxidation. Recent studies show that low concentrations of OTA induce apoptosis in renal cells and human lymphocytes without affecting protein synthesis and radical production. In addition, OTA disturbs

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phenylalanine metabolism, mostly by inhibiting the enzyme involved in the synthesis of the phenylalanine tRNA complex (McMasters and Vedani, 1999; Bunge et al., 1979; Marquardt and Frohlich, 1992)

OTA is a naturally occurring contaminant in food and animal feed in suitable conditions. Environmental factors, such as temperature, humidity, time of infection, aeration, and conditions of storage affect OTA production. OTA is found mainly in food derived from plants such as cereals, coffee, nuts, dried fruits, spices, wine and grapes. The intake of OTA through contaminated feed may lead to its occurrence in the blood, kidneys and liver of pigs and poultry (Speijers and Egmond, 1993; Krogh, 1987). When ingested as a food contaminant, OTA is found in human blood due to the long elimination half- life, as consequence of its' binding to plasma proteins, its' enterohepatic circulation and its' reabsorption from urine (Roth et al., 1988; Studer-Rohr et al., 2000). There are numerous studies from many countries detecting its presence in human population and OTA has frequently been found in human blood, urine and milk samples. These results indicate a continuous and widespread human exposure to OTA in many European countries, Canada, Japan and elsewhere in the world (Peraica et al., 2001; Palli et al., 1999; Thuvander et al., 2001; Gilbert et al., 2001; Creppy et al., 1993; Scott et al., 1998; Ueno et al., 1998; Assaf et al., 2004; Wafa et al., 1998; Sangare-Tigori et al., 2006; Pacin et al., 2008). However, exposure to OTA is not uniform, but varies significantly between individuals and geographic regions. Although there are several studies on the mycotoxin levels of different foods in Turkey, there is only one study to detect OTA levels in the patients with some kidney diseases and urinary tract tumors, as well as healthy people (Ozcelik et al., 2001). The OTA exposure levels by detecting serum or urine concentrations are not assessed in healthy people randomly selected in different areas of Turkey and there is not any evaluation on blood OTA levels according to region, season and/or age.

This study has been undertaken to investigate the regional and seasonal variability in OTA exposure of healthy population living in different regions of Turkey by measuring serum OTA concentrations by enzyme-linked immunosorbent assay (ELISA) and to evaluate the possible correlations between serum OTA levels and age. On the other hand, it was aimed to calculate the daily OTA intake levels from serum OTA concentrations in the healthy subjects and to compare with the mean tolerable daily intake (TDI) levels recommended by WHO.

2. Material and methods

2.1. Subjects and sampling

The study was conducted in the villages and suburbs of two towns (Ordu and Antalya) in Black Sea and Mediterranean Regions of Turkey. The whole study group ($n = 239$) was selected by a simple random technique among the healthy volunteers with no history of renal and hepatic diseases. The subjects were aged 6–80 and three groups according to age were composed in both regions. Dietary information, including the level and frequency of food intake was collected through a standard food- frequency questionnaire. The weights of all subjects were also recorded. Blood samples were collected into tubes without ant anticoagulant agent in July 2007 and January 2008. Centrifugation was performed at $800 \times g$ and serum was separated. All serum samples were aliquoted and stored in a freezer at $-20\ ^{\circ}C$ until analysis.

The study was approved by an Ethical Committee according to the "Declaration of Helsinki". All subjects participated in the study voluntarily and written consent (in Turkish) was obtained before blood samples were drawn.

2.2. Determination of ochratoxin A levels

2 ml of serum sample was diluted with 2.5 ml of 1 N HCl, and 4 ml of dichloromethane and shaked for 5 min. After the centrifugation at $3500 \times g$ for 15 min upper aqueous phase was removed. Dichloromethane layer was filtered by using a filter paper and 2 ml of the clear dichloromethane layer was transferred into another centrifugal screw cap vial and extracted with 0.13 M sodium hydrogen carbonate buffer, pH 8.1 (1:1). The solution was centrifuged again at $3500 \times g$ for 5 min after the shaking vigorously for 5 min. The upper sodium hydrogen layer was collected. The extraction and centrifugation were repeated. Sodium hydrogen phases were combined and the solution were diluted with 0.75 ml of 1 N HCl and 2 ml of dichloromethane and centrifuged again at $3500 \times g$ for 5 min. Dichloromethane layer was evaporated to complete dryness under the nitrogen stream. The residue was dissolved in 1 ml of 0.13 M sodium hydrogen carbonate buffer and 50 μl per well was used in the assay. The OTA measurements were done according to the procedure of RIDASCREEN OTA immunoassay for the quantitative analysis of OTA (Darmstadt, Germany). OTA standards used were ranged between 25 and 2025 ng/L. Recovery studies were performed on blank samples of plasma spiked with level of 300 ng/L of OTA and the average recovery was calculated as $100.75\% \pm 3.9$. The dilution factor was 1 and the detection limit was 25 ng/L.

