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Aflatoxin and PAH exposure biomarkers in a U.S. population with a high incidence of hepatocellular carcinoma

Natalie M. Johnson[a], **Guoqing Qian**[b], **Li Xu**[b], **Danielle Tietze**[a], **Alicia Marroquin-Cardona**[a], **Abraham Robinson**[a], **Melanie Rodriguez**[c], **Linda Kaufman**[c], **Kyle Cunningham**[c], **James Wittmer**[c], **Fernando Guerra**[c], **Kirby C. Donnelly**[a], **Jonathan H. Williams**[d], **Jia-Sheng Wang**[b], and **Timothy D. Phillips**[a,*]

[a]College of Veterinary Medicine, Texas A&M University, College Station, TX, USA

[b]College of Public Health, University of Georgia, Athens, GA, USA

[c]San Antonio Metropolitan Health District, San Antonio, TX, USA

[d]Peanut Collaborative Research Support Program, University of Georgia, Griffin, GA, USA

Abstract

The incidence of hepatocellular carcinoma (HCC) is significantly elevated in a Hispanic community in Bexar County, Texas. Chronic exposure to dietary aflatoxins (AFs) is a major risk factor for HCC; increased risk has been linked to polycyclic aromatic hydrocarbon (PAH) co-exposure and hepatitis virus infection. The aims of this study were to assess AF and PAH exposures, investigate dietary factors that may contribute to increased AF exposure, and determine the prevalence of hepatitis virus infection in Bexar Co. Blood and urine samples were collected from 184 volunteers for biomarker analyses and hepatitis screening. Serum AFB_1-lysine adduct, urinary AFM_1 and 1-hydroxypyrene (1-OHP) levels were measured using high-performance liquid chromatography. The average AFB_1-lysine adduct level detected in 20.6% of serums was 3.84 ± 3.11 pg/mg albumin (range 1.01-16.57 pg/mg). AFM_1 was detected in 11.7% of urines, averaging 223.85 ± 250.56 pg/mg creatinine (range 1.89-935.49 pg/mg). AFM_1 detection was associated with increased consumption of corn tortillas ($p = 0.009$), nuts ($p = 0.033$) and rice ($p = 0.037$). A significant difference was observed between mean 1-OHP values of non-smokers (0.07 ± 0.13) and smokers (0.80 ± 0.68) μmol/mol creatinine ($p < 0.01$). A high hepatitis C virus positivity rate (7.1%) was observed. Findings suggest that the incidence and level of AF and PAH exposure was less than that observed in a high-risk population; however, participants consuming higher amounts of foods prone to AF contamination may be more vulnerable to exposure and interactions with other environmental/biological factors (i.e., HCV).

Keywords

aflatoxin; polycyclic aromatic hydrocarbons; hepatocellular carcinoma; hepatitis C virus; biomarkers of exposure; food safety; biomonitoring

*Corresponding author: Department of Veterinary Integrative Biosciences, College of Veterinary Medicine, Texas A&M University, 4458 TAMU, College Station, TX 77843 USA. Tel.: (979)845-6414. Fax: (979)862-4929., tphillips@cvm.tamu.edu (T.D. Phillips).

1. Introduction

The incidence of hepatocellular carcinoma (HCC) in the United States has steadily increased over the recent decades (El-Serag and Mason, 1999). The state of Texas in particular has been shown to have the highest HCC mortality rate in the U.S. (Devesa et al., 1999). A Hispanic population residing within several zip codes in a community in Bexar County, TX has been disproportionately affected by a high incidence of HCC (ATSDR, 2001). Age-adjusted cancer incidence rates from the Texas Cancer Registry averaged from 2002-2006 show that Hispanics in Bexar Co. have an increased HCC incidence rate of 16.5 (95% CI = 15.0-18.0) compared to Hispanics in Texas, with an incidence rate of 10.9 (95% CI = 10.4-11.4). Incidence rates (per 100,000) are age-adjusted to the 2000 U.S. standard population, and confidence intervals (CI) are 95% for rates (Texas Cancer Registry, 2009). Notably, the HCC incidence rate for Hispanics living in Bexar Co. is considerably higher than all races in Bexar Co., with an incidence rate of 10.0 (95% CI = 9.2-10.8) and all races in Texas, with an incidence rate of 5.8 (95% CI = 5.7-6.0). Hispanic males in Bexar Co. had the highest incidence rate during this time period at 27.1 (95% CI = 24.2-30.2). Thus we were interested in exploring factors that may contribute to HCC in this community.

