Vol. 14(5) pp. 128-133, June 2020
DOI: 10.5897/AJFS2020.1935
Article Number: C2E78D064015
ISSN: 1996-0794

http://www.academicjournals.org/AJFS

ACADEMIC JOURNALS
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African Journal of Food Science

Full Length Research Paper

Health risk assessment of Brazil nut consumption by aflatoxin biomarker in urine samples

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Received 23 March, 2020; Accepted 21 May, 2020

Aflatoxins are the main mycotoxin related to the contamination of Brazil nut and an important product extracted from the Amazon region that has a high number of consumers due to its health benefits. Considering the frequent occurrence of aflatoxins in Brazil nuts, it is necessary to study the levels of exposure to these toxins associated with its consumption using biomarkers. To do this, a study was carried out with 30 volunteers of both sexes, where each volunteer received a kit containing Brazil nuts for the ingestion of 2 (two) units / day for 30 days. Urine samples were collected from each volunteer at 0 and 30 days, and the level of Aflatoxin M1 (AFM1) was determined by ELISA using the Helica Kit®. A questionnaire was used to obtain information on weight, height, age, medication use, pathological conditions, frequency of weekly consumption of certain foods more susceptible to aflatoxin contamination, and 24-h food recall. Of the 30 samples analyzed at time 0 (zero) for exposure, AFM1 presented positive results in 3 (10%) samples with a range of 2.75-70.41 ng/mL. In the analysis of the 30 days after Brazil nut consumption, the presence of AFM1 in the urine was not detected, suggesting that Brazil nut consumption did not generate impact on the urinary levels and exposure to AFM1. In conclusion, no association was found between food consumption of food susceptible to aflatoxin contamination and concentration of AFM1 in urine.

Key words: Aflatoxin, nuts, ELISA, selenium.

INTRODUCTION

Aflatoxins (AFL) belong to the group of mycotoxins and are secondary metabolites of some fungi, carcinogenic to humans (IARC, 1993) and frequently associated with Brazil nuts. They have been reported in several stages of the production chain of the native Brazil nut of the Amazon Forest (Baquião et al., 2012; Reis et al., 2012). This finding was observed in Brazil nuts shelled or inshell, for domestic and export markets, as well as for products derived from them (Moreira et al., 2016; Vargas et al., 2011; Pacheco and Scussel, 2009). However, in

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addition to AFL association with Brazil nut, it is important to evaluate the health risk related to frequent consumption due to the recent epidemiological evidence for the role of AFL in primary liver cancer, child growth impairment and immune suppression. In this context, risk analysis is a tool to evaluate the level of exposure to a contaminant (Jardim and Caldas, 2009) and in this field, the risk can be quantified as a function of the toxicity and exposure to which the consumer is exposed (IOM, 2001). Based on the risk analysis, it is possible to evaluate if the diet of a group of consumers is being affected by the presence of a chemical contaminant, such as the AFL, so it is possible to propose preventive measures to public health, either through the frequency/ingestion of food, or by improvements in the production chain. The risk from the exposure to AFL is also characterized by calculating the margin of exposure (MOE), which is defined as the ratio between the total intake and a toxicological reference, usually the lower bound of a benchmark dose level that caused 10% cancer incidence in rodents (EFSA, 2005). Andrade et al. (2013) evaluated the potential carcinogenic risks for the Brazilian population and observed in the Federal District, that total intakes for the total population and high consumers were 0.06-0.08 and 33.3-47.1 ng/kg bw/day, respectively, with Brazil nuts contributing the most to the intake for high consumers (54% for the upper bound level). Epidemiological studies have demonstrated a strong association between exposure to AFB1 and increased incidence of hepatocellular carcinoma and, another way of studying the exposure of a population is the quantification of AFL metabolites through biomarkers in blood and urine (Bando et al., 2007). After oral ingestion, aflatoxin B1 (AFB1) is absorbed and biotransformed before urinary and fecal excretion in the form of aflatoxin M1 (AFM1). Biomarkers can indicate biological processes and provide information to diagnosis of individuals who may be at risk. The aflatoxin-lysine adduct in the serum has shown correlation to the AFB1 intake, so this adduct has been used as biomarkers to quantitate exposure to aflatoxins (Benkerroum, 2019). In the United States, 1% of the population had a detectable result for aflatoxin B-lysine in a blood sample (Schleicher et al., 2013). In Malaysia, in urine samples, Redzwan et al. (2012) found a significant and positive association between milk and dairy products consumption and urinary AFM1 level. Considering that diet and its factors are constantly associated with the occurrence of cancer, and some Brazilian regions still do not have studies involving risk assessment by biomarkers, there was the need to evaluate populations of Brazil nut production areas due to data scarcity. In the northern region of Brazil, the consumption of nuts occurs also in culinary preparations and some consumers are being considered high consumers. Therefore, the objective of the study was to study a population group and the consumption of Brazil nuts for 30 (thirty) days along with the occurrence of AFM1 adducts in urine biomarkers from the volunteers.