2.3. Estimation of dietary intake of OTA

The daily intake levels of OTA were estimated from the concentration in serum samples using equation below according to Breitholtz et al. (1991).

$$\text{Daily intake levels (ng/kg b.w./day)} = C_p \times 1.34$$

C_p: plasma concentration of OTA (ng/mL).

3. Results

The total of 239 blood samples were collected from healthy people living in Mediterranean and Black Sea Regions in Turkey in the period of 2007-2008 and analysed for OTA by ELISA technique. OTA levels were detectable in 98% of the blood samples collected in summer period. 81% of winter samples in Black Sea Region were found to be

detectable and the percentage of positive winter samples in Mediterranean Region was 72%.

The mean OTA concentrations detected in all samples along with minimum, maximum and median levels are summarized in Table 1. The concentrations ranged between 0.0306 and 0.887 ng/mL with a mean of 0.137 ng/mL in all samples for winter. For summer samples, minimum and maximum levels were found to be 0.028 and 1.496 ng/mL, respectively and the mean concentration of OTA was detected as 0.312 ng/mL. OTA levels in blood samples collected in January and June were determined as 0.138 ng/mL (0.035–0.707 ng/mL) and 0.313 ng/L (0.028–1.398 ng/mL) in Mediterranean Region, respectively. In Black Sea Region, OTA concentrations were found to be 0.137 ng/mL (0.0306–0.887 ng/mL) and 0.311 ng/mL (0.043–1.496 ng/mL) in winter and summer period, respectively. The differences between all summer and winter samples were statistically significant. However, the mean concentration of OTA did not differ among the regions in the same season.

The mean serum levels of OTA according to the age in Black Sea and Mediterranean regions were given in Figs. 1 and 2, respectively. Serum OTA concentrations in winter were found to be 0.285 ± 0.101 ng/mL in children ($n = 7/7$); 0.109 ± 0.009 ng/mL in adults ($n = 37/48$) and 0.135 ± 0.055 ng/mL in elderly people ($n = 4/4$) and in summer the OTA levels were detected to be 0.877 ± 0.153 ng/mL ($n = 8/8$) in children, 0.233 ± 0.046 ng/mL in adults ($n = 47/48$) and 0.098 ± 0.028 ng/mL in geriatric group ($n = 4/4$) in Black Sea Region. In Mediterranean region, the mean OTA levels in winter period were determined to be 0.094 ± 0.02 ng/mL, 0.151 ± 0.025 ng/mL and 0.130 ± 0.030 ng/mL in children ($n = 8/10$), adult ($n = 29/39$) and elderly ($n = 7/12$) volunteers, respectively and in summer the concentrations of OTA were 0.161 ± 0.027 ng/mL in children ($n = 13/14$), 0.338 ± 0.058 ng/mL ($n = 31/31$) in adults and 0.405 ± 0.122 ng/mL in elderly ($n = 13/14$). The highest OTA concentration was determined in the children living in Black Sea Region in summer (0.877 ± 0.153 ng/mL). In Mediterranean Region, OTA levels in blood samples collected from elderly population (0.405 ± 0.122 ng/mL) in summer were higher than the other groups in the same region. Any significant differences were not found according to the sex (data not shown).

The daily intake of OTA estimated on the basis of the serum concentration of the toxin according to Breitholtz et al. was found to be in range from 0.0144 ng/kg b.w./day to 2.005 ng/kg b.w./day in both regions of Turkey. The mean daily intake levels calculated in all samples were 0.182 ng/kg b.w./day and 0.408 ng/kg b.w./day in winter and summer, respectively (Table 2). Significant differences between daily intake levels estimated for summer and winter samples were found. On the other hand, the mean daily intake levels of OTA in the same season did not differ among the regions.

