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Evaluation of aflatoxin and fumonisin co-exposure in urine samples from healthy volunteers in northern Mexico

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ABSTRACT

Aflatoxins (AF) and fumonisins (FB) are common contaminants of maize and have been associated with cancer, immune suppression, and growth stunting. In this work, AFM_1 and FB_1 were measured in urine samples of healthy volunteers from the metropolitan area of Monterrey, Mexico, while AF and FB were detected in foods collected near the sampling zone. Urine samples from 106 adults were analyzed using ultra-performance liquid chromatography-tandem mass spectrometry and toxins in foods were measured by fluorometry. The mean value of AFM_1 and FB_1 was 4.3 pg/mg creatinine from 76 samples (72 %), and 50 pg/mg creatinine from 75 samples (71 %), respectively. More than half of the samples (n = 56, 53 %) had detectable levels of both AFM_1 and FB_1. No differences in toxin levels were found between males and females or between age groups, but AFM_1 and FB_1 levels were higher ($p < 0.01$) when detected as a single exposure compared to co-exposed. Some significant results were found when comparing AFM_1 and FB_1 levels among groups of people assigned to levels of food consumption. Food samples had average concentrations of 5.3 μg/kg for AF and 800 μg/kg for FB. The results showed that co-exposure to AF and FB is common in the metropolitan area of Monterrey.

1. Introduction

Mycotoxins are common contaminants that represent important challenges to assure food security worldwide. Aflatoxins (AF) and fumonisins (FB) are frequently found as co-contaminants in many cereals, such as maize, with aflatoxin B_1 (AFB_1) and fumonisin B_1 (FB_1) being the most toxic and prevalent from their respective chemical subtypes. AFB_1 is considered a group 1 human carcinogen [1], and has been implicated in children stunting [2], immunosuppression [3], children hepatomegaly [4], and death [5]. FB_1 is a possible human carcinogen classified in group 2B [1] that has been considered a risk factor for promotion of primary liver cancers [6] as well as a contributing factor for increasing the risk of neural tube defects (NTD) development [7]. The specific mechanism of NTD development could be related to FB_1 inhibition of ceramide synthase, as positive correlations between urinary FB_1 and changes in the levels of sphinganine Sa 1-P (Sa-1P) and the Sa 1-P/Sphingosine 1-P (So-1 P) ratio in human blood are consistent with the proposed mechanism [8,9].

It has been suggested that AF and FB co-exposure causes additive toxicity effects in mice ([10]; [11]) and chickens [12], and probably

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more than additive effects in rats [13]. In trout and rodents, a higher incidence of hepatocyte nodules and liver tumors have been found as result of co-exposure, respectively [14,15]. FB_1 can alter sphingolipid signaling that in turn, modulates apoptosis and activates signaling pathways of cell proliferation in the liver, thus possibly enhancing tumorigenicity of AFB_1 in co-exposed individuals [16]. Besides the former liver cancer associations with AF-FB co-exposure, recent human studies showed the association of AF-FB co-exposure to increased risk of esophageal squamous cell carcinoma (ESCC) with a greater-than-additive interaction between co-exposures [17]. More details regarding co-exposure studies were thoroughly reviewed by Riley et al. [11].

Detection of AF and FB in maize samples is common worldwide [18]. The co-exposure to these toxins in Mexicans is suspected to be high due to regular consumption of maize, and occurrence of climate conditions that favor fungal growth and toxin production. As a comparison, according to the Food and Agriculture Organization (data from 2010), annual per capita maize consumptions were 13 and 117 kg in the United States and Mexico, respectively [19]. Maize consumption has been associated with AF presence in urine samples from a population in Texas [20] and associated with FB presence in urine samples from populations in Mexico and Guatemala [21,22]. Significant associations between urinary AFM_1 and consumed amounts of corn tortillas have been reported in a Hispanic population with high incidence of hepatocellular carcinoma (HCC) [20]. Similar associations regarding consumption of maize-based tortilla and urinary FB_1 were revealed in a cohort study from Morelos state, Mexico, in which women with "high intake" of tortillas had a 3-fold increase in urinary FB_1 compared to the "low intake" group [21]. While there are studies that reveal exposure to AF [23] and FB [21] in Mexicans, co-exposure has not been investigated. Furthermore, the specific foods acting as main sources of exposure are still unknown. Here, we report the co-exposure to AF and FB in Mexican volunteers using urinary AFM_1 and FB_1 biomarkers and the presence of both toxins in maize products from street markets.