MATERIALS AND METHODS

Standards, reagents and chemicals

For AFL M1 assay, the ELISA kits were purchased from Helica Biosystem (Santa Ana, CA, USA). The Brazil nuts supplied to the volunteer's consumption per 30 (thirty) days were purchased in the retail market of the city of Manaus-AM-Brazil from different retail lots and previously evaluated to AFL. The samples were not detected for Total AFL in the limit of detection of 0.3 µg/kg. In addition, the Brazil nut samples were evaluated for Selenium and the average was 5 µg/g.

Urine sampling and food consumption

To evaluate the AFL M1 biomarker, urine samples were collected from volunteers, from the Faculty of Pharmaceutical Sciences of the Federal University of Amazonas, Manaus- Amazonas, Brazil. The project was approved by the Research Ethics Committee under the number: 909.195 and 60 samples were collected from students and professionals consisting of men and women. The samples were collected twice in time 0 days (*N*=30) and time 30 days (*N*=30). A total of thirty (30) volunteers participated, corresponding to 50% of the total individuals available that month in the Faculty. All volunteers were informed about the study and a consent form was given for signature prior to inclusion in this study. All the participants appeared to be in good physical health and were asked to complete a questionnaire with information about weight, height, age, medication use, pathological conditions. Urine samples from each volunteer were collected during the morning in disposable urine vials. The volunteers were given a kit containing sixty (60) Brazil nuts, for ingestion of 2 units/day (20 g- extra-large size nuts). We considered the average of the amounts described by Thomson et al. (2008) of 10 g and Strunz et al. (2008) of 45 g. The frequency of sampling was based on Teng et al. (2008) to evaluate the samples on the first day and after 30 days of consumption according to the volunteer's availability (until the end of the holidays). Volunteers were asked to complete a food frequency questionnaire to record their weekly eating habits and a 24-h food recall in which all food ingested on the day prior to urine collection was recorded. The frequency of consumption of foods that are most susceptible to AFL contamination and regulated in Brazil has been classified as 0 to 8 times per week or more. The number of male participants was 9 (30%) and female 21 (70%). Of the total volunteers, 18 (60%) were students, 4 (13.3%) were employees and 8 (26.6%) were teachers. The mean age of study volunteers was 33 ± 14 years (19-67 years). There was a difference in age between the men and women sampled in this study, with women being older than men. This difference in age was around 12 years. The men had lower age dispersion (24.5 ± 2.5 years) than the women (36.6 ± 14.4 years) and there was also a difference in BMI between the men and women sampled in this study. Women have lower BMI than men.

