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OCHRATOXIN A IN HUMAN DIET AND URINE AND ITS REDUCTION BY GARLIC AND ORANGE JUICE

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ABSTRACT

Assessment of human exposure to ochratoxin A (OTA) was achieved by analysis of some foodstuff, food consumption from a series of individuals using the duplicated diet method and urinary OTA concentration. Samples of foodstuffs including cereals, legumes, dried fruits, dairy products and meats were collected from Egyptian countryside, samples of duplicate diets and urine samples were collected from 25 volunteers living in one area of the Egyptian village during one month on a weekly basis. The method uses immunoaffinity column clean-up and reversed phase high performance liquid chromatography (HPLC) with fluorescence detector for quantification of the toxin. It was found that 33.57% of foodstuff samples (a total 140 samples) contained OTA in variable quantities. All diet and urine samples contained OTA. The exposure calculated from OTA levels in the diets were below the tolerable daily intake recommended by JECFA at 1.2-14 ng /kg bw/day ; the average daily intake ranged from 1.07 to 8.43 ng/kg bw/day with a mean value 4.49±1.95 . OTA urine levels ranged from 17-61ng/L with a mean value 31.40±11.15. This suggests that the population is exposed to OTA at high frequency, since the toxin is frequently found, albeit at low levels, in a number of commonly consumed foods. High significant correlation were found between OTA consumption and urinary OTA level suggesting that urine provide a good biomarker of intake. The effect of garlic and ascorbic acid (fresh orange juice) on reducing OTA levels in human was also investigated. The results indicate that garlic and orange juice effectively reduced the levels of OTA in urine.

Keywords: Ochratoxin A; Human exposure, Food contaminants, Antioxidants

INTRODUCTION

Ochratoxon A (OTA) is a secondary metabolite of several fungal species belonging to the genera Aspergillus (e.g. A.alutaceus, formerly known as A.ochracens) and Penicillium (e.g. P. verrucosum) (Styn,1984; Ueno *et al.*, 19981). It is a potent nephrotoxic, carcinogenic, tetratogenic and immunosuppressive (Skaug, 2003). It has been suggested that severe dietary exposure to this mycotoxin in parts of Bulgaria, Romania and Yugoslavia is associated with chronic, progressive kidney disease (Balkan Endemic nephropathy) and its associated urinary tract tumors (Krogh, 1974; Vrabcheva *et al.*, 2004). A very high prevalence of nephropathy has been reported in Tunisia (Bacha *et al.*, 19936), Algeria (Khalef *et al.*, 1995); (North Africa) and in Senegal (west Africa), chronic failure associated from 87.61 of all death from urogenital disease (Kane *et al.*, 1991). The main rout of human exposure to OTA is through dietary intake of contaminated foods,e.g. cereal, bread, pork, poultry meat, coffee, beer, wine, red grape-juice and cow's milk (Speijers and van Egmond, 1993; Breitholtz-Emanuelsson *et al.*, 1993; Zimmerli and Dick, 1996; Jorgenssen *et al.*, 1996). Cereals normally account for 50-80% of average consumer intake OTA (Rizzo *et al.*, 2002). Since it is

for 50-80% of average consumer intake OTA (Rizzo *et al.*, 2002). Since it is stable to heat and other physical food processing, it can also occur in food products made from these ingredients (Subirade, 1996; BCERF, 2004). In addition to diet , exposure to OTA can possibly occur by inhalation of toxin containing conidia airborne dust (Skaug *et al.*, 2001).

