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Correlation of Ochratoxin A Exposure to Urinary Levels of 8-Hydroxydeoxyguanosine and Malondialdehyde in a Turkish Population

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Abstract Ochratoxin A is one of the most abundant food-contaminating mycotoxins in the world that is immunosuppressive, genotoxic, teratogenic and carcinogenic. Malondialdehyde is a naturally occurring product of lipid peroxidation that is mutagenic and carcinogenic. 8-Hydroxydeoxyguanosine is produced during the interaction of reactive oxygen species and DNA. In this study, Ochratoxin A, malondialdehyde and 8-Hydroxydeoxyguanosine levels of individuals in the study group were measured and results were correlated with each other. Additionally, the correlation of biomarker levels to smoking habit, alcohol and coffee consumption, age and gender of individuals was investigated. As a result of these assessments, a significant correlation was observed between Ochratoxin A exposures and malondialdehyde and 8-Hydroxydeoxyguanosine levels.

Keywords Ochratoxin A · Urine · Malondialdehyde · 8-Hydroxydeoxyguanosine

Oxidative stress is a general term used to describe the steady state level of oxidative damage in a cell, tissue, or organ, caused by the reactive oxygen species**.** Oxidative damage is associated with an imbalance between reactive oxygen species generation and antioxidant defenses.

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Ochratoxin A, 1 ((R)-N-[(5chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl)-carbonyl]-L-phenylalanine) is a naturally occurring mycotoxin produced by several species of *Aspergillus* and *Penicillium* fungi (Van der Merwe et al. 1965; EFSA 2004; Cavin et al. 2006). Ochratoxin A can be found in several food products such as cereals and cereal products, pulses, coffee, beer, grape juice, dry vine fruits and wine, cacao products and nuts. Ochratoxin A is immunosuppressive, genotoxic, teratogenic and carcinogenic. Humans are exposed to Ochratoxin A primarily through ingestion of contaminated food. Scientists hypothesize that Ochratoxin A may cause Balkan Endemic Nephropathy (BEN) seen in Southeastern European countries, fetal kidney diseases, tumors of the excretory vessels (Maaroufi et al. 1995), and the nephropathies seen in Egypt and Tunisia, the etiology of which is unidentifiable (WHO 1991). The International Agency for Research on Cancer (IARC) evaluated Ochratoxin A in 1993, and classified it as possibly carcinogenic to humans (group 2B), based on sufficient evidence for carcinogenicity in animal studies and inadequate evidence in humans (IARC 1993). Ochratoxin A inhibits protein synthesis (Creppy et al. 1984), disturbs mitochondrial respiration (Aleo et al. 1991) and causes increased lipid peroxidation (Omar et al. 1990). These adverse cellular events may represent important precursors to the development of more serious kidney disease.

Hoehler et al. (1996) reported that lipid peroxides are formed in vivo by Ochratoxin A, but the effects may vary considerably from species to species, and may also be influenced by other factors such as diet and age. Ochratoxin A enhances lipid peroxidation when added to rat liver or kidney microsomes, in vitro, or when administered, in vivo, to rats.

In contrast, Zlender et al. (2009) observed in a study with rats that the biological indicators of oxidative stress

(i.e., malondialdehyde and 8-hydroxydeoxyguanosine) remained unchanged following the administration of low doses of Ochratoxin A, even though structural indicators of oxidative stress (i.e., deranged microtubules) were observed. The degree that lipid peroxidation contributes to the overall toxicity of Ochratoxin A as well as to the reduction of physiological parameters is not known. Malondialdehyde is a naturally occurring product of lipid peroxidation and prostaglandin biosynthesis that is mutagenic and carcinogenic. Omar et al. (1990) reported that Ochratoxin A induces oxidative damages by enhancing lipid peroxidation.

Malondialdehyde formation was stimulated markedly by Ochratoxin A. Some researchers have reported that increased lesions caused by Ochratoxin A -induced lipid peroxidation could also be related with oxidative damage (Baudrimont et al. 1997). Studies have confirmed that Ochratoxin A effects malondialdehyde concentrations in both rat kidneys and liver. These studies suggest that some biomarkers of oxidative stress such as malondialdehyde can be affected even with very low doses of nephrotoxic mycotoxins that humans are exposed to (Domijan et al. 2007).