2. Materials and methods

2.1. Chemicals and reagents

AFM_1 standard was purchased from Sigma Aldrich (St. Louis, MO, USA) while FB_1 was acquired from PROMEC Unit of South Africa Medical Research Council (Tygerberg, Cape Town, South Africa). For toxins detection in urine, VICAM (Milford, MA, USA) AflaTest® WB and FumoniTest® WB immunoaffinity columns were used, while AflaTest® and FumoniTest® immunoaffinity columns were employed for food mycotoxin analyses. All other solvents were purchased from Fisher Scientific (Waltham, MA, USA) and were LC/MS grade. Ultrapure deionized water (18.2 MΩ cm) was used in all procedures that required water, except on methods that required LC/MS water.

2.2. Participants recruitment and sample collection

Institutional Review boards from Texas A&M University (College Station, TX) and Universidad Autonoma de Nuevo Leon (UANL) approved the research protocols for collection and analysis of human samples (IRB2014-0513 and COBICIS-801/2014/123-01MCAG). Enrollment of participants was achieved in 9 municipalities from the metropolitan area of Monterrey, Mexico including Apodaca, Garcia, General Escobedo, Guadalupe, Juarez, Monterrey, San Nicolas, San Pedro, and Santa Catarina. These are the most populated urban areas in the state of Nuevo Leon. From each municipality, 6 females and 6 males were recruited aiming to have urine samples from 108 participants. Inclusion criteria for participants included: age ($\geq$ 18), no history of chronic kidney disease or liver disease (based on participant's responses), consumption of maize or maize based products from street markets and signed informed consent. Potential participant's homes located near street markets (same zip code and within 300 m^2) were visited by recruitment teams from February to April 2015. After explaining the study, recruitment teams obtained informed consent, and applied a dietary questionnaire to investigate the frequency and amounts of maize and maize products consumption, based on a previous study with a Latino population exposed to AF and FB [20]. Sterile urine collection flasks and instructions for collecting a sample of first morning urine were given to each participant. Urine samples were collected by teams before 9:00 am and participants were given a gift card as appreciation for volunteering. Collected urine samples were kept in a cooler with gel packs during transfer to the Physiology, Pharmacology and Toxicology Laboratory at UANL in the city of General Escobedo. Within 5 h of urine collection, a urine subsample (2 mL) was separated and analyzed for creatinine. The rest of all urine samples were placed in 50 mL conical tubes (duplicates) for storage at −80 °C and were maintained at this temperature through shipment for analysis at Texas A&M University.

Additionally, maize food samples (a total of 90) from street markets located within neighboring distance of participant's homes, were collected to investigate as potential sources of exposure. Food samples consisted of whole corn ears (boiled, n = 3), gorditas (small thick tortilla, n = 24), masa (corn dough, n = 29) and tortillas (n = 34). An amount of 500 g of foods were collected from April to August 2015. These foods were selected based on most popular responses in the dietary questionnaires. Food samples were frozen at −20 °C until time of analysis in the Laboratory at UANL.

2.3. Determination of aflatoxin M_1 in urine

Extraction of urinary AFM_1 followed the method of Groopman et al. [24] with modifications of Sarr et al. [25] and Wang et al. [26]. Briefly, urine samples were centrifuged at 887 xg, and 5 mL of supernatant was collected and diluted with 5 mL of water. Diluted samples (10 mL) were then loaded onto a 3 mL AflaTest® WB immunoaffinity column at a flow rate of 1 mL/min. After column washing, the AF fraction was eluted from the column with 2 mL of 80 % methanol, dried under N_2 gas and re-suspended in 200 μL of methanol-water solution (50/50 v/v%). For toxin quantification, an Acquity H-Class UPLC-MS/MS instrument equipped with a tandem quadrupole mass detector (TQD) with a wide range of ionization options, and a 2.1 × 50 mm Acquity UPLC BEH C18 column with a particle size of 1.7 μm (Waters Corporation, Milford, Massachusetts, USA) were used. Water-acetonitrile (ACN) (70/30 v/v%) solution was buffered with 1% formic acid and used for isocratic separation. Injection sample volume was set to 10 μL and was run through the column with an elution rate of 0.325 mL/min. The column effluent was coupled to the MS/MS detector, operated in the electrospray positive ion mode with conditions optimized for AFM_1 based on Warth et al. [27]. The precursor ion was set to 329.00 Da and the two product ions were 273.00 Da (quantifying ion) and 259.1 Da (qualifying ion). Urinary AFM_1 concentrations were expressed as pg/mg creatinine to correct for urine dilution. External AFM_1 standards were prepared weekly and injected following every five injections of samples. The limit of detection (LOD) was calculated at 3 ppt. A greater than 85 % recovery was achieved from extractions with a relative standard deviation of less than 5%.

