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Original Article

Investigation of ochratoxin A (OTA) in bakers' urine samples in Istanbul by immunoaffinity column clean-up and high-performance liquid chromatography

Sultan Mehtap Buyuker

Üsküdar University, Department of Pharmacy Services, Istanbul, Türkiye

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Corresponding Author: Sultan Mehtap Buyuker, Üsküdar University, Department of Pharmacy Services, Istanbul, Türkiye
Email: sultanmehtap.buyuker@uskudar.edu.tr

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Abstract

Aim: Ochratoxin A (OTA), is the most toxic mycotoxin in the ochratoxins produced by Penicillium and Aspergillus species. It is known that this mycotoxin has carcinogenic, nephrotoxic, teratogenic, and immunotoxic effects. The aim of our study is the inhalation exposure of bakery workers to ochratoxin A, which can be found in flour.

Materials and Methods: In this study, OTA occurrence and level in urine samples collected from bakers in Istanbul were analyzed by $NaHCO_3$ dilution, IAC (Immunoaffinity Column) clean-up, and high-performance liquid chromatography with fluorescent detection. Creatinine and malondialdehyde levels were measured spectrophotometrically in collected urine samples. Also, 8-hydroxy 2-deoxyguanosine which is a biomarker of DNA damage was tested by ELISA (Enzyme-Linked Immunosorbent Assay).

Results: As a result of our study, the limit of detection (LOD) and the limit of quantification (LOQ) of the method were measured as 0.06 $ngmL^{-1}$ and 0.2 $ngmL^{-1}$, respectively. Of the total collected urine samples 185, 4.9% were contaminated with OTA. Also, the statistically significant difference between OTA levels has not been determined in urine samples of bakers working at the production department and the bakers working at the other departments of the bakery ($p>0.05$). In order to define the exposure profile of OTA in bakers a questionnaire was conducted among the volunteers as well. But after the evaluation of the answers related to gender, age, dietary habits, coffee consumption, smoking, and alcohol consumption, no correlation was found with the OTA exposure ($p>0.05$).

Conclusion: In the present study although OTA presence was not statistically significant, OTA detection in the urine of bakers' indicates the necessity of regular follow-up for these workers.

Keywords: Creatinine, HPLC method, malondialdehyde, ochratoxin A, urine

INTRODUCTION

Consumption of animal feed contaminated with OTA can pass to humans through the food chain. For this reason, animal health and food safety pose a potential risk to human health. Another route of transmission is through direct consumption or indirect exposure to OTA-contaminated food. In our study, we aimed to analyze the amount of OTA found in the urine of bakers' who were exposed to flour dust due to their occupation.

Ochratoxins, A.ochraceus (A.alutaceus), A.melleus, A.alliaceus, A.ostianus, A.sclerotorium, A.albertensis, A.wentii, A.auricomus, A.niger var. niger, A.sulphureus (A.fresenii) and P.viridicatum (P.verrucosum) are mycotoxins produced by molds (1-4). Ochratoxin was identified by Van der Merwe et al. in 1965 and received this name because it is a producer mold (5). The most well-known and toxic metabolite of ochratoxins is ochratoxin A (OTA). Ochratoxin A is a mycotoxin commonly found in cereals (6). OTA has been published as a "Group 2B" probable carcinogen by the International Agency for Research on Cancer (IARC) (7-9). OTA has also been studied as an epigenetic carcinogen to show a clustered carcinogenic effect. However, it is considered a direct carcinogen due to its direct binding to DNA (8,10).

Ochratoxins are produced by *Penicillium* and *Aspergillus* molds. *Aspergillus ochraceus* is important because it was the

first species to produce OTA. In 2004, a second OTA-producing species with similar characteristics was found and named *A.westerdijkiae* (11). In the following years, the mold type *A.steynii* with a similar structure was defined (12). It has been determined that these mold species produce OTA at a much higher rate than *A.ochraceus*. Among *Aspergillus species, Nigri Aspergillus carbonarius* is known to be the major OTA producer. It forms OTA in grapes and its products, grape juices, wines (13), and some coffee beans (14). The proportion of *ochratoxygenic* isolates belonging to *A.niger* is much less than those belonging to *A.carbonarius* species (15). It has been reported to produce OTA in *A.lacticeffeatus and A.sclerotioniger* species (16).