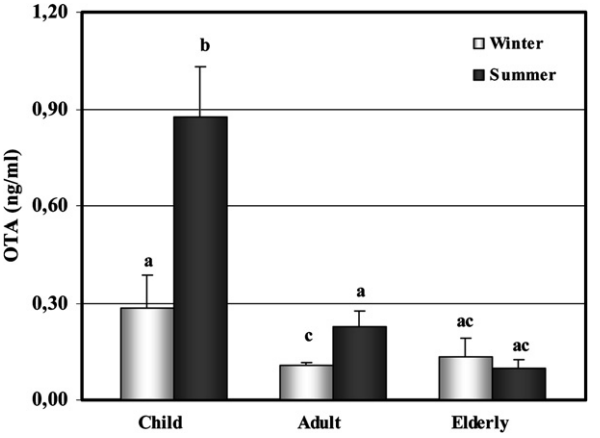

Fig. 1. Seasonal variations of serum OTA levels in different age groups in Black Sea Region. Superscripts of different letters differ significantly ($p < 0.05$) from each other.

The mean serum concentrations of OTA and daily intake levels of all age groups in both regions were also given in Figs. 3 and 4, respectively. No significant differences were observed between the age groups in winter period. The levels of OTA in children were higher than the others in summer, but the difference was not statistically significant. On the other hand, the highest daily intake levels of OTA were observed in overall children in summer.

4. Discussion

The occurrence of OTA in biological fluids in general population has been evaluated in many countries in the world. The results of the studies consistently indicate that humans are continuously exposed to OTA. However, the mean concentration of OTA in healthy population does not exceed 1 ng/mL in most European countries. The extensive investigations on seasonal variability of OTA levels in human blood are limited (Palli et al., 1999; Peraica et al., 1999, 2001).

Palli et al. reported that 85% of the serum samples collected from healthy Italian adults contained 0.2–1.0 ng/

Table 1
OTA levels in healthy population living in Black Sea and Mediterranean Regions.

Region		Number of samples		Serum OTA concentrations (ng/mL)			
		Non-detectable	Detectable	Min	Max	Median	Mean ± SEM
Mediterranean	Winter	17	44	0.0346	0.707	0.095	0.138 ± 0.018^{a}
	Summer	2	57	0.0279	1.398	0.181	0.313 ± 0.044^{b}
Black Sea	Winter	11	48	0.0306	0.887	0.098	0.137 ± 0.019^{a}
	Summer	1	59	0.0431	1.496	0.127	0.311 ± 0.051^{b}
Overall	Winter	28	92	0.0306	0.887	0.099	0.137 ± 0.013^{a}
	Summer	3	116	0.0279	1.496	0.146	0.312 ± 0.034^{b}

[a,b] Values in columns not sharing a common superscript alphabet differ significantly, $p < 0.05$.

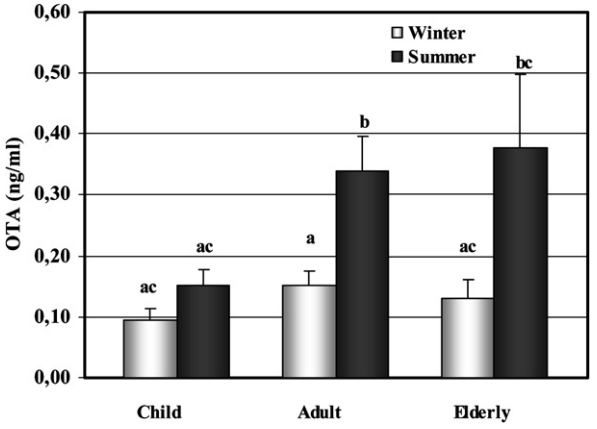

Fig. 2. Seasonal variations of serum OTA levels in different age groups in Mediterranean Region. Superscripts of different letters differ significantly ($p < 0.05$) from each other.

mL OTA (mean: 0.56 ng/mL). They found a strong positive correlation between OTA concentrations and male gender (Palli et al., 1999). They also reported higher OTA levels in summer period in agreement with our results. The authors suggested that the higher values of OTA in serum samples collected during the summer could have been related to particular climate conditions and seasonal variations in dietary and drinking habits of Italian people.

Peraica et al. (2001) measured plasma OTA levels of healthy people living in five Croatian cities in different regions. The highest mean concentration was 0.39 ng/mL in June. The lowest frequency of positive samples and the lowest mean concentration of OTA (0.19 ng/mL) were found in December. On the other hand, regional differences were also determined possibly due to separate nutritional habits of inhabitants.