8-hydroxydeoxyguanosine is an oxidatively modified guanosine, which has been widely used as an oxidative DNA damage marker in various diseases (Boonla et al. 2007). Age-associated increases of 8-Hydroxydeoxyguanosine have been shown in human heart mitochondrial DNA and human leukocytes. In addition, increased 8-Hydroxydeoxyguanosine levels in age-related diseases, such as diabetes mellitus and hypertension, have also been reported (Kouda et al. 2001). Cigarette smoking has been reported to be associated with an increase of urinary 8-Hydroxydeoxyguanosine (Hakim et al. 2003). In this study, we determined the nature of the correlations between Ochratoxin A, malondialdehyde and 8-Hydroxydeoxyguanosine levels of individuals in the study group with each other and investigated possible relationships between Ochratoxin A, malondialdehyde and 8-Hydroxydeoxyguanosine levels with various demographic and lifestyle factors.

Materials and Methods

A total of 180 samples of human urine were collected from healthy individuals from different cities of Turkey. All volunteers were asked to fill out a questionnaire to collect data about their sex, age, coffee consumption, alcohol consumption and smoking habits. From 180 participants involved in the study 43.3% (n = 78) were female and 66.6% (n = 102) were male. As for the age distribution of volunteers, the percentage of ≤35 was 51.6 and >35 is 48.4. The volunteers were given clean 100 mL non- sterile plastic vessels with screw cap and were requested to fill vessels up with the first morning urine of the day on which they participated in the survey. The filled plastic vessels were returned to the laboratory on ice in an ice chest, and kept at −20°C in a deep-freeze until the analysis for creatinine, Ochratoxin A, malondialdehyde and 8-Hydroxydeoxyguanosine.

Due to different excretion rates, spot urine concentration may vary, but correction by expression in ng g^{-1} creatinine allowed us to study spot urine instead of 24-h samples. A SIGMA 3K30 refrigerated centrifuge (SIGMA Laborzentrifugen GmbH, Osterode am Harz, Germany) was used for centrifugation of urine samples for analysis of creatinine and Ochratoxin A. Creatinine concentrations were determined by a spectrophotometric method based on the reaction of creatinine with picric acid in an alkaline medium. The absorbance of the yellow–red color produced is measured at 520 nm wavelength.

The presence and levels of Ochratoxin A in urine samples collected from the volunteers involved in this study were analyzed by a method using $NaHCO_3$ dilution, IAC clean-up and HPLC with a fluorescence detector (Akdemir et al. 2010). The results were calculated using creatinine adjustment for each urine sample. The limit of detection (LOD) and limit of quantification (LOQ) of the method were measured as 0.006 and 0.018 ng/ml^{-1} respectively.

An Agilent 1,100 HPLC system (Germany) and an Agilent 1,200 series fluorescence detector (FLD) (G1321A), software (Chem Station Rev A 10.2 (1757) Copyright® Agilent Technologies 1990–2003) was used. The column used was a ZORBAX Eclipse XDB C18, 250 × 4.6 mm, 5 μm particles (Agilent Technologies®, Germany).

Malondialdehyde and 8-Hydroxydeoxyguanosine levels in human urine were measured by spectroscopic method using "NWLSS™ Malondialdehyde Assay" test kits (American Northwest Life Science Specialist, LLC) and ELISA technique utilizing from "Highly Sensitive 8-Hydroxydeoxyguanosine Check" test kits (Japon JalCA Company) respectively. Measurements were implemented at 532 nm wave length and the results were calculated using creatinine adjustment for each urine sample.

The materials used in this study were of high quality. The Ochratoxin A standard (CAS No. 303-47-9) used was of Supelco brand (50 μg mL^{-1} in benzene: acetic acid (99:1), pkg of 1 mL). The immunoaffinity columns (IAC) containing specific antibodies against Ochratoxin A and LP (OchraTest™WB) (VICAM Science Technology (Watertown, MA, USA)) were used for getting the data more accurate and sensitive. Acetonitrile and methanol were High-Performance Liquid Chromatography (HPLC) grade (Merck, Germany). Glacial acetic acid and 5% $NaHCO_3$ (Merck, Germany) in distilled water were of analytical

grade. Water was purified by distillation and passage through a milli Q system (Millipore, Bedford, MA).

For statistical analyses, database management and statistical analysis was performed with SPSS Microsoft version 13.0 software (SPSS, Chicago, IL, USA). Ochratoxin A, malondialdehyde and 8-Hydroxydeoxyguanosine results gained from the analysis were assessed with regard to age, gender, smoking habit and coffee consumption by student t-test. Pearson correlation test was used to reveal whether there is a correlation between Ochratoxin A, malondialdehyde and 8-Hydroxydeoxyguanosine levels. All the data of this study were normally distributed. A p-value < 0.05 was accepted as statistically significant.