OTA has a nephrotoxic effect in the vast majority of mammals (17). Studies with experimental animals reveal that OTA has nephrotoxic, teratogenic, immunosuppressive, hepatotoxic, genotoxic, apoptosis-inducing, and lipid peroxidation-increasing effects (18-20).

OTA has been classified as Group 2 B (possible carcinogen) by the International Agency for Research on Cancer (IARC) (7,21,22). It is also called epigenetic carcinogen through its indirect carcinogen mechanism (23). However, it is also considered a direct carcinogen because it can bind to DNA (8).

MATERIALS AND METHODS

The urine samples analyzed in this study were obtained from bakery workers working in bakeries in different districts of Istanbul. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Research Ethics Committee of Marmara University (B.30.2.MAR.0.01.02/AEK/20, approval date 03/03/2011). Urine samples were analyzed by using the high-pressure liquid chromatography (HPLC=High-Performance Liquid Chromatography) method with the fluorescent detector, which has a very good separation and purification feature and can perform sensitive analysis up to the picogram level, after the extraction of OTA by passing through immunoaffinity columns. In addition, the amounts of malondialdehyde and creatinine in urine samples were measured by spectrophotometric methods and the amount of 8-hydroxy-2-deoxyguanosine, which is an indicator of DNA damage, was determined by the ELISA method.

Analysis of Urine Samples for OTA

Urine samples collected from bakery workers working in bakeries in various regions of Istanbul were transported to the laboratory in disposable urine containers and stored in a deep freezer at -20 ºC until analysis. A sensitive method, HPLC, was used to measure the OTA level in the urine samples of bakery workers. The method of Fazekas et al. (2005) was used, but minor changes were made during the application (24).

The modified analysis method is given below.

- 15 ml urine sample + 15 ml 5% $NaHCO_3$ solution
- Centrifuge at 2500 rpm for 5 minutes in an ultracentrifuge device in 50ml Falcon tubes.
- 25 mL of solution taken from the top of the centrifuged extract is passed through the immunoaffinity column at a rate of approximately 1 drop per second.
- Then, 10 ml of bidistilled water is passed through the column once again at a rate of 1 drop per second.
- Take 3 ml of methanol and OTA in the column into a vial.
- The eluate taken into the vial is evaporated under nitrogen gas.
- It is re-dissolved with 150 µL of mobile phase solvent and stored at +4 ºC until injection.

In the investigation of the presence of OTA in urine samples, an Agilent 1100 model HPSK device with a fluorescent detector in the Marmara University Faculty of Pharmacy Central Research Laboratory was used.

Spectrophotometric Measurement of Urine Creatinine Levels

Creatinine forms an orange-yellow color with picric acid in an alkaline environment. The intensity of the color formed is directly proportional to the creatinine concentration. The colorimetric reaction, also known as the "Jaffe Reaction", was measured kinetically at 490 nm. A commercially available reagent kit (BIOLABO REAGENTS) was used to determine urinary creatinine levels.

Determination of 8-Hydroxy 2-Deoxyguanosine Levels in Urine Samples

ELISA kit (Cayman Chemical Company) was used to determine the levels of 8-OH-dG in urine. This assay is based on a comparison of oxidatively damaged guanine species and 8-OH-dG-acetylcholinesterase compounds with monoclonal antibodies with limited DNA/RNA oxidative damage. The Cayman Chemical ELISA kit detects three types of oxidized guanines in the determination of DNA/RNA oxidative damage. These; It is 8-hydroxy-2-deoxyguanosine from DNA, 8-hydroxy-guanosine from RNA, and 8-hydroxyguanine from DNA or RNA. Collected urine samples were stored at -20 °C in order to determine the levels of 8-hydroxy-2-deoxyguanosine in the urine. All urine samples stored at -20 °C before the study was brought to room temperature and then diluted from 1:250 to 1:1000.