2.4. Determination of fumonisin B_1 in urine

Urinary FB_1 was extracted following methods by Robinson et al. [28]. Urine samples were centrifuged at 887 xg and supernatant (5 mL) was passed through a FumoniTest® WB immunoaffinity column (VICAM) with 1 mL/min as flow rate. Column was washed with 6 mL of PBS, followed by a 6 mL H_2O wash. Then, FB_1 fraction was eluted from the column with 2 mL of methanol and dried under N_2. After drying, the FB_1 pellet was re-suspended in 1 mL of ACN-water solution (50/50 v/v %). Detection and quantification were performed with the same

equipment and column as for AFM_1. Protocol eluents, water (eluent A) and ACN (eluent B) contained 1% formic acid. After an initial time of 2.69 min at 90 % A and 10 % B, the proportion of B was increased linearly to 90 % within 1.71 min, followed by a hold-time of 1.4 min, then a steep return and column re-equilibration for 1.10 min, and 5 min wash before the next injection. Flow rate was set to 0.4 mL/min. The column was directly coupled to the MS, which was operated in the electrospray positive ion mode. MS/MS conditions were optimized for FB_1 as reported by Warth et al. [27]. The precursor ion was set to 722.5 Da and the two product ions were 334.4 Da (quantifying ion) and 352.2 Da (qualifying ion). FB_1 concentrations in urine were also expressed as pg/mg creatinine. External FB_1 standards were prepared and injected daily. LOD stablished for the method was 40 ppt. Recovery from extractions was the same as for AFM_1.

2.5. Mycotoxins in food samples

Total AF and FB in food samples were detected following VICAM AflaTest® and FumoniTest® fluorometric procedures, respectively. Frozen samples were thawed at room temperature and dried at 60 °C overnight. Samples were then ground using a blender and passed through a No. 20 sieve (Fisher Scientific, Waltham, MA, USA) with 850 μm of pore size. For AF extraction, a 50 g of ground sample was blended with 5 g of NaCl and 100 mL of methanol-water solution (80/20 v/v%). The sample was filtered twice, and filtrate was passed through a VICAM immunoaffinity column following procedure for corn (0−100 ppb). Similarly, FB were extracted from a 50 g sample according to test procedure for corn (0−10 ppm). Powder sample was blended with 100 mL of methanol-water solution (80/20 v/v%) and then filtrated. Extracted solution was passed through the immunoaffinity column and fumonisins were then eluted with 1 mL of methanol. Ortho-phthalaldehyde (OPA) and 2-mercaptoethanol were then added for FB derivatization. AF and FB levels were read in a VICAM series-4EX fluorometer using appropriate standards. LOD for AF and FB were 1 ppb and 0.25 ppm, respectively.

2.6. Statistical analysis

Analyses were performed using SAS® University Edition (SAS Institute, Cary, NC, USA). Values below detection limits (non-detectable) were handled as missing data. Treating missing data incorrectly may introduce bias when estimating the mean and variance of the distribution which may consequently reduce power in hypothesis tests [29]. Therefore, we used the MI Procedure (Multiple Imputation) to compute maximum likelihood estimates that represent a random sample of the missing values. In this study, we used a fully conditional specification method [30] to impute all missing values, using the maximum number of imputations (i. e. 100) allowed in the procedure. After imputations, we used the MIANALYZE and GLIMMIX Procedures (Generalized Mixed Model, with a lognormal distribution) to combine means and their differences for the various levels of the explanatory variables. This methodology allowed us not to drop the missing data and to generate one, reliable, single p value for all relevant comparisons. The imputation process was performed using the toxin levels already adjusted by creatinine because the use of the raw data for imputation would introduce additional bias in the analysis (i. e. all 100 imputed values from each missing sample would have to be divided by a single number of creatinine level). The comparisons were adjusted by the Tukey's multiple comparison test. The variable age (in years) was used to classify the data in three groups ('young', 18−32 years, n = 35; 'adult', 33–52 years, n = 35; 'senior', 53−81 years, n = 36). Groups were also created depending on the frequency (e.g., once a week) and the quantity of types of food consumed. A chi-squared test was used to determine whether there was a statistically significant difference between the observed frequencies and the expected frequencies of subjects between categorical variables (e. g. co-exposure status vs food consumption groups).