Results and Discussion

Demographic data including age, sex and lifestyle factors such as smoking and coffee consumption and subjects' urinary Ochratoxin A, 8-Hydroxydeoxyguanosine and malondialdehyde levels are presented in Table 1. We did not find any relationship between Ochratoxin A levels or 8-Hydroxydeoxyguanosine levels and demographic and lifestyle factors. However, there was a statistically significant enhancement in malondialdehyde levels in the subjects older than 35 years compared with those younger than 35 years ($p < 0.05$), and malondialdehyde levels in women were significantly higher than those of men ($p < 0.05$).

The urinary Ochratoxin A exposures and 8-Hydroxydeoxyguanosine and malondialdehyde levels of the subjects are shown in Fig. 1. Results show that a significant correlation between Ochratoxin A exposures and 8-Hydroxydeoxyguanosine and malondialdehyde levels ($p < 0.05$ and $p < 0.001$ respectively) (Fig. 1). A significant correlation was found also between 8-Hydroxydeoxyguanosine and malondialdehyde levels ($p < 0.001$).

Malondialdehyde has been used for many years as a convenient biomarker for lipid peroxidation because of its facile reaction with thiobarbituric acid to form an intensely colored chromogen (Janero 1990). Malondialdehyde, the major product of lipid peroxidation can react with DNA macromolecule to form adducts to deoxyguanosine, deoxyadenosine and deoxycytidine and it is first shown to be carcinogenic in 1972 following topical administration to mice (Shamberger et al. 1974). Mukai and Goldstein reported the mutagenic activity toward *Salmonella typhimurium* in 1976 (Mukai and Goldstein 1976).

It is generally accepted that the level of 8-Hydroxydeoxyguanosine (modified base/nucleoside) in urine may be a good indicator of oxidative DNA insult and a general index of the level of the oxidative stress in the organism. Therefore, measurement of 8-Hydroxydeoxyguanosine excreted via urine is important to determine an integrated measure of total oxidative stress, DNA damage and the repair activity of DNA in the organism (Wu et al. 2004). Many studies have shown that various demographic,

Table 1 Demographic information and urinary Ochratoxin A, 8-Hydroxydeoxyguanosine and malondialdehyde levels of the subjects (mean ± S.D.)

	N = 180 (%)	Ochratoxin A (ng/g creatinine)	8-Hydroxy deoxyguanosine (ng/mg creatinine)	Malondialdehyde (nmol/mg creatinine)
Total	180 (100)	9.68 ± 4.21	2.91 ± 1.05[b]	5.80 ± 3.67[a,c]
Age (year)				
≤ 35	93 (51.6)	9.33 ± 6.62	2.87 ± 1.79	7.37 ± 4.27[d]
> 35	86 (48.4)	10.05 ± 5.55	2.94 ± 1.09	10.33 ± 5.39
Sex				
Men	102 (56.7)	8.85 ± 4.55	2.83 ± 1.62	7.46 ± 3.39[d]
Women	78 (43.3)	10.76 ± 4.08	3.01 ± 2.33	10.56 ± 4.71
Smoking				
Non-smokers	109 (60.6)	10.05 ± 6.35	2.90 ± 1.12	9.68 ± 4.71
Smokers	71 (39.4)	9.10 ± 4.15	2.91 ± 2.00	7.45 ± 3.51
Coffee				
Non-consumer	72 (40)	9.90 ± 6.61	2.55 ± 1.12	8.12 ± 3.22
Consumer	108 (60)	9.53 ± 4.63	3.15 ± 1.52	9.26 ± 3.28

[a] $p < 0.001$ correlation between malondialdehyde levels and Ochratoxin A levels

[b] $p < 0.05$ correlation between 8-Hydroxydeoxyguanosine levels and Ochratoxin A levels

[c] $p < 0.001$ correlation between malondialdehyde levels and 8-Hydroxydeoxyguanosine levels

[d] $p < 0.05$ between malondialdehyde levels of two different age groups and of two different sex groups