Urine samples diluted in accordance with the kit procedure were applied to the plate. It was incubated for 18 hours and washed 3 times with 1:400 diluted buffer solution at the end of the incubation period. The samples were read in the microplate reader at 405 and 420 nm wavelengths without waiting.

Determination of Malondialdehyde (MDA) in Urine

The "NWLSS™ Malondialdehyde Assay" test kit was used for the measurement of MDA, the final degradation product of lipid

peroxidation. The test principle is based on the reaction of MDA with thiobarbituric acid.

Statistical Analysis of Data

NCSS (Number Cruncher Statistical System) 2007&PASS (Power Analysis and Sample Size) 2008 Statistical Software (Utah, USA) program was used for statistical analysis.

Descriptive statistics such as mean, standard deviation, median, frequency, ratio, minimum and maximum were used in the representations of the study data in tables and text.

The compatibility of the variables with the normal distribution was determined by examining the Kolmogorov-Smirnov test and box plot graphic representations. While our age and creatinine variables show normal distribution, OTA (ng/ml) and MDA measurements do not show normal distribution. Student-t Test in the comparison of the variables that can be measured quantitatively and that show normal distribution; the Mann-Whitney U test was used to compare two groups of variables that did not show normal distribution.

The Kruskal Wallis Test was used to evaluate the non-normally distributed OTA variable according to the type of smoking defined under three groups.

While Pearson Correlation Analysis is used in the relations with the MDA variable showing normal distribution in the evaluation of the relations between the parameters; Spearman Correlation Analysis was used for the OTA variable that did not show normal distribution.

Linear Regression analyses were used to interpret the results of the Validation Results of the Detection Method of OTA in urine with HPLC, the calibration curve of MDA, and the 8-hydroxy-2-deoxyguanosine (pg/ml) variables measured by the ELISA method.

The lowest level of significance was accepted as $p<0.05$.

RESULTS

In the study, the amount of OTA was determined in the urine samples of 185 bakery workers collected from different bakeries in Istanbul. The standards prepared at six different concentrations were injected into the HPLC device twice, and a regression curve was drawn against the area of OTA at this concentration measured by the device (Figure 1).

The correlation between the area obtained and the concentration was investigated in the analyzes performed on 5 different urine samples (spiked samples) to which OTA was added at different concentrations. Concentrations (0.25ng/mL, 0.5ng/mL, 2.5ng/mL, 5ng/mL, 7.5ng/mL) were added. The graph showing the correlation between the area and the concentration obtained from the analysis of OTA-added urine samples is shown in Figure 2.

The retention time (Rt) for OTA was found to be 5.67 minutes. OTA-free urines were extracted, and no impurities or foreign peaks were observed at the indicated retention time (Figure 5). The chromatogram of urine with OTA added is shown in Figure 6. The chromatogram of the naturally contaminated urine sample is given in Figure 3.

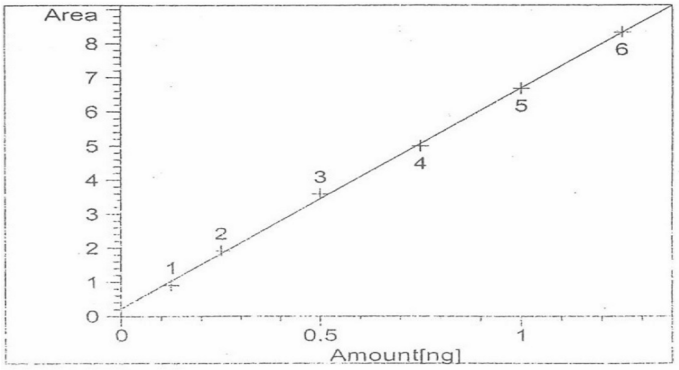

Figure 1. Regression curve versus area of OTA measured by the device at 6 different concentrations (0.125ng/mL, 0.25ng/mL, 0.5ng/mL, 0.75ng/mL, 1ng/mL, and 1.25ng/mL)