3. Results

Urine samples from two subjects of the original 108 recruited participants could not be obtained (one person could not provide enough urine sample and the other was not available at time of collection). Hence, only 106 urine samples were available for analysis. Study population statistics are presented in Table 1. Males (n = 51) and females (n = 55) were evenly represented in this study. All were of Mexican nationality with an average age of 44 years old (range: 18–81 years). Most participants were originally from Nuevo Leon (n = 89, 84 %), the state in which the study was conducted. Eighty-seven participants (82 %) claimed an average monthly income of less than 10,000 pesos (≈ 500 dollars). Few participants were on a special diet (i.e., reduced sugar/salt, gluten free or arthritis diet) (n = 11, 10 %). There was only one report of a child with a birth defect, but it was unrelated to neural tube development.

General distribution of biomarker data is presented in Fig. 1. Table 2 shows the descriptive statistics for each mycotoxin analysis independent of exposure status. AFM_1 was detectable in 76 samples (72 %; n = 30 non-detectable) at an average level of 4.3 pg/mg creatinine (detectable range: 0.3−26 pg/mg creatinine). FB_1 was detectable in 75 samples (71 %; n = 31 non-detectable) with an average level of 50.1 pg/mg creatinine (detectable range: 2.2−248.5 pg/mg creatinine). There was no difference in urinary levels of AFM_1 (p = 0.09) and FB_1 (p = 0.90) between males and females. There was also no difference between age groups for AFM_1 (p = 0.15) or FB_1 (p = 0.14).

Table 3 shows the descriptive statistics for samples indicating co-exposure. More than half of samples with detectable levels of AFM_1 or FB_1 (n = 56, 53 %) had detectable levels of both toxins. Average AFM_1 levels (pg/mg creatinine) were higher when detected as a single exposure compared to the urine samples containing both AFM_1 and FB_1 (p = 0.05). There were also higher levels of FB_1 when detected as a single

Table 1
Demographic characteristics of study participant.

Characteristic	n (%)
Sex	
Male	51 (48)
Female	55 (52)
Age	
18−32	35 (33)
35−52	35 (33)
53−81	36 (34)
Nationality	
Mexican	106 (100)
State	
Coahuila	5 (5)
Nayarit	2 (2)
Nuevo Leon	89 (84)
San Luis Potosi	2 (2)
Tamaulipas	6 (6)
Veracruz	1 (1)
No Answer	1 (1)
Income	
< 10,000	87 (82)
10,000−20,000	14 (13)
20,000−30,000	3 (3)
No Answer	2 (2)
Special diet	
Reduced Sugar and Salt	1 (1)
Reduced Sugar	4 (4)
Reduced Salt	1 (1)
Reduced Fat	1 (1)
Reduced Carbohydrates	2 (2)
Arthritis Diet	1 (1)
Gluten-Free	1 (1)
No special Diet	95 (89)
Children with birth defects	
Yes	1 (1)
No	102 (96)
No Answer	3 (3)

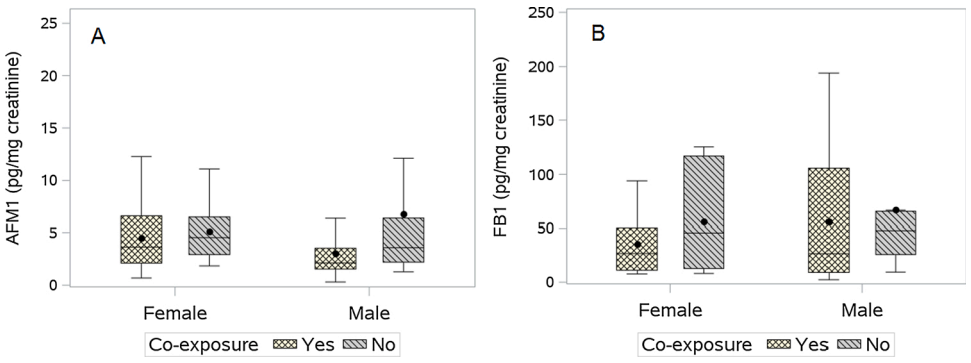

Fig. 1. Boxplots illustrating AFB_1 (A) and FB_1 (B) concentrations (pg/mg creatinine) in urine samples for females and males for both co-exposure and single exposure. The filled circles represent the mean and the horizontal lines inside the boxes represent the median. Average AFM_1 and FB_1 levels where higher in samples with single exposure (no co-exposure) compared to the levels in samples that had both mycotoxins (co-exposure), but there was no significant difference between females and males (see main text for more details).