Aflatoxin M1 assay

The urine sample analysis was performed using the Helica Biosystem ELISA kit for urinary AFM1 (Santa Ana, CA, USA). All samples were analyzed according to instructions from the kit manual. A calibration curve was generated based on the absorbance values of standard solutions provided with the kit containing 0; 150; 400; 800; 1500; 4000 ng AFM1 / ml. The

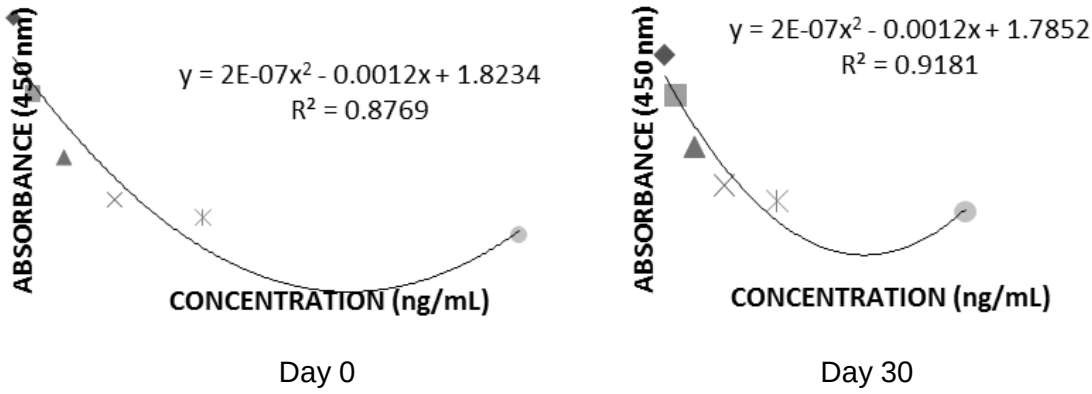

Figure 1. Calibration curve of AFM1 standard in day 0 and day 30.

absorbance was measured at 450 nm with a microplate reader. The urine samples were centrifuged at room temperature at 1500 rpm for 10 min to remove the debris and precipitates; thereafter, a 1:20 dilution (50 µL of supernatant + 950 µL of distilled water) was made. The validation steps were done with the samples measured against an arbitrarily chosen urine standard. They were spiked with either 0.5 or 2.0 ng/mL AFLM1 in three separate experiments and the recovery was measured. The fortified samples were covered from light and kept overnight at 2-8°C. The calibration curves and the straight equation are shown in Figure 1. The standard solutions provided by the Helica Kit AFM1 showed linearity of (a) time 0: 0-4000 ng/mL range for the analyte with a coefficient of determination (R^2) of 0.8769 and (b) time 30: range of 0-4000 ng/mL for the analyte with a coefficient of determination (R^2) of 0.9181.

Statistical analysis

The correlation between the consumption of more frequent food groups and the Brazilian nut consumption with the concentrations of AFM1 in the urine was carried out by Spearman test (Genest et al., 2013). This was also used for correlation between age, weight, height and BMI. Wilcoxon test (Fay et al., 2010) was performed to verify the statistical significance between genres while Bayesian analysis (Kruschke, 2014) was used to study the age difference and BMI between the sexes.

RESULTS AND DISCUSSION

AFM1 in urine samples

Among the samples analyzed at time 0 (zero), 3 (10%) presented positive results for the exposure of AFM1 in the urine with levels of 2.75-70.41 ng/mL, according to Figure 2.