The presence of OTA in human plasma and its transmission to the mother' milk has been reported in several countries (Gareis *et al.*, 1988; Frohlich *et al.*, 1991; Thuvander *et al.*, 2001). However , human exposure to OTA is not uniform but varies significantly between individuals and geographic regions. It was shown that the OTA levels were higher in human milk samples from women living in region with extensive agriculture, than in area with little or no agriculture (Skaug *et al.*, 1998). Moreover, a study from France reported higher levels of OTA in human blood samples from rural areas than from urban areas (Creppy *et al.*, 1991). Heterogeneous OTA exposure can be due to individual and regional dietary differences. In Bulgaria, the exposure of population to OTA was supported by a very high prevalence of OTA levels exceeding 2ng/ml in the blood of affected population (Petkova-Bocharova *et al.*, 1988; Petkova *et al.*, 1991). OTA has also been found more often in the urine of people living in BEN-endemic villages than in those in non-endemic village, and the highest levels were seen in patients with BEN or Urinary tract tumors (Castegnaro *et al.*, 1991). Gilbert *et al.*, 2001 and Petkova-Bocharova *et al.*, 2003 reported that measurement of OTA in urine is a good marker of exposure and it will be less invasive than blood for follow up of population exposure.

The occurrence of OTA is widespread across both temperate and tropical region but it less recognized in Africa and very little data is currently available so more research is needed to accurately assess the extent of OTA contamination in foodstuffs and human exposure (BCERF, 2004). Given the paucity of information about dietary exposure to OTA in Egypt, this study aimed to investigate the levels of this toxin in foodstuffs, diet and urine from healthy volunteers selected in the age 17-25 years selected from Egyptian village. The effect of daily garlic intake and fresh orange Juice on reducing OTA levels was also investigated.

MATERIAL AND METHODS

1.Material

1.1.Food samples

A total of 140 samples (10 each) including cereals (corn, wheat, rice and macaroni); legumes (Faba bean, beans and lentils); dried fruits (apricot, dates and fig); dairy products (milk and cheese) and meats (cow's meat and chicken) were collected from Egyptian countryside to determine ochratoxin A (OTA) residues.

1.2 Duplicate diet study

The method used based on a previously published method described by Gilbert *et al.*, 2001 Twenty five healthy 17-25 years old volunteers were selected from Egyptian countryside. All subjects had clinically normal blood biochemistry, hematology and urine analysis and exhibited negative results to

hepatitis B surface antibodies. Non of them displayed renal disability; volunteers a history of hepatic, renal or disorder or cardiovascular or gastrointestinal diseases were excluded. Also excluded were subjects who undergone any surgical operation on the digestive tract other than an appendectomy. Participants declared that they would consume their normal diet for the duration of the study (one month). Participants were asked to prepare duplicates of the normal amount of food that they ate and drank and to store them in a refrigerator until collection; samples were prepared on weekly basis, i.e four samples per subject. The constitutes and weights of each weekly diet (food and drink) were recorded. The four samples were blended together using an overhead homogeniser before a sub-samples was taken for analysis.

1.3 Urine collection

A-Urine samples were collected during twenty- four hours; aliquot (50ml) of these samples were taken from each subject on days 0, 1, 6, 13, 20 and 27 of the same month and stored at –80 C until OTA analysis as described by Petkova-Bocharova *et al.*, 2003.

B-Urine collection after some treatments

Ten participants were chosen to investigate the effect of garlic and ascorbic acid (orange juice) on reducing OTA levels in human urine. Five of the subjects consumed daily morning for two weeks 20g garlic and other group taken a cub of fresh orange juice(approximately 250ml) . The urine sample were collected on the 14^{th} day and stored as described above.

1.4 Chemicals

All reagent and solvents for the extraction were obtained from Sigma-Aldrich and they were of analytical grade quality.

2 Ochratoxin A analysis

2.1 Extraction and chromatographic separation

2.1.1 Food samples

Fifty grams of samples were put into high speed blender, 25 ml phosphoric acid (0.1M) and 250ml chloroform were added and blended for 3 min. Near end of blending 10 g diatomaceous earth were blended, then filtered through filter paper whatman No. 4. Fifty ml of portion were collected and transferred to separatory funnel, 10ml sodium bicarbonate (3%) were added and shake gently. Then, the upper phase was collected for column separation (AOAC, 1995). The whole diluted extract was applied to an ochratest immunoaffinity column at a slow steady flow rate of 2-3ml/min. After washing the column with 10ml of distilled water, OTA was eluted with 4ml of methanol, then the elute was evaporated to dryness under a steam of nitrogen at 40 C (Pittet *et al.*, 1996).