Table 2
Levels of urinary AFM_1 and FB_1 in study participants independent of co-exposure status.

AFM_1 levels (pg/mg creatinine)		FB_1 levels (pg/mg creatinine)	
Number Positive	76	Number Positive	75
Mean ± SD	4.3 ± 3.9	Mean ± SD	50.1 ± 51.5
Gmean*	3.1	Gmean	28.6
Median	2.9	Median	29.4
Range	0.3–26	Range	2.2–248.5
Percentiles		Percentiles	
25	1.9	25	10.8
50	2.9	50	29.4
75	6.2	75	68.6

* Geometrical mean was calculated to facilitate comparisons with other investigations.

Table 3
Levels of urinary AFM_1 and FB_1 in study participants by co-exposure status.

AFM_1 levels (pg/mg creatinine)		FB_1 levels (pg/mg creatinine)	
Number Positive	56	Number Positive	56
Mean ± SD	3.7 ± 2.9	Mean ± SD	46.4 ± 48.8
Gmean*	2.7	Gmean	25.6
Median	2.5	Median	26.6
Range	0.3–12.3	Range	2.2–194
Percentiles		Percentiles	
25	1.7	25	9.7
50	2.5	50	26.6
75	4.8	75	67.1

* Geometrical mean was calculated to facilitate comparisons with other investigations.

exposure compared to samples containing both toxins ($p = 0.01$). Chi-squared tests did not show any significant association in the number of subjects between co-exposure status and dietary groups (e. g. rice consumption).

Regarding dietary questionnaires, data revealed that 78 % ($n = 83$) of the participants consumed traditional Mexican food between one to five times a week. Maize tortillas were the most frequently consumed with 41 % ($n = 43$) of the population consuming them more than twice a day. Samples were organized into categories based on consumption of different foods to evaluate any possible difference in urine toxins levels.

With few exceptions, the data did not suggest an association between food category and detectable toxin levels (Table 4). For instance, more AFM_1 was detected in participants consuming Traditional Mexican Food less than once a week than in participants consuming it more than once a week ($p = 0.05$). A similar observation was noted for corn in can ($p = 0.03$) and corn chips ($p = 0.02$) were participants reporting to never consume the foods had higher toxin levels than participants consuming more than once a week. Also, interesting differences were noted regarding flour tortilla consumption, where participants reporting no

Table 4
Summary of results of comparisons based on consumption of different foods.*.

Food	Groups	Results for AFM_1	Results for FB_1
Traditional Mexican food	Three (A: less than once a week, n = 23; B: at least once a week, n = 38; C: more than once a week, n = 45)	A > B (p = 0.08) A > C (p = 0.05)	NS
Corn in can	Four (A: never, n = 20; B: less than once a week, n = 47; C: once a week, n = 25; D: more than once a week, n = 13)	A > D (p = 0.03)	NS
Maize products	Three (A: less than once a week, n = 43; B: once a week, n = 35; C: more than once a week, n = 27)	NS	NS
Corn tortilla	Three (A: less than once a day, n = 23; B: once a day, n = 40; C: more than once a day, n = 43)	NS	B > A (p = 0.09)
Flour tortilla	Five (A: never, n = 13; B: less than once a week, n = 20; C: once a week, n = 24; D: from 2 to 5 times a week, n = 26; E: more than once a day, n = 23)	A > E (p = 0.02)	C > B (p = 0.07)
Rice	Four (A: less than once a week, n = 17, 58.8 % co-exposure; B: once a week, n = 20, 55 % co-exposure; C: from 2 to 5 times per week, n = 50, 46% co-exposure; D: more than once a day, n = 19, 57.9% co-exposure)	A > B (p = 0.02) A > C (p = 0.05) A > D (p = 0.01)	NS
Peanut butter	Three (A: never, n = 60; B: less than once per week, n = 24; C: more than once per week, n = 19)	NS	NS
Nuts	Four (A: never, n = 14; B: less than once per week, n = 33; C: once a week, n = 29; D: more than once per week, n = 29)	A > C (p = 0.03) A > D (p = 0.06)	NS
Corn chips	Four (A: never, n = 28; B: less than once a week, n = 27; C: once a week, n = 25; D: more than once a week, n = 26)	A > D (p = 0.02) C > D (p = 0.07)	B > A (p = 0.09)