The mean plasma level of OTA was determined to be 0.2 ng/mL in 406 Scandinavian healthy people and the daily intake of OTA was calculated as 0.26 ng/kg b.w./day from plasma levels by the method used by Breitholtz et al. (Thuvander et al., 2001; Breitholtz et al., 1991). No gender differences were observed and seasonal variations were not evaluated. Scott et al found a higher mean level of OTA (0.88 ng/mL) in 144 Canadian subjects due to the intake of locally produced food with a high consumption of pork meat (Scott et al., 1998). Creppy et al. (1993) reported higher frequency of OTA positive plasma samples in rural than in urban populations in France indicating that locally produced food may significantly influence the exposure of OTA.

In Japan, plasma OTA levels were detected in healthy volunteers ($n = 184$) living around Tokyo. 85% of the samples analysed was found to be positive, but the mean value of OTA was 0.068 ng/mL ranging from 0.004 ng/mL to 0.278 ng/mL. This level was less than that reported in Europe as well as Turkey possibly due to completely different dietary habits (Ueno et al., 1998).

Serum OTA levels in all samples collected from two regions in our study were determined as 0.137 ng/mL and 0.312 ng/mL in winter and summer period, respectively. These results are in agreement with the distribution reported in several European countries. We did not observe any significant regional differences. However, the frequency of samples with OTA concentrations above 1 ng/mL was 12% in Black Sea Region and 5% in Mediterranean Region during the summer period and the highest OTA concentration was 1.496 ng/mL in Black Sea Region. In winter, OTA levels in all samples were below 1 ng/mL. Dietary habits of inhabitants in two regions are not same completely. Although the populations in these regions consume vegetables, fruits, and meat as well as locally produced food, the people living in Black Sea Region consume more cereals including corn and corn products. It was generally accepted that the main contributors to OTA intake are cereals and cereal products. We also found that aflatoxin and OTA levels in corn samples grown and consumed in this region were higher than the other regions (Giray et al., 2009). On the other hand, the climate in Mediterranean region is proper for the occurrence of mycotoxins due to hot and humid conditions especially in summer period. The humidity in Black Sea Region can also cause favourable conditions for the growth of moulds and production of toxins.

There is not any study in order to evaluate the OTA levels only in the healthy population in Turkey. Our study is the first to assess the blood OTA concentrations and daily intake levels in healthy people along with seasonal and regional variations and the correlations with age and gender. Ozcelik et al analysed serum OTA levels in the patients with different kinds of urinary disorders (hemodialysis patients $n = 35$; peritoneal dialysis patients $n = 28$; bladder cancer patients $n = 15$; renal stone patients $n = 15$; totally $n = 93$) in Isparta, Turkey and compared to the healthy control group ($n = 40$) living in the same area. OTA

Table 2
Dietary daily intake levels of ota in healthy population living in Black Sea and Mediterranean Regions.

Region		Estimated daily intake levels (ng/kg bw/day)			
		Min	Max	Median	Mean ± SEM
Mediterranean	Winter	0.0464	0.947	0.128	0.185 ± 0.024^{a}
	Summer	0.0238	1.873	0.242	0.406 ± 0.058^{b}
Black Sea	Winter	0.0144	1.188	0.132	0.180 ± 0.058^{a}
	Summer	0.0312	2.005	0.167	0.411 ± 0.068^{b}
Overall	Winter	0.0144	1.188	0.132	0.182 ± 0.045^{a}
	Summer	0.0238	2.005	0.192	0.408 ± 0.045^{b}

[a,b] Values in columns not sharing a common superscript alphabet differ significantly, $p < 0.05$.

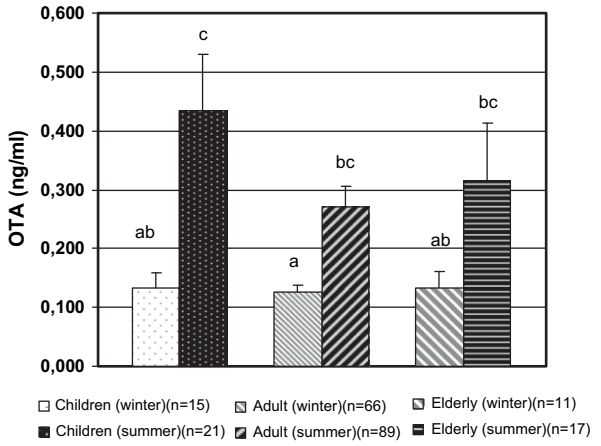

Fig. 3. Seasonal variations in serum OTA levels of different age groups in all subjects. Superscripts of different letters differ significantly ($p < 0.05$) from each other.

concentrations were found to be 0.4 ± 0.28 ng/mL in healthy adults in the study (Ozcelik et al., 2001) and this value is higher than the mean OTA level of all adults in both seasons in our study.