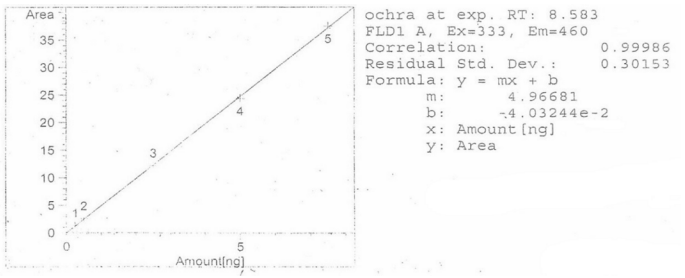

Figure 2. Graph showing the correlation between area and concentration obtained from analyzes of OTA added urine samples

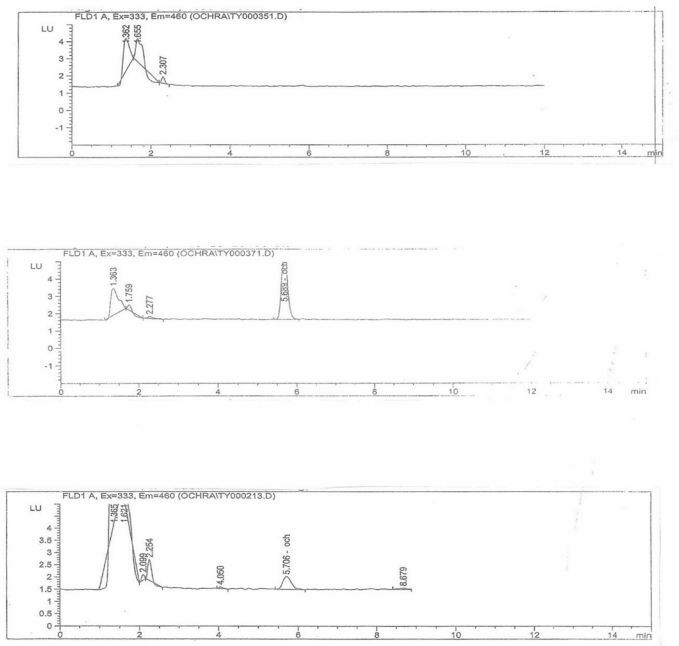

Figure 3. Chromatogram of urine sample without OTA

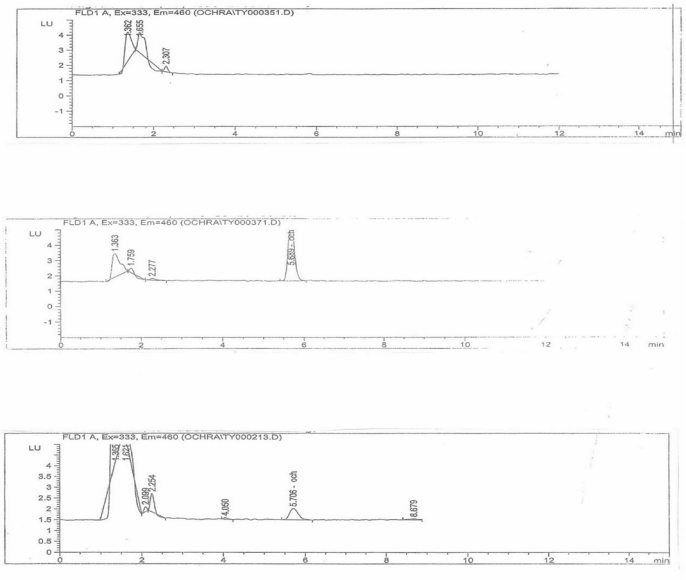

Figure 4. Chromatogram of urine sample containing 1ng/mL OTA

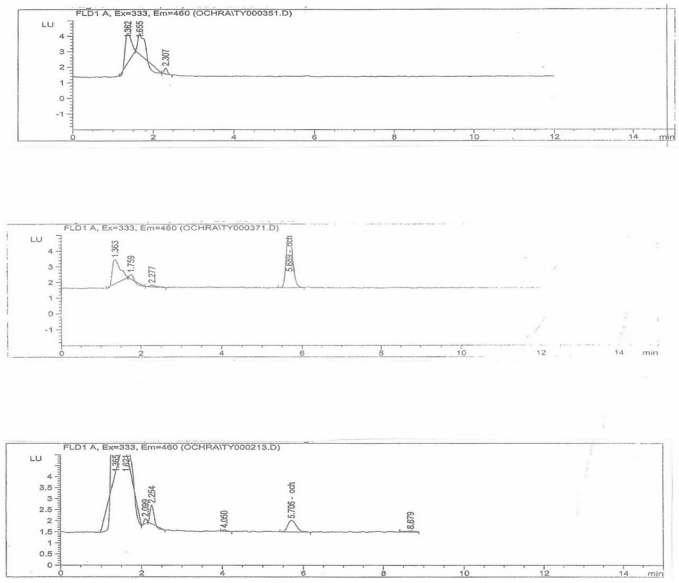

Figure 5. Chromatogram of 0.141 ng/mL natural contaminated urine sample

Table 1. 8-Hydroxy 2-Deoxyguanosine measurement results

Sample No	8-hydroxy-2-deoxyguanosine pg/mL	Sample No	8-hydroxy-2-deoxyguanosine pg/mL	Sample No	8-hydroxy-2-deoxyguanosine pg/mL	Sample No	8-hydroxy-2-deoxyguanosine pg/mL
1	222.446	**25**	437.122	**49**	219.673	**70**	371.034
2	382.044	**26**	298.749	**50**	271.255	**71**	380.407
3	935.89	**29**	194.371	**51**	272.096	**77**	231.286
4	395.053	**30**	548.523	**52**	178.584	**78**	341.184
5	711.606	**31**	448.986	**53**	157.415	**79**	325.59
6	425.473	**32**	294.441	**54**	445.074	**80**	374.194
7	621.182	**33**	366.591	**55**	212.771	**81**	306.644
8	1270.456	**34**	238.344	**56**	445.074	**82**	326.878
9	2102.291	**35**	594.301	**57**	294.441	**83**	377.396
10	690.068	**36**	388.464	**58**	261.976	**84**	319.44
11	290.224	**39**	869.28	**59**	297.624	**85**	193.757
12	503.15	**40**	804.855	**60**	410.605	**87**	299.882
