Serum Levels of Ochratoxin A in Healthy Adults in Tuscany: Correlation with Individual Characteristics and between Repeat Measurements \(^{1}\)

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Abstract

Ochratoxin A (OTA), a mycotoxin widely contaminating staple foods and beverages, has been classified as a “possible human carcinogen (Group 2B)” by the IARC. Serum levels of OTA were measured in a group of 138 healthy adults (age, 35–65 years) living in the area surrounding Florence (Tuscany, central Italy) and detected in all but four samples (97%). After the exclusion of one subject with a peak value of 57.2 ng/ml, OTA levels ranged between 0.12 and 2.84 ng/ml, with mean and median values of 0.56 and 0.48 ng/ml, respectively. OTA levels were significantly higher in men than in women (0.64 versus 0.50) and correlated positively with height. A strong association was found with the season in which blood samples were obtained, with summer values higher than autumn values. On the other hand, OTA levels tended to be negatively associated with blood pressure, either systolic or diastolic; no association was evident with age, weight, body mass index, and smoking history. The associations with height and season persisted in a multivariate regression analysis.

A subgroup of subjects provided a repeat blood sample approximately 1 year later. The Spearman correlation coefficient between 68 pairs of original and repeat measurements was practically null (\(r = 0.05\)). Only two subjects (2.9%) had OTA levels of >1 ng/ml on both occasions.

These results suggest that OTA contamination is widespread in foods consumed by this population, in agreement with previous reports from Italy and other countries. A strong seasonal variation, which possibly differs from year to year, was observed. OTA serum levels are a short-term biomarker with a high within-subject variability; therefore they have limited use at the individual level but can be used to characterize populations or subgroups of subjects. Additional analyses are needed to explore the dietary determinants of OTA levels in this population.

Introduction

The consumption of foods contaminated by mycotoxins, widespread toxic compounds produced by mold secondary metabolism, can represent a relevant source of danger to humans. OTA, \(^{3}\) produced by \(P\)enicillium \(v\)iridicatum and several \(A\)spergillus \(o\)chraceus strains, is commonly found in foods such as cereals, oleaginous seeds, coffee, beer, wine, and meat products (including cured meats, sausages, pork, and chicken meat) as a result of carryover from contaminated animal feed.

The nephrotoxic effects in animals and the correlation between OTA occurrence in food and the incidence of both Balkan endemic nephropathy (1) and, possibly, urinary tract tumors have been established. More recently, the IARC has classified OTA as a “possible human carcinogen (Group 2B)” based on sufficient evidence for carcinogenicity in experimental animal studies and inadequate evidence in humans (2). The genotoxicity of the toxin has long been a topic of debate (3, 4).

As a consequence of the possible health hazards related to the ingestion of OTA, efforts to assess OTA levels in foods have been made by researchers worldwide and by governmental authorities in many countries. On the other hand, the evaluation of OTA levels in biological fluids represents an alternative approach to assess the presence of this contaminant in foods and its risk for humans. Research dealing with OTA and its metabolites in biological fluids can also improve our knowledge of the biological effects of this toxin.

The current study was performed to correlate the serum levels of OTA with individual characteristics of a group of healthy adults participating in an epidemiological study in Tuscany, a region of central Italy, and to estimate within-subject variability by measuring OTA levels in repeat samples collected 1 year later.

Materials and Methods

Serum Samples. Serum samples were obtained from healthy volunteers residing in the Florence district, collected in disposable vials, stored at \(-80^\circ\)C, and shipped on dry ice to the laboratory in Rome for analysis at the end of the study. A group

\(^{1}\) The abbreviations used are: OTA, ochratoxin A; BMI, body mass index; TDI, tolerable daily intake.

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of 138 volunteers (age, 35–64 years) was randomly identified among participants enrolled on selected days in the large prospective European Prospective Investigation into Cancer and Nutrition study and provided a blood sample in the months of July (n = 82) and October (n = 56) 1994. A subgroup of 69 participants, equally distributed among those enrolled in the two periods (42 of 82 and 27 of 56 volunteers, respectively), also agreed to provide a repeat blood sample approximately 1 year later, with most samples obtained in the period between October 1995 and January 1996. After the exclusion of one subject with a peak value (and his repeat sample), 137 samples and 68 repeat measurements were available for statistical analyses.