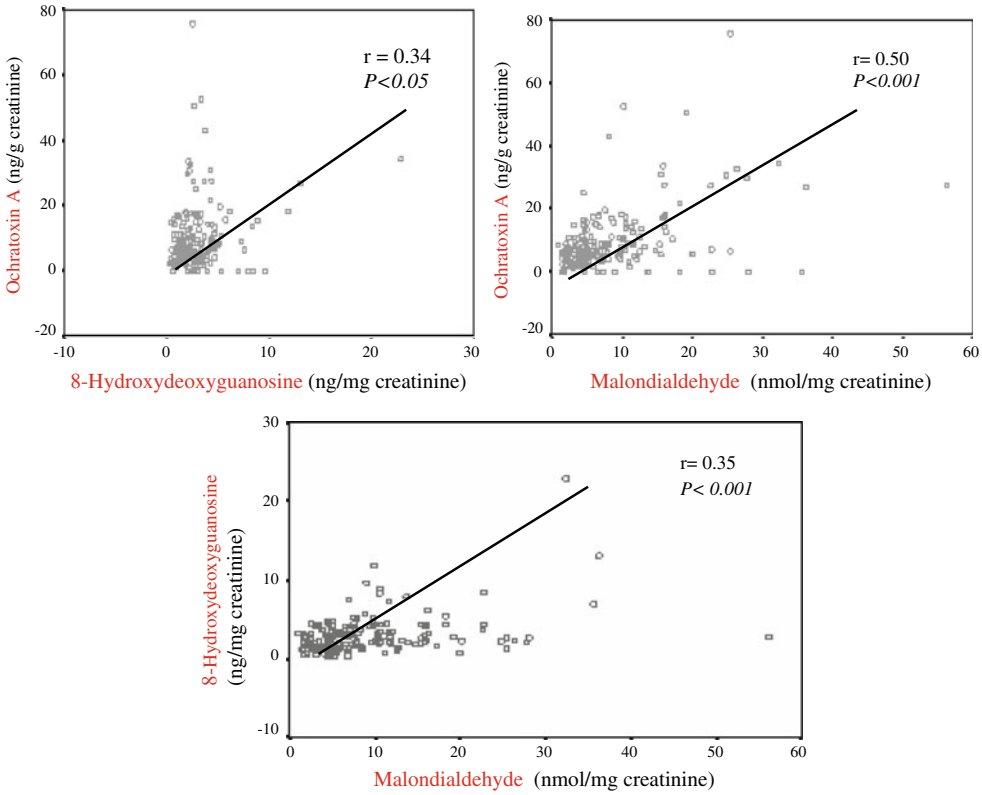

Fig. 1 Correlations between urinary levels of Ochratoxin A, 8-Hydroxydeoxyguanosine and malondialdehyde levels

occupational and lifestyle factors such as age, sex, smoking, alcohol consumption and the use of dietary supplements are associated with the urinary excretion of 8-Hydroxydeoxyguanosine (Wong et al. 2003; Irie et al. 2005; Kimura et al. 2005). In agreement, we also found statistical significance between the levels of Ochratoxin A and 8-Hydroxydeoxyguanosine. Irie et al. (2005) found a significant relationship between age and 8-Hydroxydeoxyguanosine levels, but others did not (Kimura et al. 2005; Boonla et al. 2007). In our study, we only found a significant relationship between age and malondialdehyde levels ($p < 0.01$). Irie et al. (2005)and Kimura et al. (2005)found a significant relationship between 8-Hydroxydeoxyguanosine levels and sex. In our study, we found a significant relationship between malondialdehyde levels and sex ($p < 0.01$). But contrary to these findings Boonla et al. (2007) have not found any relationship between sex and both 8-Hydroxydeoxyguanosine and malondialdehyde levels. Irie et al. (2005) have found a significant relationship between smoking and 8-Hydroxydeoxyguanosine levels, but on the other side both Kimura et al. (2005) and we have not found any relationship. We also have not found any relationship between coffee consumption and both 8-Hydroxydeoxyguanosine and malondialdehyde levels. Additionally to these data, we found a significant enhancement between Ochratoxin A levels and 8-Hydroxydeoxyguanosine levels ($p < 0.01$).

To our knowledge, there are not many studies evaluating Ochratoxin A exposures in the population. Furthermore, we could not find a study investigating malondialdehyde and 8-Hydroxydeoxyguanosine levels to reveal the Ochratoxin A's enhancing effect on lipid peroxidation. It is so interesting that whether our subject's Ochratoxin A levels are under the limits, we found a correlation between Ochratoxin A levels and malondialdehyde and 8-Hydroxydeoxyguanosine levels but due to the limited size of the study groups, further extensive studies involving a large number of subjects are needed. However, there are several analytical methodologies used for quantifying Ochratoxin A in food products, new methods are highly recommended in order to evaluate the food safety. Also, because the incidence and level of Ochratoxin A contamination may vary greatly from year to year, continual monitoring of Ochratoxin A is important. It will be valuable to use average contamination levels obtained from multiyear surveillance studies to better characterize the exposure levels of humans. Further animal and human health studies will also help in developing safe exposure guidelines.

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