The AFM1 values in urine observed in this study were higher than the ones previously reported in China by Qian et al. (1994), in which 317 urine samples were analyzed and levels of AFM1 detected ranged from 0.17 to 5.2 ng/ml. In another study, Zhu et al. (1987) cited the range from 0 to 3.2 ng/mL and Jonsyn-Ellis (2000) cited from 0.1 to 374 ng/mL. In Taiwan, the 250-person urinary excretion analysis evaluated by Hatch et al. (1993a, b) resulted in AFM1 concentrations ranging from 0.003 to 0.108 ng/mL. These evidences show that most of the volunteers in this study appear not to have been exposed to toxic levels of AFL in food. In addition, AFM1 is also found in the milk of lactating cows and other mammalian species, including humans that consumed food products contaminated with AFB1. In a study conducted by Chen et al. (2018), plasma samples from 114 children less than 36 months of age from Tanzania were collected and analyzed for the presence of AFL. About 72% of the children had detectable levels of AFB1-lys, with an average level of 5.1 (95% CI: 3.5-6.6) pg/mg albumin. In Malaysia, the population appeared to be exposed to AF-B1, but at levels below enough to cause cancer (Leong et al., 2012). In Brazil, a study was conducted by Jager et al. (2016) and the authors estimated the probable daily intake (PDI) of aflatoxin by using the results from analysis of food products collected by the time of samples collection, and data from a 24-hour dietary recall questionnaire. In urine samples from volunteers of the University of São Paulo, 65% (*n*=74) presented levels of urinary AFM1 at mean levels ranging from 0.37±0.23 to 1.70±2.88 pg/mg creatinine. A modest but significant correlation (r=0.45; p=0.03; N=23) was found by the authors for the first time in Brazil between the concentration of AFM1 in urine and the PDI for total AFL (AFB1+AFM1). Urinary AFM1 was confirmed as sensitive to monitor human exposure to AFL by diet. In Baghdad, Ali et al. (2017) analyzed 218 urine samples collected from two localities and 40% of the samples from the Rajshahi District showed urinary AFM1 levels in the range of 1.7-104 pg/mL in the summer and in a range of 1.8-190 pg/mL in the winter season. In the Dhaka District, urinary AFM1 was detected in 31% of the samples in a range of 1.7 to 141.5 pg/mL. In the present study after thirty (30) days, all samples presented negative results for the presence of AFM1 in the urine. This can be explained by changes in eating habits as hepatic enzymes may form hydroxylated metabolites of AFB1 including AFM1, which can be excreted in the urine and

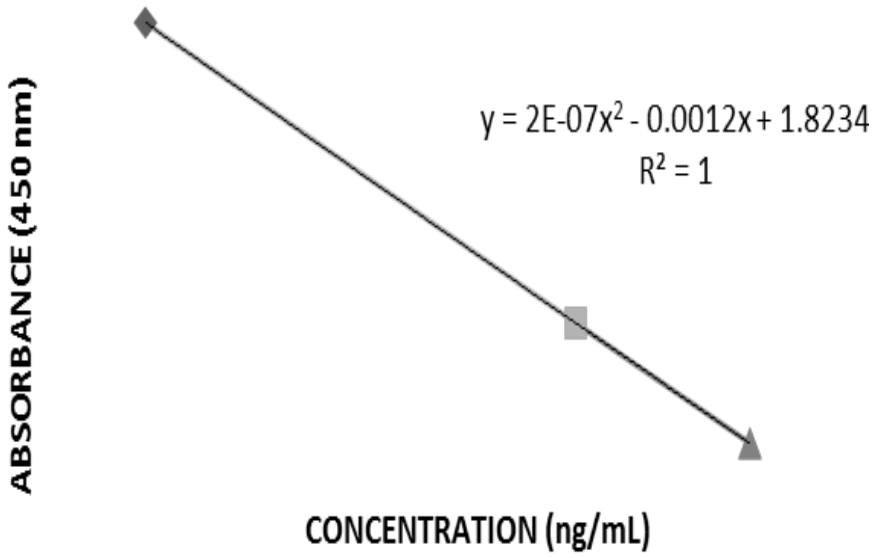

Figure 2. AFM1 in the urine samples.