2.1.2 Urine samples

The analytical method used based on a previously published method described by Zimmerli and Dick, (1995) A solution containing 33.7ml of 85% orthophosphoric acid and 118g (2 mol) sodium chloride per liter was prepared. A 10 ml volume of this solution was added to 10 ml urine and mixed for 1 minute using vortex mixer. After addition of 5ml of chloroform and intensively mixing for 2-3min., the mixture was centrifuged at 2500g for 15

min. The clear organic phase at the bottom of the tube was carefully withdrawn by Pasteur pipette and transferred to a pear-shaped flask (150ml). The extraction was repeated with another 5ml of chloroform and the combined extracts were evaporated to dryness at 30-40C using rotavapor. The extract re-dissolved in 5% acetonitrile in phosphate buffer saline and cleaned-up using an immunoaffinity column.

2.2 Determination of OTA by High Performance Liquid Chromatography (HPLC)

OTA was determined by using HPLC technique according to the method described by Studer-Rohr *et al.*, (30). HPLC analysis was carried out with liquid chromatography equipped with solvent delivery system (Shimadzu) and a reverse phase analytical column packed with C18 material (Spherisorb 5µm O D52, 15cm x 4.6nm). the mobile phase consisted of acetonitrile :water :acetic acid (99:99:2). The separation was performed at ambient temperature at a flow rate of 1.0ml/min, the injection volume was 20ul for both standard solutions and operated extracts. The fluorescence detector was operated at an excitation wave length of 460 nm.

RESULTS AND DISCUSSION

Ochratoxin A levels in Foodstuff

The results in Table 1 shows that of 140 food samples including plant based products and animal products OTA (33.57%) were detected in variable levels. The highest OTA levels were found in cereals and legumes followed by dried fruits, dairy products and meats. Cereals (corn, wheat, rice and macaroni) were contaminated with the toxin with concentration ranged from (18-421µg/kg) and legumes samples (Faba bean, beans and lentils) were contaminated with 15-276 OTA µg/kg. Residues of OTA have been detected in the samples of milk, cheese and meats with a range (20-50 ng/ml), (10-33µg/kg) and (20-56µg/kg) respectively. The OTA levels in dried fruits (apricot, fig and dates) ranged from 30-190 µg/kg. The occurrence of OTA in foodstuffs is influenced by environmental factors such as humidity and temperature. Thus OTA contamination of foodstuffs vary with geographical condition production and storage methods and also with the type of foods (Breitholtz-Emanuelson *et al.*, 1991; Handlock. 1993).

Around the world, OTA is found most frequently in stored beverages, particularly cereals and grains, also it found in dried fruits, wine coffee, beer, cocoa, juices, spices, pork, poultry and dairy products(BCERF, 2004).In Egypt, Zohri and Abdel-Gawad, 1993 reported that all the samples tested of dried fruits were contaminated by OTA and the concentration range between 50-110 µg/kg of apricots, 6-120 µg/kg of figs and 210-280µg/kg of prunes. Also Abdel-Satar and Saber, 1999 detected OTA in two samples of dates in Assiut, Egypt with level 36-450µg/kg. Ninety samples of foods and agricultural commodities collected from Egyptian market revealed that OTA were detected in 21.1% of the samples with variable quantities (Aziz and Youssef, 2002).

Table (1): Natural occurrence of OTA in some Egyptian plant and animal products

Commodity	No. of positive samples	OTA Range (µg/kg)
(10 samples each)		
I) Plant - base products		
1- Cereals		
Corn	4	40-421
Wheat	3	23-295
Rice	2	26-310
Macaroni	2	18-185
2-Legumes		
Faba bean	3	15-198
Bean	3	17-213
Lentils	4	20-276
3- Dried Fruits		
Figs	6	50-140
Apricot	5	30-100
Dates	5	60-190
II) Animal products		
1-Dairy products		
Milk	3	20-50
Cheese	2	13-70
2-Meats		
Cow's meat	2	14-33
Poultry meat	3	20-56
Total of positive samples	47	
Percentage	33.57	