15	267.789	**41**	302.001	**61**	315.816	**88**	202.313
16	578.357	**42**	1011.999	**62**	306.644	**89**	194.371
17	545.597	**43**	768.566	**63**	192.018	**188**	604.026
18	1309.244	**44**	106.421	**64**	248.031	**92**	149.89
20	565.754	**45**	1389.323	**65**	208.077	**93**	85.396
22	624.816	**46**	707.079	**66**	203.617	**94**	109.645
23	363.529	**47**	374.194	**68**	349.228	**98**	250.509
24	201.112	**48**	417.826	**69**	590.959	**101**	239.862

Limit of Detection (LOD) and Low Limit of Measurement (LOQ)

The detection limit is the smallest undetermined concentration of analyte detected, unlike the background noise (25). As a rule, the signal-to-noise (S/N) ratio is used in chromatographic measurements. The value where the S/N ratio is 3 is the LOD value (26).

The lower limit of measurement is the lowest amount of analyte in the sample that can be quantitatively detected within the limits of appropriate accuracy and precision. The lower limit of measurement (LOQ); S/N is the value where the ratio is 10. The minimum detectable OTA concentration (LOD) was determined as 0.06ng/ml, and the minimum detectable OTA concentration (LOQ) value was determined as 0.2 ng/ml as a result of the additional experiments.

Measured Creatinine and MDA Values

The calibration curve of MDA is given in Figure 6.