The PROC GLIMMIX in SAS was used to compare AFM_1 and FB_1 levels between dietary groups. P values come from Tukey's multiple comparisons test. *Here we only show the results from the comparisons that reached ($p < 0.05$) or were close to reach ($0.05 < p < 0.1$). NS: non-significant p values to report.

regular consumption of this food had higher levels of AFM_1 than participants consuming more than once a day ($p = 0.02$). The case of rice consumption also appeared to be noteworthy. People that consumed rice less than once a week had significantly higher levels of AFM_1 compared to all the other groups of participants that consumed rice more frequently. Additional analysis showed that low consumption of rice was associated with high consumption of other products (e.g., $p < 0.05$ for rice and Mexican traditional food frequency consumption, chi-squared test), which may explain our findings. Although marginally significant, some differences were noted for FB_1, where higher levels of this toxin were detected in participants consuming flour tortilla once a week than on participants consuming it less than once a week ($p = 0.07$). A similar finding was noted for FB_1 and corn chips consumption, where more FB_1 was detected in participants consuming less than once a week compared to participants that reported to never consume corn chips ($p = 0.09$).

Most of the food samples (97.8 %) collected from street markets neighboring participant homes contained AF and FB. Average concentrations for AF and FB were 5.3 μg/kg and 800 μg/kg, respectively. AF values ranged from 0 to 41 μg/kg while for FB values fluctuated from 10 to 6000 μg/kg. The highest content of AF was found in a food sample of tortilla collected from Santa Catarina while a sample of masa collected in General Escobedo registered the highest content of FB.

4. Discussion

Single exposure to AF and FB is a public health concern and co-exposure deserves even more attention. This paper is the first to investigate co-exposure to both toxins in a Mexican population that relies on commodities vulnerable to mycotoxin contamination. Even though levels of mycotoxins were low, a high prevalence of AFB_1 and FB_1 co-exposure was observed. AFM_1 levels were detected in 72 % of our samples with a mean concentration of 4.3 pg/mg creatinine. Similar studies from a Hispanic population in Texas show a contrasting 12 % presence of AFM_1 in urine samples with an average concentration of 223.9 pg/mg creatinine [20]. Compared to other high-risk populations such as Ghana [31], our average levels for AF were also lower (4.3 vs 1, 800.1 pg/mg creatinine). One previous report of AF biomarkers in a population from Monterrey showed mean levels of AF-adduct of 2.7 pmol AF/mg albumin, these values were said to be consistent with daily dietary exposure to AFB_1 at concentrations between 2−14 μg [23]. From our biomarker data, by calculating the AF urine levels in a total of 100 mL and assuming excretion percentages of AFM_1 from 1.2 to 2.1 % of AFB_1 ingested in males and 1.3–1.7 % in females, as reported by Zhu et al. [32], our population was likely exposed to AFB_1 average concentrations of 32 ng a day. The lowest and highest values calculated for males were 4 ng and 146 ng, while values for females were 7 and 101 ng, respectively. To estimate the average AFB_1 exposure, we only used the samples with AFM_1 levels above detection limits. By doing so, the average AFM_1 in males was calculated to be 524.5 pg/dL whereas 459.9 pg/dL was calculated for females. This gave us ranges from 42.6–24.1 ng of AFB_1 for males and from 35.4–25.8 ng of AFB_1 for females, according to the excretion % previously discussed by Zhu et al. [32]. For an average adult (70 kg/b.w.), our calculated average daily exposure to AF in the diet (32 ng) falls below the 1 ng/kg/b.w. This aflatoxin daily intake has been used to estimate cancer potency per 100 000 in human populations, resulting in central estimates of 0.01 additional cancer cases per 100 000 for hepatitis B virus surface antigen negative (HBsAg-) populations and 0.3 additional cancer cases per 100 000 for HBsAg + populations [33]. Moreover, liver cancer is a chronic disease and correlation with dietary AF consumption through urinary AFM_1 is not entirely appropriate as this is a short-term biomarker of exposure. Additionally, protective effects against AF toxicity have been associated to the consumption of ingredients from plant origin such as chlorophyllin, broccoli, and green tea polyphenols, as these compounds have impacts on AF absorption or metabolism in humans [34]. All these ingredients are widely available in the Mexican modern diet and are expected to ameliorate AF exposure and toxicity to some extent.