Ochratoxin A levels in duplicate diet and human exposure

The results in table 2 revealed that OTA was detected in all the composite diet samples. The levels found ranged from 0.1 to 0.65 ng OTA/g diet, with a mean value, 0.32±0.12. The results also show that the amount of food consumed by the subjects per week varied considerably from 3889.2 to 10500 gm and the average amount of OTA ingested per week ranged from 696.15 to 4200 ng with a mean value 2178.53±946.15. It was noticed from the recorded ingredients used for the preparation of participants' meals (breakfast, lunch and dinner) consists mainly from carbohydrates including cereals and cereals products (bread, rice and macaroni), potatoes, legumes (Faba beans, lentils and beans) and cheese. From the description of the individual meals components and the results in Table 1, it is possible to trace the potential sources of contamination by OTA. Over the world OTA has been reported in a number of food components or cooked food; bread (Osborne *et al.*, 1996; Subirade, 1996); wheat (Vrabcheva *et al.*,2000); beans (Petkova-Bochrova *et al.*, 1991); spices (El-Kady *et al.*, 1995; Vrabcheva rt al 1999); potatoes (Abouzied *et al.*, 2002); poultry and dairy products (BCERF, 2004).

The calculated daily intake which corresponding to OTA consumed by individuals ranged from 1.07 to 8.43 ng/kg bw/day (mean 4.49±1.95) based on the actual body weights of each subject (Table 2). These values are

lower than that proposed by the World Health Organization/Food and Agricultural Organization Joint Expert Committee on Food Additives (JECFA) as the tolerable daily intake (TDI), (1.2- 14 ng/kg bw/day) (WHO, 2002). This means that the calculated value corresponding to 31% of the TDI; this exposure may be not seemed to represent a health hazard. The scientific committee for food (SCF, 1998) recommended that it would be prudent to reduce exposure toward the lower end of the TDIs of 1.2-14 ng/kg bw/day which have been estimated by other bodies e.g. below 5 ng/kg bw/day. The results in present study are higher than that reported in a similar study performed in the United Kingdom by Gilbert *et al.*, 2001. They reported that the daily intake ranged from 0.26 to 3.54 ng/kg bw/day with a mean value 0.94 ng/kg bw/day. While it lower than that obtained from healthy individual living in Balkan Endemic Nephropathy area using the same method (duplicate diet method).

Table (2) Levels of ochratoxin A in duplicate diets and urine of subjects

Subject	Average weight of diet (g) week	OTA level in diet ng/g	OTA consumed ng	Body weight kg	Daily Intake ng/kg bw/day	Average OTA urine Level ng/L
1	5191.90	0.21	1090.30	68.00	2.29	22.00
2	5320.00	0.38	2021.60	74.00	3.90	29.00
3	6395.20	0.15	959.28	70.00	1.96	20.00
4	7385.00	0.28	2067.80	77.00	3.84	28.00
5	7730.80	0.42	3246.94	55.00	8.43	58.00
6	9800.00	0.35	3430.00	73.00	6.71	39.00
7	7840.00	0.33	2587.20	69.00	5.36	33.00
8	10500.00	0.40	4200.00	76.00	7.89	61.00
9	9465.00	0.19	1798.35	76.00	3.38	25.00
10	9163.00	0.30	2748.90	75.00	5.24	31.00
11	7350.00	0.41	3013.50	71.00	6.06	36.00
12	6339.90	0.36	2282.36	70.00	4.66	26.00
13	4165.00	0.65	2707.25	58.00	6.67	43.00
14	5110.00	0.53	2708.30	64.00	6.05	42.00
15	6440.00	0.38	2447.20	66.00	5.30	35.00
16	5915.00	0.10	591.50	79.00	1.07	17.00
17	9555.00	0.37	3535.35	78.00	6.48	38.00
18	6790.00	0.25	1697.50	70.00	3.46	27.00
19	6370.00	0.28	1783.60	69.00	3.69	24.00
20	3889.20	0.30	1166.76	63.00	2.65	23.00
21	7280.00	0.26	1892.80	75.00	3.61	29.00
22	4095.00	0.17	696.15	68.00	1.46	18.00
23	5443.20	0.22	1197.50	52.00	3.29	20.00
24	6160.00	0.26	1601.60	71.00	3.22	26.00
25	9065.00	0.33	2991.45	78.00	5.48	35.00
Mean	**6910.33**	**0.32**	**2178.53**	**69.80**	**4.49**	**31.40**
Std.	**1878.66**	**0.12**	**946.15**	**7.10**	**1.95**	**11.15**