**Analytical Methods.** OTA determination was performed by high-performance liquid chromatography according to validated methods in the National Institute of Health laboratory (Rome, Italy; Ref. 5). Briefly, serum samples were extracted by ethyl acetate at pH 2, centrifuged at 10,000 × g and injected into high-performance liquid chromatography after reconstitution with 2% acetic acid–water/acetonitrile solution [43:57 (v/v)]. The quantitative analysis was performed by fluorescence detection, and the OTA identity was confirmed by postcolumn derivatization with the formation of OTA-methyl ester. The repeatability and accuracy of the method were verified by recovery experiments on human serum samples at OTA spiking levels of 0.5 and 2.0 ng/ml. The average recovery values ranged from 85–90%, with a SD and a coefficient of variation of 2.6 and 2.0%, respectively.

**Questionnaire and Individual Information.** Information on individual characteristics (including lifestyle and dietary habits) was obtained by using questionnaires filled out by each participant (6). Anthropometric measurements (height and weight) and blood pressure were also carried out during the period following a standardized protocol that was verified periodically. BMI was calculated according to the following formula: (weight in kilograms)/(height in meters)². Overall, 13,597 volunteers were enrolled in the period between 1993 and 1997 (approximately 15 per day) into the Florence European Prospective Investigation into Cancer and Nutrition study cohort. All participants signed an informed consent form and provided a fasting blood sample and detailed demographic information for follow-up procedures. Biological samples have been preserved and are currently stored in liquid nitrogen tanks for future nested case-control studies on selected outcomes, including cancers and other chronic diseases.

**Statistical Analyses.** Tertile levels for continuous variables were calculated based upon the whole series of 137 subjects. The Wilcoxon rank-sum tests for paired and unpaired data were performed for statistical evaluation of the significant difference in OTA distributions of two groups. Kruskal-Wallis tests were used if more than two groups were compared. The Spearman rank-correlation coefficient was used to determine the correlation between repeated values of OTA. The relative importance of the various variables collected was assessed using a stepwise discriminant procedure based on the multiple regression model, with OTA levels as dependent variables. The natural logarithm transformation of OTA values was used to improve normality. OTA values below the detection level of 0.10 were considered as 0.05 ng/ml. Starting from a full model with all variables included, nonsignificant variables were progressively deleted with a step-down procedure based on a likelihood ratio test.

**Estimation of Dietary Intake of OTA.** The plasma clearance of OTA through renal filtration is restricted due to binding of the toxin to plasma macromolecules. On the basis of the renal filtration rate of inulin and the free fraction of OTA in plasma, it is possible to calculate the renal filtration for OTA in humans: 0.033 ml/min corresponds to 0.67 ml/kg body weight per day for an average 70-kg person, according to the Klaassen formula \( K_r = C_p \times C/A \), where \( K_r \) represents continuous dietary intake, \( C_p \) represents plasma clearance, OTA, represents the plasma concentration of OTA, and \( A \) represents bioavailability (7). We used serum concentrations as an approximation of plasma concentrations and used individual anthropometric data to estimate daily intakes for each subject.

**Results**

OTA was detected in the serum of all but four participants (97%) and ranged between 0.12 and 57.2 ng/ml. After the exclusion of the subject with this peak value, statistical analyses showed that the mean and median values were 0.56 and 0.48 ng/ml, respectively, among the remaining 137 subjects; the highest value was 2.84 ng/ml.

The distribution of OTA values is reported in Fig. 1: 9.5% of participants had a value higher than 1.0 ng/ml; 84.7% ranged from 0.20–0.39 ng/ml; 18.2% from 0.40–0.59 ng/ml; 27.7% from 0.60–0.79 ng/ml; 8.0% from 0.80–1.0 ng/ml; and 5.8% from 1.0–2.84 ng/ml.
between 0.2 and 1.0 ng/ml; and 5.8% were below 0.2 ng/ml. The distribution of OTA values according to gender, season, and tertile levels of other individual characteristics is reported in Table 1. Women (n = 86) had a mean value of 0.50 ng/ml and a median value of 0.45 ng/ml with a percentage of positive samples of 96.5%. Men (n = 51) had a significantly higher mean value of 0.64 ng/ml (P = 0.04); the median value was 0.57 ng/ml with the proportion of positive samples of 96%. Significantly higher OTA values were found in blood samples collected during the summer period of the study year (P = 0.001). OTA values were also higher among taller subjects (P = 0.01) and tended to be higher among those subjects with lower values of blood pressure, either systolic or diastolic (P = 0.06). No association was evident with age, weight, BMI, or smoking history (Table 1).