Regarding FB_1, there is only one other study reporting this toxin in urine samples from Mexico. Women from Morelos state with reported "low" intake of tortillas (eating 1–5 tortillas in each meal), had a mean FB_1 urine value of 44 pg/mg creatinine [21]. This is very similar to what we report here for FB_1 in our co-exposed population (mean of 46.4 pg/mg creatinine) with most participants reporting to consume more than 2 tortillas in each meal and to consume tortilla more than 2 times a day. When comparing FB_1 levels in our study with values from a Guatemalan population, our toxin concentrations were lower than what was found for that population with means of 0.04 ng/mL vs 0.9 ng/mL-unadjusted for creatinine [22]. Based on reports that only 0.5 % of the FB_1 ingested dose is eliminated through the urine [22], our population was likely exposed to 941 ng daily, a value under the 2 μg/kg b.w./day Provisional Tolerable Daily Intake (PTDI) reported for this mycotoxin. Although urinary FB_1 is a validated biomarker of exposure commonly used in humans, it only reveals recent fumonisin exposure with elimination time likely lasting no more than 5 days [9]. This is further supported by Collins et al. [35] that mentioned the fast elimination of FB_1, along with low bioavailability and interindividual variations as the main limitations when trying to relate levels of urinary FB_1 to an individual's dietary intake. Hence our population may have been exposed to the levels calculated only for a short amount of time and that exposure was likely modulated by other factors.

The role of food preparation techniques such as nixtamalization may offer a possible explanation to the reduced mycotoxin exposure in the Mexican population. As a public health intervention, nixtamalization holds sufficient evidence for implementation as a post-harvest method to reduce both AFB_1 and FB_1 exposure [34]. Most maize products consumed by our participants, except for fresh maize ears and canned maize, were expected to be nixtamalized. Nixtamalization is an alkaline-lime treatment for maize products and is widely practiced in Latin America. In this treatment, maize is boiled in a calcium hydroxide water solution and left there to soften the grain and facilitate removal of pericarp, then maize is removed from the alkali solution, rinsed, and processed to make masa (maize dough), dry flour, tortillas, and other foods [16]. The hydrolytic opening of the AFB_1 lactone ring is the reason behind reduction in toxicity achieved in the nixtamalization process [36, 37], this structural change increases its solubility and allows toxin extraction into the cooking-liquid, which is typically poured off. Evidence of lower AF toxicity achieved through nixtamalization has been reported in poultry and rodents. For example, eight-year-old chickens fed contaminated nixtamalized masa (AFB_1 = 260 μg) for five days showed not important differences from the control animals (non-contaminated diet), while chickens receiving contaminated non-nixtamalized masa died after five days [38]. Additionally, juvenile Wistar rats (22-day old) fed with tortillas prepared from nixtamalized-contaminated-maize exhibited decreased weight gain and food consumption compared to control animals that died within two weeks [39]. Nonetheless, there is evidence that the open lactone structure can revert to the original configuration under low pH conditions, such as the ones found in the stomach [40]. Nixtamalization has also been shown to reduce FB concentrations in maize-based foods [41] and to reduce toxicity in animal models [42–44]. However, variations in nixtamalization methods can account for variations in FB reduction during the process, for instance initial concentrations of FB in maize, amount of calcium hydroxide as well as interactions of FB_1 with reduced sugars may all influence the amount of FB_1 bioavailable through nixtamalized maize products [45].

Co-exposure status can be another possible explanation to our observations regarding low levels of mycotoxins detected in urine. Previous work in rats demonstrated a statistically significant reduction of AFM_1 urinary output in animals dosed with both AFB_1 and FB_1 compared to AFB_1 only control [46]. Urinary FB_1 was also lowered when animals were dosed with both toxins. Possible antagonism during gut

adsorption along with a modulatory effect of FB on AF metabolism have been proposed as explanations for reduced excretion levels of both mycotoxins in co-exposure situations [46]. It is also known that mycotoxins have effects on Na^+ co-transport of sugars and amino acid carrier systems and these effects could also be responsible for its own reduced absorption in the gastrointestinal tract [47].