The average weekly intake of OTA varies from 1.86 to 92.7 ng/kg bw/week, some of this levels approach the provisional tolerable weekly intake established by the JECFA at 100ng/kg bw (Vrabcheva *et al.*, 2004). It was reported that OTA exposure is affected by geographical location (Hadlock, 1993).

Ochratoxin A levels in participants urine

OTA was found in all urine samples examined. The OTA levels ranged from 17-61 ng/L with a mean value 31.4±11.15. There are few studies about the level of OTA in urine. Gilbert *et al.*, 2001 found 10-58 ng OTA/L in urinary healthy individuals from UK. These results are very similar to those found in this study. Higher levels have been detected for healthy individuals in Bulgaria, the OTA levels in the urine ranged from 10-1910 ng/l. In Africa urinary OTA levels in urine samples from boys and girls in Sierra Leone (this country has a high morbidity and infant mortability rates; UNICEF, 1989) ranged from 70-59000 ng/L and 80-148000 ng/L respectively in dry seasons; one girl had OTA 148000ng/L. In rainy season the OTA ranged from 600 to 72200 ng/L and 70 to 4900 ng/L for boys and girls respectively (Jonsyn- Ellis, 2000).

The above results suggests that the population is exposed to OTA at high frequency, since the toxin is frequently found, albeit at low levels, in a number of commonly consumed foods. The statistical analysis show that there was high significant correlation ($P<0.05$) between OTA consumption and urinary OTA level suggesting that urine provide a good biomarker of intake as hypothesized by Gilbert *et al.*, (2001) and Petkova-Bcharova *et al.*, (2003)

Effect of garlic and fresh orange Juice on OTA level in human

The results in Fig 1 indicate that garlic and fresh orange Juice effectively reduced the levels of OTA in urine. After two weeks of consumption 20 gm garlic and a cup of orange daily morning the reducing percentage of OTA in participants urine were 100% and 87.78% respectively. Antioxidants such as vitamin A, C and E have been shown to reduce the toxic effects of OTA in animals (Bose and Sina 1994; Atroshi *et al.*, 2000).

CONCLUSIONS

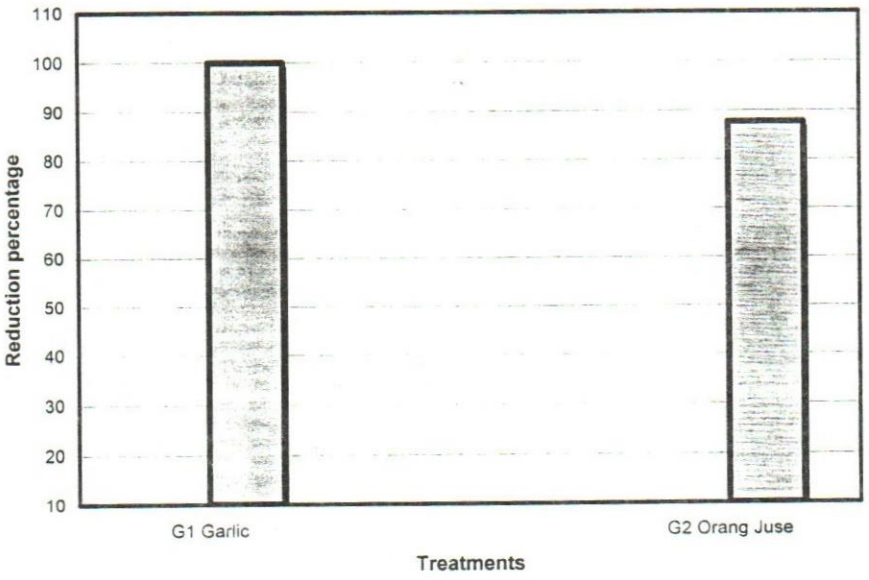
1- The results in this study and data reported from other countries consistently indicate that humans are continuously exposed to OTA, and there is no doubt that this compound is toxic; it has been shown tetragenic and immunotoxic properties; a nephrotoxic and genotoxicity. In view of these characteristics, regular control should be enforced and exposure to OTA should be kept to a minimum, avoiding the consumption of heavily contaminated foods.