Multivariate statistical analysis (Table 2) showed that height and season of blood collection (autumn versus summer) were the strongest determinants of OTA and, taken together, accounted for about 16% of the variance of OTA (R² = 0.158).

Analyses stratified by sex showed the same pattern of associations among females, the largest group.

**Repeat Measurements.** A subgroup of 68 subjects (approximately 50% of the original participants) agreed to provide a repeat blood sample approximately 1 year later: OTA levels were detected in all but five samples (92.7%). OTA values ranged between 0.10 and 2.77 ng/ml; the mean and median values were 0.57 and 0.44 ng/ml, respectively; 20.6% of the values were below 0.2 ng/ml, and 13.2% of the values were higher than 1.0 ng/ml. Practically no correlation was found between the first and second measurements (Spearman correlation coefficient = 0.05; Wilcoxon rank-sum tests for paired data, P = 0.8). Correlation was very poor, even when only subjects whose blood samples had been obtained in the same season 1 year apart were considered. Only 2 subjects (2.9%) had values of >1.0 ng/ml on both occasions, 13 subjects (19.1%) had one value of >1.0 ng/ml, whereas all of the others had values of <1.0 ng/ml on both occasions (Fig. 2). In the study period in which repeat blood samples were collected (from the summer of 1995 until the winter of 1996), OTA levels showed a high variation, following a seasonal pattern that appeared to be partially different from that observed in the previous year. The one subject with the peak value at the first determination in July 1994 (57.2 ng/ml) also provided a repeat blood sample (15 months later) that showed a much lower OTA value (0.38 ng/ml).

**Estimation of Dietary Intake of OTA.** According to the Klaassen formula, the mean daily intake of OTA estimated on the basis of the serum concentration of the toxin was 0.77 ng/kg body weight for the whole series of participants (average weight, 69.2 kg); the individual estimates ranged between 0.05 and 3.13 ng/kg body weight/day, with a median value of 0.69. A total of 34 subjects (24.8%) had a daily intake estimated to

| Table 1 | Mean and median values of OTA serum levels (ng/ml) according to gender, season, smoking history, and tertile levels of selected individual characteristics in 137 healthy volunteers (Florence, Italy)a |
|---------|----------------------------------|----------------|----------------|----------------|----------------|
| Characteristics | N | Mean | SD | Median | Range | Pb |
| Gender | | | | | |
| Male | 51 | 0.64 | 0.45 | 0.57 | ND–2.84 | |
| Female | 86 | 0.50 | 0.30 | 0.45 | ND–1.89 | 0.04 |
| Period | | | | | |
| Summer | 81 | 0.65 | 0.42 | 0.54 | ND–2.84 | |
| Autumn | 56 | 0.43 | 0.21 | 0.37 | ND–0.96 | 0.001 |
| Age (yr) | | | | | |
| <47 | 46 | 0.56 | 0.45 | 0.45 | ND–2.84 | |
| 47–52 | 44 | 0.57 | 0.36 | 0.51 | ND–1.89 | |
| >53 | 47 | 0.55 | 0.29 | 0.48 | ND–1.48 | 0.7 |
| Weight (kg) | | | | | |
| <63.0 | 46 | 0.52 | 0.34 | 0.47 | ND–1.89 | |
| 63.0–72.0 | 45 | 0.52 | 0.32 | 0.41 | ND–1.48 | |
| >72.0 | 46 | 0.64 | 0.43 | 0.56 | 0.12–2.84 | 0.1 |
| Height (cm) | | | | | |
| <160 | 43 | 0.45 | 0.30 | 0.36 | ND–1.36 | |
| 160–169 | 47 | 0.60 | 0.32 | 0.52 | 0.17–1.89 | |
| >169 | 47 | 0.62 | 0.45 | 0.52 | ND–2.84 | 0.01 |
| BMI (kg/m²) | | | | | |
| <23.5 | 45 | 0.58 | 0.36 | 0.49 | ND–1.89 | |
| 23.5–26.0 | 46 | 0.55 | 0.45 | 0.43 | ND–2.84 | |
| >26.0 | 46 | 0.55 | 0.28 | 0.48 | ND–1.24 | 0.7 |
| Systolic blood pressure (mmHg) | | | | | |
| <118 | 46 | 0.62 | 0.50 | 0.48 | ND–2.84 | |
| 118–130 | 44 | 0.61 | 0.30 | 0.54 | 0.18–1.25 | |
| >130 | 47 | 0.45 | 0.22 | 0.41 | ND–0.9 | 0.06 |
| Diastolic blood pressure (mmHg) | | | | | |
| <74 | 45 | 0.63 | 0.49 | 0.51 | ND–2.84 | |
| 76–86 | 47 | 0.59 | 0.32 | 0.53 | ND–1.48 | |
| >86 | 45 | 0.45 | 0.22 | 0.41 | ND–1.04 | 0.06 |
| Smoking history | | | | | |
| Current smoker | 33 | 0.49 | 0.32 | 0.37 | 0.12–1.89 | |
| Ex-smoker/never smoker | 104 | 0.58 | 0.38 | 0.49 | ND–2.84 | 0.2 |