The ratio of AFM_1 and FB_1 levels quantified in our population (1:11) was comparable to previous reports supporting a higher frequency of FB exposure in adults for which ratios of AF:FB ranged from 1:12.6 to 1:15 [48,49]. A similar trend has been reported for children, although at higher ratios of AF:FB such as 1:66 [50]. This can be directly related to the higher allowance limits for FB in foods (800−4000 μg/kg) as opposed to AF (4−20 μg/kg) ([51,52]; [53]). Greater FB presence in foods could also reflect competition between the mycotoxigenic fungi during growth and harvest of the contaminated commodity. Marín et al. [54] reported that some *Fusarium* species can reduce the presence of *Aspergillus* species, particularly at 15 °C. A clear overlap of niches between the fungi may exists, although the mechanism of how this overlap affects mycotoxin production is unknown.

Regarding possible associations between consumption of maize products and toxin levels in urine, we directed our questionnaire to focus mainly on maize due to its high consumption status in the Mexican population. However, several samples showed non-detectable levels of toxins and had to be treated as missing values. A common practice in the analysis of non-detectable data is to substitute the missing values with a constant value, such as the half the LOD, the LOD divided by the square root of 2, or zeros. Another alternative involves the use of multiple imputations, which has shown more advantages over other methods that deal with non-detectable data [55]. In this study, the use of this method revealed some differences in urine toxin levels between subjects with different consumption behaviors. Other studies have also shown associations between food consumption and toxin levels in urine [21,22] by using chi-square tests. Differences among our current study can be related to differences in handling non-detectable data and the number of categories used in chi-square analyses. Our dietary questionnaire also included a few queries related to rice consumption for comparisons with previous investigations [20]. Rice is a cereal for which aflatoxin contamination is gaining attention as there are reports showing contamination at levels ranging from 0.1–32.9 μg/kg in samples from markets, supermarkets, and mills [56–59]. Presence of FB in rice has also been demonstrated at concentrations of 4.3 μg/g of FB_1 in whole rice kernels [60]. Although rice cannot be ruled out as a source of exposure to both mycotoxins, our data does not support rice consumption as an important factor modulating urinary levels of AF or FB in our population. In the current study, it is not clear why in several cases, a reported "never" consumption of a particular food was associated with higher urine toxin values and not with other consumption frequencies. We believe the dietary questionnaire had inherent limitations including the accuracy of responses given by participants.

Results of toxins in food samples showed a 5.3 μg/kg average concentration for AF and a higher average concentration for FB (800 μg/kg). In Mexico, the maximum allowed content for AF in foods is 12 μg/kg and no regulations exist for FB. Our average AF level was found below what was detected in a study measuring AF in tortillas sold in Mexico City which reported an average of 20.3 μg/kg (3−385 μg/kg range) [61]. Our study is the first one to measure AF or FB in foods from Mexican street markets, nonetheless when comparing to a study from the state of Veracruz where AF and FB were measured in tortilla samples collected over 3 years, our AF and FB average concentrations were higher. Results from that study revealed average AF and FB concentrations of 1.3 μg/kg and 106.7 μg/kg, respectively [62]. In our study, the content of AF and FB in the foods cannot be related with the biomarker data from our population, as sampling periods between biomarker data and food samples from street markets occurred in different months (from 2 to 5 months apart). Nonetheless, toxin determinations in foods demonstrated the common occurrence of AF and FB and reveal the latent risk of co-exposure.

5. Conclusion

This work is the first report of co-exposure to AF and FB in a Mexican population. Compared to other populations with similar dietary patterns, the exposure to both toxins occur at low concentrations but with high frequency. Implications of co-exposure for human health could be numerous, but one aspect of concern is the potential of FB_1 to modulate AFB_1 hepatocarcinogenicity by altering mediators of cell death and survival through the inhibition of ceramide synthase. The administered questionnaire showed limitations to capture relationship between specific foods and exposure. However, maize products sold in street markets were found to be contaminated with both mycotoxins and need to be considered a potential source of exposure.

Author statement

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Declaration of Competing Interest

The authors report no declarations of interest.

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