2- High significant correlation were found between OTA consumption and urinary OTA level suggesting that urine provide a good biomarker of exposure.

3- Consuming garlic and fresh orange Juice appears to be effectively reduce the toxicity of OTA in human.

4- More research is needed to accurately assess the extent of OTA contamination in food and human exposure in many of the world, particularly Africa and Asia.

Fig 1 Effect of some treatments on OTA levels in urine



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مستويات الأوكراتوكسين أ في وجبات وبول الإنسان ومحاولة خفضها باستخدام عصير البرتقال والثوم.

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المركز القومي للبحوث – شعبة الصناعات الغذائية والتغذية – قسم علوم الألبان

الأوكراتوكسين أ هي من نواتج التمثيل الثانوية لنمو العديد من الفطريات التي تنتمي إلي جنس *Aspergillus* (علي سبيل المثال *A alutaceus.* والمعروف بإسم *A ochrations.* وجنس ال Penicillum (علي سبيل المثال *P. verrucosum*) ذو تأثير سام علي الكلي وتأثير مسرطن وكذلك تأثير نقص المناعة ولوحظ أن التعرض الحاد عن طريق استهلاك وجبات ملوثة بهذا النوع من السموم الفطرية في أجزاء من بلغاريا ورومانيا ويوغسلافيا مرتبط بأمراض الكلي المزمنة وكذلك بأورام المسالك البولية.

ولقد هدفت هذه الدراسة إلي التعرف علي مدي تواجد سم الأوكراتوكسين في الأغذية المصرية ومدي التعرض له باستهلاك تلك الأغذية وتم ذلك بتقدير هذا السم في بعض الأغذية وكذلك في وجبات مجموعة من الأفراد وذلك باستخدام طريقة (duplicate diet method) وكذلك تقدير تركيزه في البول المجمع من هؤلاء الأفراد . ولقد شملت عينات الأغذية تحت الدراسة الحبوب والبقوليات والفواكه المجففة واللحوم والتي تم تجميعها من إحدى قري الريف المصري وعينات من الوجبات والبول لخمسة وعشرون متطوع أصحاء ويتراوح أعمارهم بين ١٧-٢٥ سنة لمدة شهر يقطنون نفس المكان المأخوذ منه العينات وتم تقدير التوكسين باستخدام جهاز HPLC . ولقد أظهرت النتائج أن ٣٣,٥٧% من عينات الأغذية (مجموع ١٤٠ عينة) احتوت علي تركيزات مختلفة من الأوكراتوكسين تراوحت بين ١٣- ٣١٠ ميكروجرام/ كجم . وكذلك احتوت جميع الوجبات المختبرة وعينات البول علي التوكسين .وكان المتناول من التوكسين في الوجبات أقل من المدى المسموح به حيث تراوح بين ١,٨٧-٨,٤٣ نانوجرام / كجم /وزن جسم الإنسان /يوم بمتوسط بلغ ٤,٤٩ ± ١,٩٥ وتراوح مستوي التوكسين في البول من١٧ إلي ٦١ نانوجرام/لتر بمتوسط ٣١,٤± ١١,١٥ . لذا يمكن القول بأن العينة تحت الدراسة تعرضت بصفة مستمرة علي مدار شهر للتلوث بسم الأوكراتوكسين أ وبالتالي تعرضت للإصابة بأمراض الكلي ونقص المناعة والسرطان وفي ضوء ذلك يجب بذل الجهود حتى يكون التعرض للتوكسين أقل ما يمكن وفي أضيق الحدود الممكنة