Multiple factors including diet, environment, lifestyle, health status, gender and genetic susceptibility play a role in the etiology of HCC. Chronic dietary exposure to aflatoxins (AFs), fungal contaminants commonly detected in grain and nut crops (i.e., corn and peanuts), has been established as a major risk factor for HCC development (CAST, 2003; Wogan, 1992). AFB_1, the most prevalent of the AFs, is a potent hepatocarcinogen in animals and humans (IARC, 2002). Biomarkers of AF exposure, e.g., AFB_1-lysine albumin adduct and AFM_1 metabolite, are reliable indicators of chronic and acute exposure, respectively, that have been correlated with elevated HCC risk in several human populations (Groopman et al., 2005). Hoque et al. (1999) previously demonstrated the presence of AFB_1-lysine adducts in a small number of HCC patients (5/5 sera samples) registered at the University of Texas (U.T.) M.D. Anderson Cancer Center, prompting the question ‘does AFB_1 play a role in the etiology of HCC in the U.S.?’ Populations may be at increased risk for HCC due to additional biological factors, namely hepatitis virus, and/or environmental carcinogen exposures. Wu et al. (2007) documented an elevated HCC risk associated with exposure to a class of environmental contaminants known as polycyclic aromatic hydrocarbons (PAHs). In that report, the greatest risk was found among participants concurrently exposed to high levels of AFs and chronically infected with hepatitis B virus (HBV). While a variety of biological indicators of PAH exposure exist, the urinary biomarker 1-hydroxypyrene (1-OHP) has been validated in many human populations and is widely accepted due to the presence of pyrene in most PAH mixtures (Bouchard and Viau, 1999). In recent work, we reported that a population in Ghana (highly exposed to AFs) was co-exposed to PAHs based on the presence of 1-OHP in the majority of urines collected (Johnson et al., 2009). Cancer mortality patterns in Ghana show liver cancer is the leading cause of cancer mortality in men and the third highest in women (Wiredu and Armah, 2006). In areas of high HCC incidence, such as sub-Saharan Africa, China, and Southeast Asia, HCC occurrence is closely related to HBV infection. Ross et al. (1992) formerly demonstrated a synergistic interaction between HBV and AFB_1 in the development of liver cancer. Subsequently, Sun et al. (1999) followed a cohort of Chinese men with chronic HBV for 10 years and found the relative risk of HCC was significantly increased in subjects with detectable AFM_1 levels. In addition, co-infection with hepatitis C virus (HCV) further increased HCC risk, indicating HBV and HCV interact as risk factors.

While HBV is endemic to parts of the world with high HCC cases, the frequency of HBV infection in the U.S. is far lower. Conversely, an association between HCV infection and HCC incidence has been demonstrated in the U.S., particularly in Texas (Davila et al.,

2004). Records from U.T.M.D. Anderson Cancer Center have shown that more than 50% of HCC cases observed in Texas could be attributed to HCV infection (Hassan et al., 2002). In recent work, Chen et al. (2007) showed AF biomarkers of exposure were associated with advanced liver disease in HCV patients in an endemic area in Taiwan. While it is well-established that a viral-chemical interaction exists between the hepatitis virus and AFs, the possible contribution of AFs in the human diet has not yet been assessed in Bexar Co. Due to the disproportionate occurrence of HCC observed in a minority community in Bexar Co., an environmental health study was conducted as a preliminary survey to 1) assess AF and PAH exposures; 2) investigate dietary factors that may contribute to increased AF exposure, and 3) determine the prevalence of HBV and HCV infection.

2. Materials and Methods

2.1. Participant recruitment and sample collection

Study participants were recruited from three zip codes (where the incidence of liver cancer is significantly elevated) located within the San Antonio metropolitan area of Bexar Co. This area encompasses nearly 11% of Bexar Co.'s population, and residents are predominantly Hispanic (90.2%). Like other southern regions of Texas, corn and corn-based products represent a staple food source; hence, we were interested in assessing AF exposure in this community. A total of 186 participants were recruited at the San Antonio Metropolitan Health District (SAMHD) from October 2007 to May 2008. Volunteers (males and females) who qualified as study participants met the following criteria: 1) at least 18 years of age and 2) a minimum of two years residency (within the last 12 months) in one of the three specified study zip codes. The study protocol was approved by the Institutional Review Board at Texas A&M University, and all participants were provided written informed consent, as well as an oral explanation of the study protocol prior to beginning the study. Upon enrollment, SAMHD public health officials administered an environmental and personal health questionnaire (in English or Spanish) and collected demographic information through in-person interviews. Biological samples, including venous blood and urine, were collected and stored frozen (-20°C) until transport to Texas A&M University and the University of Georgia. Following sample collection, it was noted that two participants did not meet the eligibility criteria concerning residency, and data collected from these subjects were not included. Thus, 184 participants comprised our study population.