There are also some studies on blood OTA levels of healthy population and nephropathy patients in Middle East and North African countries. Assaf et al. reported that mean plasma OTA concentration was 0.17 ± 0.01 ng/mL in healthy blood donors ($n = 250$) living in Lebanon and that the frequency of OTA-positive samples was found to be 33%. Sex and age differences were not observed. However regional differences on plasma OTA levels were determined possibly due to specific local alimentary habits. On the other hand, daily intake level of OTA was calculated to be 0.23 ng/b.w./day from serum OTA levels according to the equation described in Breitholtz (Assaf et al., 2004; Breitholtz et al., 1991).

Mean OTA levels in blood from the healthy population in Tunisia varied with location, dietary habits, the way of food storage and/or climate. Incidence of positive samples and mean concentrations were found to be different. Bacha et al. (1993) and Maaroufi et al. (1995) determined an overall incidence of OTA contaminated sera of 52% in the control population (range 0.1–8.8 ng/mL). In the other study, the average OTA concentration was found to be 0.53 ± 1.0 ng/mL in 62 healthy people (Grosso et al., 2003). On the other hand, Abid et al. (2003) performed a study in the people having kidney disease and healthy population from 1991 to 2000 to follow up the situation of human exposure to OTA in Tunusia. It was reported that mean blood OTA levels were 3.35 ng/mL in 1991, 2.25 ng/mL in 1994, 2.6 ng/mL in 1997 and 1.22 ng/mL in 2000 in the healthy groups. The percentage of OTA contaminated people in the healthy control group significantly decreased, however all values are higher than the mean OTA levels in Turkish healthy population living in both regions possibly due to climate and geographical conditions or dietary habits.

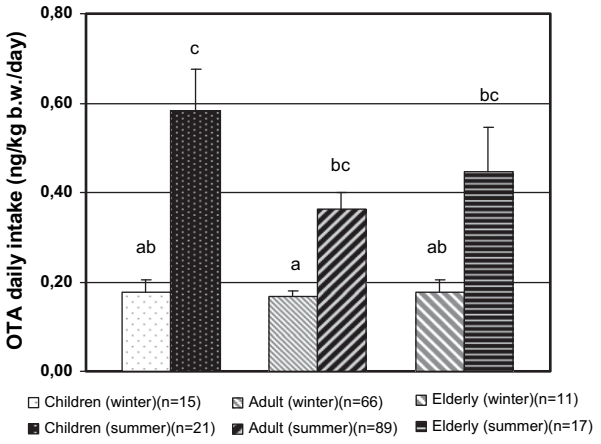

Fig. 4. Seasonal variations in OTA daily intake levels of different age groups in all subjects. Superscripts of different letters differ significantly ($p < 0.05$) from each other.

In Egypt, Wafa et al. (1998) reported to find low OTA levels with the mean of 0.01 ng/mL in urine samples of healthy controls ($n = 25$) and they did not detect OTA in the serum samples. In an Algerian study, 67% of the population was contaminated with OTA and the mean OTA level was determined as 2.8 ng/mL (Khalef et al., 1993). The Moroccan population seems to be less contaminated than their neighbors and the mean plasma OTA concentration was reported to be 0.29 ng/mL. No gender differences have been observed between the male and female populations like our study (Filali et al., 2002). The OTA levels in this population as a function of age showed that the people aged between 40 and 50 were the most exposed (~70% of positive), but average value (0.42 ng/mL) in the younger group (18–30 years) was higher than the others. However, OTA levels were not measured in pediatric and geriatric populations in Morocco.