a One subject with a peak value of 57.2 ng/ml was excluded from analysis.
b Wilcoxon test or Kruskal Wallis test.
c ND, not detected.
be higher than 1 ng/kg body weight. According to gender, daily intakes results were quite similar: 0.79 and 0.74 ng/kg/day for males and females, respectively (average weight: males 77.4 kg; and females, 64.4 kg).

Discussion

Our results show that OTA is detected in almost all serum samples tested and suggest that OTA contamination is widespread in foods and beverages consumed by this group of healthy adults selected from an Italian population. OTA levels in human blood have been investigated in several countries, and a high proportion of samples with a low concentration of this toxin have been reported in several studies, indicating a widespread exposure of humans to OTA. In our study, 85% of the human sera contained 0.2–1.0 ng/ml OTA, in agreement with the distribution reported in several European countries (8). Only one sample contained an extremely high level of OTA, but a repeat blood sample provided 1 year later by the same subject showed an OTA value that was lower than average. Overall, the within-subject variation was very high, with almost no correlation between repeat samples collected 1 year apart.

We found a strong positive association between the levels of OTA and male gender, height, and the summer period and an inverse one with blood pressure. The latter, although only borderline, is particularly interesting in view of the role supposedly played by OTA in the Balkan endemic nephropathy. Blood pressure measurements in our study were well standardized according to an international protocol. The association with male sex disappeared in a multivariate analysis, whereas with height persisted, suggesting a possible correlation with a higher amount of contaminated foods consumed by taller individuals. This finding, however, should be confirmed before excluding the role of chance or differences in metabolism. The higher values found in blood samples collected during the summer of that year could have been related to two factors. Particular climatic conditions could have been responsible for a higher contamination of animal feed in the period immediately preceding collection. A strong seasonal variation was detected by Ominski et al. (9) in sera samples obtained from a large series of pigs in Canada: in the month of July, 65% of the samples contained detectable levels of OTA, as compared with 38%, 21%, and 17% in April, October, and January, respectively. On the other hand, dietary and drinking habits show a clear seasonal variation in Italy, and this could lead to a different intake during different periods of the year. Evaluation of average and specific short-term individual dietary information is necessary to clarify this issue.

In Bulgaria, Petkova-Bocharova et al. (10) have found a higher mean value of OTA in a group of patients with endemic nephropathy and/or urinary system cancers compared to a group of healthy volunteers. However, it is not clear how relevant current OTA levels are in view of the short half-life of OTA in human serum (approximately 20–50 days; Ref. 7). In addition, high OTA levels in patients with chronic renal failure might be due to a reduced clearance; prospective studies using baseline OTA levels could better clarify these issues.