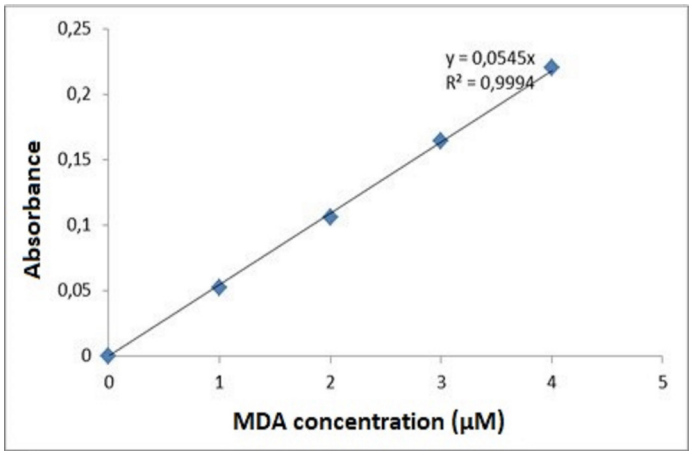

Figure 6. Calibration Curve of MDA

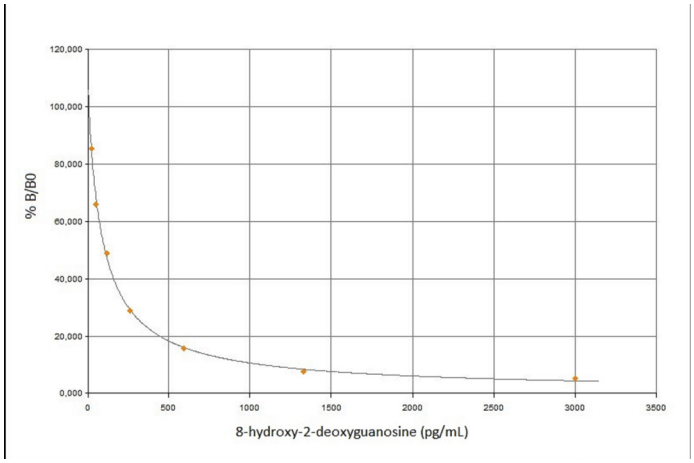

Figure 7. Calibration curve for 8-hydroxy-2-deoxyguanosine (pg/ml)

8-Hydroxy 2-Deoxyguanosine Measurement Results in Urine Samples

Calibration curve for 8-hydroxy-2-deoxyguanosine (pg/ml) is given Figure 7 and the results are given in Table 1.

DISCUSSION

Molds that can reproduce in foodstuffs and which can be contacted very frequently in daily life in this way, and especially the toxic metabolites they form, are one of the research topics that are emphasized.

Molds multiply in raw and processed materials under suitable conditions, causing the quality and quantity of the product to be changed and deteriorated, and on the other hand, they create toxic substances that have negative effects on human health. Among these toxic substances, ochratoxin A, which is very common, is a type of mycotoxin detected in various cereal species (27).

Karagözlü and Karapınar (1998) determined OTA at the level of 0.27-9.84 ppb in 4 of 100 food samples consisting of wheat, corn flour, oat flakes, oatmeal, oat flour, and muesli and the result did not indicate any danger in terms of OTA. However, they emphasized the necessity of doing more studies on OTA (28).

Baydar et al., (2007), in their study to determine the levels of aflatoxin and OTA in foods consumed in Ankara, found that only 3 of 25 grain samples contained OTA above 3 ppb, which is the acceptable limit in Turkey (3.45, 3.69 and 4.07 ppb) reported (29).

In the study of Akdemir (2008), OTA values were measured in 233 urine samples collected from 4 cities in Turkey, and the urine with the highest contamination was found in Ankara. (Positive sample rate: 90.1%, mean OTA concentration: 75.60 ng OTA/g creatinine). They found that the OTA content of urine samples from Ankara was significantly higher than the OTA content of urine samples from Diyarbakır, İstanbul, and İzmir (30).

Pena et al., (2006) conducted a validation study of an analysis method for OTA analysis in urine samples and analyzed OTA in human urine samples collected from various cities in Portugal with this method. OTA values ranging from 0.021ng/mL to 0.105ng/mL were found on the LOQ in 42 of 60 urine samples collected from residents of Coimbra, Portugal (31).