2.2. Chemicals and laboratory analysis

Authentic AFB_1, AFM_1 and 1-OHP standards were purchased from Sigma Chemical Co. (St. Louis, MO). Blood specimens were analyzed for complete blood count, HBV surface antigen (HBsAg) and anti-HCV antibodies according to standard laboratory operating procedures at the SAMHD.

2.3. Serum aflatoxin B_1-lysine adduct analysis

Serum AFB_1-lysine adduct levels were measured by a modified high-performance liquid chromatography fluorescence (HPLC-f) method (Qian et al., 2009). In brief, serum samples (150 μl) were digested by Pronase (Calbiochem, San Diego, CA) and loaded onto a Waters Oasis Max cartridge (Milford, MA). Cartridges were sequentially washed and eluted with 2% formic acid in methanol. The eluents were evaporated to dryness and reconstituted in 150 μl of 10% methanol prior to HPLC injection. Analysis was carried out on an 1100 liquid chromatography system (Agilent Technologies, Wilmington, DE), and chromatographic separation was performed on a 250 × 4.6 mm Agilent C18 column, particle size 5 μm. The mobile phase consisted of 20 mM ammonium phosphate monobasic (pH 7.2) and methanol in a linear gradient profile. The concentration of AFB_1-lysine adducts was monitored at 405

nm (excitation) and 470 nm (emission). Peaks for authentic AFB_1-lysine adduct standard and samples were co-eluted at retention times averaging 12.7 min. The detection limit of this method was 10 pg/ml. Results were adjusted for serum albumin levels.

2.4. Urinary aflatoxin M_1 and 1-hydroxypyrene analyses

Urinary AFM_1 levels were analyzed using immunoaffinity column purification followed by HPLC-f using methods previously described by Wang et al. (2008). Urinary 1-OHP levels were also measured with an HPLC-f method based on a procedure developed by Gardiner et al. (1992), as previously described by Johnson et al. (2009). Quantification of AFM_1 and 1-OHP were based on peak area and retention times as compared to external standards run daily. The limit of detection for urinary AFM_1 and 1-OHP using these methods was 0.5 pg/ml and 0.25 nmol/L of urine, respectively. Creatinine concentrations were measured at St. Joseph's Regional Health Center Laboratory in order to correct for variations in urine dilution.

2.5. Statistical analyses

Median, mean, standard deviation (SD) and detectable range were calculated for concentrations of all biomarkers measured. Statistical analyses were done using SPSS software version 15.0 (Chicago, IL). For comparisons, t-tests or Wilcoxon tests were used as appropriate to examine differences between biomarker data. Chi-square tests were performed to examine demographic data and variables assessed by the questionnaire. Crude odds ratio estimates for the relationship between various dietary factors and aflatoxin biomarkers were determined by generating 2×2 contingency tables. A p-value ≤ 0.05 (two-tailed) was considered significant.