A high frequency of OTA contamination in human serum samples collected in Italy has been reported in two studies (11, 12), both in healthy volunteers and in nephropathic patients living in central and southern Italy. High values in human blood were also found in Tunisia (13), where the range of contamination was 0.7–7.8 ng/ml for the general population and 12–55 ng/ml for patients suffering from chronic renal failure. In Hungary (14), 52 of 100 human blood samples and 38 of 92 colostrum samples were reported to contain OTA (0.2–12.9 ng/ml in blood samples; 0.2–7.3 ng/ml in colostrum samples). Also, a previous study carried out in Italy showed the presence of the toxin in 22 of 111 (19.8%) human milk samples (15); the highest peak value found was 21.9 ng/ml. The values we have found in this study are also quite similar to those recently collected within the European SCOOP task on OTA (16) reporting surveys from Denmark (144 plasma samples, 49% positive with a mean value of 1.8 ng/ml and a maximum value of 13.2 ng/ml), France (3070 plasma samples, 19% positive with a mean value of 0.4 ng/ml and a maximum value of 161 ng/ml), Germany (309 plasma samples, 86% positive with a mean value of 0.34 ng/ml and a maximum value of 7.9 ng/ml), and Sweden (39 plasma samples, 100% positive with a mean value of 0.34 ng/ml and a maximum value of 1.88 ng/ml). Our results were also in agreement with the OTA values reported in Hungary by Solti et al. (17); the concentration of 355 sera samples varied from <0.2 to 10 ng/ml OTA, but 75% of the samples contained 0.2–1.0 ng/ml OTA. Another recent study (18) reported the contamination of almost all samples of food and feed collected in a Croatian village with endemic nephropathy; OTA blood levels in the range of 2–50 ng/ml were detected in 4.5% of this population, and lower values were found in another nonendemic village of the same area (2.4% positive samples; range, 2–10 ng/ml).

According to the Klaassen empirical formula, the mean daily intake of OTA estimated on the basis of the serum concentration of the toxin in our series was similar to previous estimates for Italy and other European countries. The Klaassen formula is based on several assumptions, but it might be an alternative approach to estimate the dietary intake of OTA. Several international organizations have defined a wide range

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**Table 2** Multivariate regression analysis*

| Variable | Parameter estimate (b) | SE(b) | Partial R² | Pr
|---------|----------------------|-------|-----------|---
| Height (cm) | 0.0109 | 0.00434 | 0.0757 | 0.01
| Period (summer) | 0.2136 | 0.06087 | 0.0827 | 0.0006

*The regression model also included terms for age and gender.

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**Fig. 2.** Correlation between repeat measurements of OTA serum levels in 68 subjects (Florence, Italy). Solid line, linear regression function.
of TDI for OTA because of the different toxic effects taken into consideration (4). A TDI of 5 ng/kg body weight has been suggested by The Nordic Working Group on Food Toxicology and Risk Evaluation (4); none of our study subjects (except the subject with the peak value) exceeded this TDI level according to Klaassen estimates.

Data from several countries consistently indicate that humans are continuously exposed to OTA, and there is no doubt that this compound is toxic. OTA has shown teratogenic and immunotoxic properties; a nephrotoxic effect is evident in all mammalian species tested (2), and genotoxicity has been reported recently (3). Its possible causal role in urothelial tumors has yet to be confirmed (19). In view of these characteristics, regular controls should be enforced, and exposure to OTA should be kept to a minimum, avoiding the consumption of heavily contaminated foods. National authorities as well as producers and their organizations should agree on defining maximum levels of OTA and other mycotoxins in foods; a policy based on Hazard Analysis and Critical Control Points (HACCP) procedures to protect consumers health seems highly advisable.

From an epidemiological point of view, OTA serum levels are a short-term biomarker with a high within-subject and seasonal variability; therefore, they have limited use at the individual level. However, OTA measurements can be used to characterize populations or subgroups of subjects, particularly in prospective studies storing blood samples taken at baseline, well before possibly related outcomes occur. A positive association with height emerged in this study. Additional studies of dietary determinants of OTA intake in humans are urgently needed, and the correlation between OTA levels and the consumption of specific food items and beverages should be explored.

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