OTA levels in the blood were investigated in a study conducted by Özçelik et al., (2001), with blood samples taken from 93 healthy people with various kidney diseases and 40 healthy people in and around Isparta. Of the 93 blood samples, 35 belong to chronic renal failure patients undergoing hemodialysis, 15 to bladder cancer, and 15 to kidney stone patients. According to the results of the study, the highest OTA value in the blood was found in kidney patients receiving hemodialysis treatment (2.1±1.2 ng/mL). The amount of OTA in the blood samples of healthy people was 0.4±0.28 ng/mL. This result shows that OTA plays a role in urinary diseases in humans (32).

Fazekas et al., (2005) investigated OTA levels in urine by collecting urine samples from a total of 88 healthy people from 5 settlements in 3 districts in Hungary. They found an average OTA concentration of 0.013ng/mL (ranging from 0.006ng/mL to 0.015ng/mL) in 61% of the urine samples. OTA concentration in urine samples collected from the Heves district was found to be quite high compared to samples collected from Hajdu-Bihar and Somog districts ($p<0.003$). This study revealed that the OTA levels in the urine of Hungarian people differ by region. It has been determined that people living in rural areas are more exposed to OTA. It was also concluded that Hungarian people had low exposure to OTA, <1ng/kg/day (24).

In a study conducted by İlker Ateş et al., (2011) to measure whether there is a correlation between 8-hydroxydeoxyguanosine and MDA levels in terms of OTA in urine samples, the amounts of 8-hydroxydeoxyguanosine and MDA were measured in urine samples collected from different regions of Turkey and they were determined as OTA/creatinine. The amount in the urine was calculated. In the study, they did not find a relationship between demographic characteristics and living conditions, and OTA levels in urine. However, they found that the MDA levels in the urine samples were higher in the study group over 35 years of age compared to those younger than 35 years old. Studies have shown that there is a significant correlation between the presence of OTA in urine samples and the levels of 8-hydroxydeoxyguanosine and MDA. Again, a significant correlation has been reported between 8-hydroxydeoxyguanosine and MDA (33).

OTA formation in agricultural products is a factor that threatens human health. Due to the fact that it is a contagious disease that is commonly found in different foods, especially grain types, people are exposed to OTA significantly. In the study of Giray et al., (2009), serum OTA levels were measured in children living in different regions of Turkey; Based on these levels, daily OTA intake levels were calculated and evaluated in terms of its relationship with age, nutrition, gender, and body mass index. Serum OTA level was found above 1ng/mL in 25% of the study group. The data show that there is no relationship between OTA levels in the blood and age ($p>0.05$) (34). Many studies have been conducted on OTA in the world and our country. However, most of these studies are on foodstuffs or animals. Studies conducted in urine or blood in terms of monitoring the exposure in the population are more limited both in our country and in the world. As it is known, ochratoxins have different metabolites. The reason for examining OTA is that it is one of the most important and toxicologically important toxins among the ochratoxins group. For this reason, the detection and diagnosis of OTA, which is the most common ochratoxin type with the highest toxic effect for humans, has been made (35).

Many toxic effects of OTA, including nephrotoxic, teratogenic, genotoxic, hepatotoxic, and neurotoxic, have been detected in human and animal studies (36).

Detection of OTA in human biological fluids is minimal. Therefore, the work requires extreme precision. The fact that the people we selected as the study group was exposed to OTA by inhalation and this exposure was analyzed in the urine makes our study much more meaningful. The exposure of bakery workers to OTA by inhalation causes the exposure dose to be very low. However, considering how toxic OTA is, we can say that even very small doses are very important for human health.

Studies on exposure to spores through inhalation are limited in the literature in the world and our country. This clearly demonstrates the importance of our study.

There are many methods for detecting OTA in the literature. Since OTA measurement from human biological fluids is at low doses, HPLC, which is a very sensitive method, was preferred. Before, OTA was passed through the immunoaffinity column and the extraction process, which is the most sensitive stage of the study, was performed in 2005 by Fazekas et al. (24).

Some modifications have been made to the methods in order to provide precision and ease of operation. By using the centrifugation technique instead of filtering the sample through filter paper, a clearer extract was obtained, and the process was carried out faster. The manifold was used when passing the IAK through the column. In the stage of collecting OTA from the IAK column to the vial, unlike the method of Fazekas et al., (2005), 3 ml of methanol was used as the elution solvent instead of 2 ml of methanol in order to remove all of the toxins in IAK (24).