3. Results

Table 1 provides the descriptive characteristics and HBV and HCV status in our study population. Slightly more than one fourth (26.6%) of the participants were male and 73.4% were female. The average participant age was 48 (median: 49; range: 18-83 years). The majority of participants (97.3%) were of Hispanic ethnicity; the remaining percentage of the study population (1.1 and 1.6%) was Native American and African American, respectively. Serum analysis at SAMHD included screening for HBsAg and anti-HCV antibodies. None of the participants were HBsAg+, while 7.1% (13/184) of the study population was anti-HCV+. Fig. 1A and 1B show the distribution of AF biomarkers of exposure in the study population. Of the total serums analyzed for AFB_1-lysine adduct (n = 170), 20.6% had detectable levels with the average level at 3.84 ± 3.11 pg/mg albumin (median: 2.96; detectable range: 1.01-16.57 pg/mg albumin). Urinary AFM_1 was detectable in 11.7% of samples analyzed (n = 179) with the average level at 223.85 ± 250.56 pg/mg creatinine (median: 141.53; detectable range: 1.89-935.49 pg/mg creatinine). Characteristics described above did not differ significantly among the participants in the AF-detectable and non-detectable groups.Fig. 1 (C) illustrates the distribution of 1-OHP in study participants stratified by smoking status. Of the samples available for 1-OHP analysis, 51.2% of 125 non-smokers and 100% of 35 tobacco-smokers had detectable levels of 1-OHP with median excretion values of 0.01 and 0.17 μmol/mol creatinine, respectively. Moreover, there was a significant difference between mean 1-OHP levels measured in non-smokers (0.07 ± 0.13 μmol/mol creatinine) and smokers (0.26 ± 0.33 μmol/mol creatinine) ($p < 0.01$). While a slight lack of concordance between the total number of study participants and amount of samples analyzed for AF and PAH biomarkers arose due to unforeseeable events during sample collection and transfer, adequate amounts of samples for each biomarker were analyzed to validate statistical analysis. A primary aim of the environmental and personal health questionnaire administered at SAMHD was to investigate dietary factors that may

contribute to increased AF exposure in the study population. Results from questions on food consumption showed that > 98% of participants reported that they ate commodities prone to AF-contamination (e.g., corn, nuts, rice and a variety of corn/peanut-based foods) at varying frequencies. For instance, a large percentage of the population consumed corn tortillas (44.8%) and rice (30.1%) frequently (3-14 times per week); the vast majority ate ≥ 1 tortilla (97.6%) or ≥ ½ cup of rice (82.7%) at each time of consumption. When food consumption was examined according to AF biomarker detection, the amount of corn tortillas ($p = 0.009$), rice ($p = 0.037$), and nuts ($p = 0.033$) consumed was found to be significantly associated with urinary AFM_1 detection (Table 2). No association was found between food consumption and detectable AFB_1-lysine adduct, except a marginally significant association with rice consumption ($p = 0.053$).

4. Discussion

Interaction between dietary AFs and hepatitis virus infection increases the risk for HCC development. Areas located between latitudes 40° N and S (which includes Texas) encompass populations at risk for chronic AF exposure based on suitable temperature, humidity, and vulnerability of staple commodities for mycotoxin contamination (Williams et al., 2004). Since contamination has been reported in foods from Texas and surrounding areas (Torres et al., 1995; Wood, 1992), particularly after periods of drought, our primary objective was to assess AF exposure in a predominantly Hispanic population in Bexar Co. with an increased incidence of HCC. McCoy et al. (2008) compared three procedures (ELISA, HPLC-f and HPLC with isotope dilution mass spectrometry) for measuring human AF-albumin adduct levels and found a good correlation between the three independent methods. In our study, serum AFB_1-lysine adduct levels, quantified by HPLC-f, revealed that the majority of participants had a non-detectable AF exposure, which was confirmed by urinary AFM_1 data. While the correlation between serum and urine AF biomarkers was not significant, results indicate that some individuals showed low levels of chronic exposure whereas others exhibited low to moderate to high short-term AF exposure. This may be due to the differences in half-life of the two biomarkers. The AFB_1-lysine adduct has a longer *in vivo* half-life reflecting integrated exposures over weeks to months compared to AFM_1 excretion representing recent exposure (i.e., 24 to 48 hours) (Wang et al., 1996).

Though there was a measurable AF exposure in Bexar Co., the percentage and levels were lower than those we previously observed in the Ashanti Region of Ghana, which represents a population at high risk for aflatoxicosis due to frequent and high level consumption of contaminated foods (Jolly et al., 2006; Wang et al., 2008). For instance, in two surveys, 91.2% and 88.1% of 91 and 159 participants, respectively, had detectable AFM_1 levels ranging from 0.66 to 13,297.67 pg/mg creatinine. Findings from our environmental health survey in Bexar Co. suggest that participants overall had a lower exposure to AFs than participants from a developing country (i.e., Ghana). Proper production, storage and processing of foods and effective enforcement of regulations all contribute to reduced AF exposures in developed countries. Further work delineating the source of food (e.g., store bought, home-grown crops, human food grade quality, etc.) may be of importance since low socioeconomic conditions in rural and metropolitan communities in developed countries may necessitate the use of lower quality foodstuffs. Importantly, no tolerable daily intake has been set for AFB_1, as determined for other (less carcinogenic) mycotoxins. The U.S. Food and Drug Administration has set an action level of 20 ppb in foods intended for human consumption, which corresponds to ~30 μg AFs/day, assuming that an average adult consumes approximately 1500 g of food/day. Using a urinary excretion rate of 2 - 5% (Cheng et al., 1997) and a metabolic excretion rate of 1500 ml urine/day, it can be estimated that the mean AFM_1 excretion in Bexar Co. corresponds to an average daily AFB_1 consumption ranging from 9.8 - 24.6 μg/day. Although the estimated average daily AFB_1