used as a biomarker of short-term exposure to AFB1. Another possibility concern was the presence of protective agents and antioxidants such as selenium in the nut itself. He et al. (1993) concluded in the study that 0.4 mg/kg dietary supplement with selenium may protect the humoral immune function of the ileum mucosa of the AFB1-induced deficiency. Limaye et al. (2018) cited the ability of selenium to decrease immunosuppression, hepatic dysphonia, and apoptotic damage induced by AFB1 which is central to conferring anti-AFB1 protection through dietary supplementation. Due to the prevalence of AFB1 in animal feed and its diverse range of hosts, protective mechanisms highlighting the role of dietary selenium in relieving toxicity has been reported in many animals including swine, poultry and ducks. These works corroborate the idea that selenium may play a protective role against AFL. In the present study, the selenium level of the nuts was according to previous studies and necessary to provide the recommended daily intake, as the volunteers consumed an average of 18 g of nuts per day. If the average Selenium content of nuts was 5 ug/g, the daily dose was not toxic, although it exceeded the recommended level of 55 ug/day. Although urinary AFM1 dosage is a validated biomarker to assess AFL exposure, in this study, urinary AFM1 was a biomarker of short-term AFB1 exposure. In this context, it is suggested that more studies be conducted that correlate the selenium content and other items of the diet with the presence of AFM1 in the urine.

Consumption data versus AFM1

This study correlated the levels of urinary AFM1 with the frequency of consumption of certain foods more susceptible to AFL contamination reported in the literature. The most consumed foods reported by all subjects were rice and milk, but there was no statistical ($p > 0.34$) significance between these foods and the level of urinary AFM1 (Table 1). The lack of association between urinary AFM1 levels and rice and milk consumption suggested that these foods do not make a significant contribution to dietary AFL exposure. This result was similar to the study by Romero et al. (2010), where the correlation analysis for maize, peanut and milk-based food intake and the detected levels of AFM1 in urine did not show a statistically significant relationship. Becker-Algeri et al. (2016) cited the AFM1 as the most frequent AFL in milk and is thermally resistant and - not completely inactivated by pasteurization, sterilization, or other milk treatment processes. Milk and dairy products are foods with a high probability of AFL contamination reported in several studies (Beltran et al., 2011; Heshmati and Milani, 2010). Concerning rice, Majeed et al. (2018) reported that in rice samples from Pakistan, 56% were contaminated with AFB1. Besides, there is the possibility that other foods not reported in the questionnaire are responsible for exposure to AFL in individuals, for example coffee, beans, corn derivatives are also associated with mycotoxins (Bui-Klimke and Wu, 2015; Nugraha et al., 2018). Individuals may present variable AFM1 excretion rates, since factors inherent to the organism may or may not interfere with the metabolism of AFB1 to AFM1 (Jager et al., 2014). Thus, the variability in the use of this metabolite by the individuals participating in this study may also have contributed to a lack of correlation between the consumption of risk products and the concentration of urinary AFM1. Only female

Table 1. Spearman's correlation test between AFM1 in urine and Rice and Milk consumption.

Group	*r*	*p*
Rice	0.12555149	0.5085629
Bovine Milk	0.17798320	0.3467183

Table 2. Spearman's correlation test between AFM1 in urine, age, weight, high and BMI.

Group	*R*	*P*
Age	0.09836534	0.6117010
Weight	0.23123550	0.2557164
Height	0.23380625	0.2311253
BMI	0.12711667	0.5360408

participants presented levels of urinary AFM1. Analysis of the statistical data did not show significant differences between the sexes (p=0.1363). There was also no significant (p>0.23) difference between age, weight, height and BMI (body mass index) (Table 2).

Conclusion

The objective of the study was to study urine samples from volunteers before and after consumption of Brazil nuts. We observed that urinary AFM1 concentrations at t=0 that ranged from 2.75-70.41 ng/mL, indicating a low exposure of the study population to aflatoxins. The consumption of Brazil nuts and by-products did not increase the urinary levels of AFM1. Further studies using urinary biomarkers are required to estimate AFL exposure in populations of higher food (Brazil nut) intake susceptible to AFL contamination in Brazil and Amazon region, such as vegan, elderly and health diet consumers.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

This work was supported by grants from The National Council for Scientific and Technological Development-CNPq- 402776/2016-0.

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