There are limited studies on the evaluation of blood OTA levels according to age (Filali et al., 2002; Assaf et al., 2004; Pena et al., 2006; Grosso et al., 2003; Palli et al., 1999; Ueno et al., 1998) and it was reported that there is little or no significant correlation between age and levels and/or incidence of OTA in human blood (Palli et al., 1999; Ueno et al., 1998; Grosso et al., 2003; Assaf et al., 2004). However, most of these studies were carried out in adult populations. Gilbert et al (Gilbert et al., 2001) showed that plasma OTA levels appear to depend on age, with higher levels in 30–44 years old compared to <30 and >45 years old. In Moroccan population the OTA concentrations were found to be higher in 18–30 years old than older age (Filali et al., 2002). The other study comparing men and women aged <25, 25–39 and >40 years suggested the presence of a positive correlation between age and OTA levels in men and the opposite effect for women (Hadlok, 1993). The greater percentages of individuals with OTA levels >1 ng/mL for two genders aged 31–40 and females over 60 years were reported compared to other age groups in Czech Republic (Ruprich and Ostry, 1993). It cannot be found any evaluation on the role of age on OTA exposure in children. Serum OTA levels were determined as 0.4–8.2 ng/mL in the children under 5 years old living in Sierra Leone, but the values were not compared to adults or elderly (Jonsyn, 1999).

In our study, the mean OTA concentrations and the mean daily intake levels of OTA were not significantly different between age groups of all study population in winter period. Although the levels in children were higher than the others in summer, the differences were not significant statistically. On the other hand, the OTA concentrations in all children were found to be enhanced three times approximately in summer period compared to winter in our study. The elevations observed in summer were 2 and 2.5 times compared to winter samples in adults and elderly, respectively. Furthermore, the OTA levels of children in Black Sea Region were found to be higher than adults significantly in both seasons. In Mediterranean Region, there is no significant difference in the OTA levels of all age groups in winter, but children have lower OTA levels than the other age groups in summer by contrast with Black Sea Region and it suggests that the children in Mediterranean Region prefer to consume less contaminated food like vegetables in summer.

Blood OTA concentration has been reported to be a good index for predicting OTA intake. Therefore, the daily intake of OTA was estimated from the mean concentration of serum samples according to Breitholtz et al. (1991). The method includes assumptions on plasma clearance and bioavailability. A good agreement between this method and the estimation method from the intake of OTA-contaminated food was reported (Breitholtz et al., 1991). The mean daily intake levels of OTA are calculated as 0.182 ng/b.w./day in winter and 0.40 ng/b.w./day in summer for all subjects and the highest value was found 2.005 ng/b.w./day.

TDI of OTA in human was assessed by several organizations. The Nordic Working Group on Food Toxicology and Risk Evaluation accepted the TDI of 5 ng OTA/kg b.w./day based on their calculations on the carcinogenicity studies with safety factor of 5000 (The Nordic Working Group on Food Toxicology and Risk Evaluation, 1991). The Canadian expertise also evaluated OTA and suggested Provisional TDI (PTDIs) of 1.2–5.7 ng/kg b.w./day for a risk level of 10^{-5}(Kuiper-Goodman, 1990). WHO proposed the provisional TDI of 16 ng OTA/b.w./day based on the lowest adverse effect level for kidney damage in pigs and a safety factor of 500 (WHO, 1991). Joint Expert Committee on Food Additives (JECFA) established a Provisional Tolerable Weekly Intake (PTWI) of 112 ng/kg b.w. per week (equivalent to 16 ng/kg b.w./day) at its 37th meeting (WHO, 1996) and reconfirmed this value, but rounded it off to 100 ng/kg b.w. per week corresponding to approximately 14 ng/kg b.w./day (WHO, 1996). All daily intake values estimated in our study are below the TDI.

In conclusion, the mean serum concentrations of OTA in healthy population in the Black Sea and Mediterranean Regions were found not to be exceeding 1 ng/mL in agreement with the distribution reported in most European countries. The daily intake levels of OTA were calculated below the TDI levels given by regulatory authorities. However, our results suggest that Turkish population living in both regions is continuously exposed to OTA and that the exposure levels are also elevated in summer period compared to winter. The accurate prediction of the possible health impact of individual mycotoxins is difficult, especially in susceptible populations such as children and elderly. Possible additive or synergistic effects of multiple mycotoxins make the task far more complex and long-term effects are beyond foresight. Therefore, surveillance on food contaminants including OTA should be conducted by government and related ministry continuously. Different types of foods should be routinely tested for OTA presence prior to use and contaminated products should be eliminated. On the other hand, the training programs on this problem should be developed especially for agricultural producers. Using the scientific knowledge and improved techniques for harvesting, handling and storage will reduce or eliminate contamination problem with mycotoxins and prevent the threat to human health and the risk of economic loss.

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