As a result of the preliminary trials related to the method, a 25 mL sample was taken and centrifuged at 2500 rpm to prevent compression in the colon in IAK. No problems were encountered during the extraction from the IAK column.

According to the findings we obtained as a result of our study, the OTA concentration in the urine of the bakery workers is between 0.06-0.26 ng/mL, the MDA is between 0.66-17.97 µM, and the creatinine value is between 1.86-58.50 mg/dL. but it was seen that there was no significant correlation between these values. Again, there was no significant difference between the amount of OTA in the urine samples of the workers in the non-manufacturing department, which we allocated as the control group, and those working in the manufacturing department ($p>0.05$).

During the study, questionnaire forms were filled in by the volunteers. To evaluate the participant group in terms of nutrition and socio-cultural aspects and to conduct a wider statistical study on exposure, the questionnaire form was arranged in a very comprehensive way. The fact that the survey was conducted one-on-one with the participants by personally going to the bakeries enabled the working conditions of the bakery workers to be examined more closely. When we evaluated the results of the questionnaire, no significant result could be found between the nutritional habits, genetic differences, gender and age characteristics of the participants, and the amount of OTA in their urine.

Fazekas et al., (2005) method was used in our study. Unlike this method, the sample size was taken as 25 mL to provide ease of passing through IAK. HPLC was preferred because it was thought to be the most appropriate method for the measurement

of low-dose OTA in the urine (24).

After quantifying OTA with HPLC, creatinine, and MDA levels were also measured in the same urine samples. It is known that the major toxic effect of OTA is on the kidneys. The creatinine values in the urine and the MDA level, which is an indicator of free radical damage, were measured spectrophotometrically, and the correlation of MDA with the amount of creatinine and OTA was examined. However, no correlation was found between MDA, creatinine, and OTA levels ($p>0.05$). Since the amount of OTA detected in urine samples is very low and it is difficult to detect MDA in urine samples, very significant results could not be obtained. When we analyzed OTA, creatinine, and MDA levels according to the study group, no significant differences were observed again ($p>0.05$).

While evaluating the amount of OTA in urine samples, we cannot say that this is only the dose formed by the inhalation of spores. Because it is known that people from whom we take urine samples can be exposed to OTA through nutrition. For this reason, when creating the working group, it was divided into two those working in the manufacturing department and non-manufacturing employees.

Those working in the non-manufacturing department were accepted as the control group. However, considering that both groups were exposed to OTA through nutrition, it was not observed that there were very high differences in the presence of OTA among those working in manufacturing.

OTA was found in the urine samples collected in our study. However, there was no evidence that the inhalation exposure was very significant. As we can see in the statistical results we obtained from the surveys, when the economic conditions of the bakery workers are considered, it is seen that their eating habits are mostly in favor of grain products. It is thought that future studies should include different parameters, be more comprehensive, and OTA exposure caused by different exposure routes should be measured by comparing it with nutrition and working in parallel. Thus, it will be ensured that more accurate estimations and objective results for the application will be obtained in risk analysis studies that can be carried out effectively.

During our study, it was observed that the working conditions of the bakery workers were not suitable for both general health conditions and exposure to OTA. It is recommended to empty the flour and use a mask, which is a very simple but very preventive measure during work.

CONCLUSION

During the storage and storage of cereals, which are of great importance in nutrition, depending on environmental conditions such as humidity and temperature, mold formation and the formation of OTA, which has an important place among them, is of great importance for health. The most important cause of human exposure to mycotoxins is the consumption of mycotoxin-containing foods. This study is a public health study. The degree of exposure of bakery workers due to their occupation was determined. The ochratoxin A analysis, which is an important biomarker in monitoring the exposure of bakery workers to a toxic substance, constituted the study.

Conflict of interests

The authors declare that there is no conflict of interest in the study.

Financial disclosure

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Ethical approval

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Research Ethics Committee of Marmara University (B.30.2.MAR.0.01.02/AEK/20, approval date 03/03/2011).

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