intake is below 30 μg, individuals in the 75th and 95th percentiles may have estimated daily AFB_1 consumptions ranging from 15.3 - 38.2 and 49.2 - 122.9 μg/day, respectively. Thus, human health hazards associated with such AF exposure over time cannot be ruled out.

Of additional concern in Bexar Co. was assessing co-exposure to PAHs, which may increase the risk for HCC in the presence of AFs and hepatitis virus infection (Wu et al. 2007). Findings from this portion of our study illustrated that all study participants classified as tobacco-smokers had detectable levels of urinary 1-OHP, whereas approximately half of non-smoking participants did not show a measurable exposure to PAHs. Data further demonstrated a significant difference in mean 1-OHP concentrations when participants were stratified by smoking status. This is in agreement with previous work showing statistically significant increases in 1-OHP excretion in tobacco-smokers exposed to background levels of environmental PAHs (Levin et al., 1995; Viau et al., 1995). Conversely, in our population in Ghana smoking failed to produce any differences in urinary 1-OHP levels, compared to not smoking, indicating a predominant environmental PAH exposure (Johnson et al., 2009). Moreover, 1-OHP levels measured in Bexar Co. were considerably lower than those previously recorded in Ghana. Findings in this U.S. population were comparable or lower than those previously recorded for non-smoking individuals in numerous developed countries (Levin, 1995). Overall, results suggest that non-tobacco smokers in Bexar Co. are not at high risk for PAH exposure, based on this short-term biomarker.

An additional objective of our environmental health study in Bexar Co. was to determine the prevalence of HBV and HCV since hepatitis virus infection clearly contributes to the overall burden of HCC. Previous findings from a study in a Texas male prison population indicated that inmates who were older, Hispanic, and infected with HCV or HBV had elevated rates of both HCC prevalence and mortality (Baillargeon et al., 2009). While no participants in our study population were HBsAg+, 7.1% were positive for HCV. HBV infection is closely linked to HCC in developing countries; however, its impact may be far less in areas of the U.S. where HBV vaccination is common. In contrast, the prevalence of HCV is of significant importance, especially since no vaccination is currently available. Data from this Bexar Co. community was higher than the overall prevalence in Texas, reported to be 1.79% (varying from 1.25-2.63% across Texas counties) (Yalamanchili et al., 2005). Thus, the implementation of biomonitoring and intervention strategies, particularly in vulnerable individuals may play an important role in reducing the overall negative public health impact of dietary AF exposure. This is especially relevant in individuals at high risk for HCV-induced HCC, such as women considered to be at risk in their child bearing years and infants that acquire the virus early on as a result of perinatal infection.

A limitation of our study was the uneven recruitment of females due to the lack of male participation in the recruitment process. In future studies in this area, partnerships with local non-profit groups have been established and will serve to recruit participants with emphasis on equal gender participation. These mutually beneficial partnerships and collaborations with researchers at Texas A&M University and the San Antonio Metropolitan Health District will facilitate future work in this area.

5. Conclusions

The primary goal of this pilot study was to gain insight into the current public health of this vulnerable community and gather information to support further exploration of potential factors that can contribute HCC risk. Biomarkers measured in this study reflect current exposures to AFs and PAHs. Results from our environmental health study showed a significant association of increased consumption of certain foods and the excretion of AFM_1

in a minority population in Texas. In addition, the HCV positivity rate is considerably high in this community and warrants attention.

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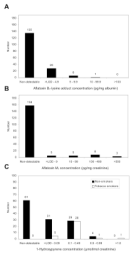

Fig. 1.

Distribution of aflatoxin B_1-lysine adducts in serum (A), aflatoxin M_1 metabolite in urine (B), and 1-hydroxypyrene in urine of smokers and non-tobacco smokers (C) from Bexar Co., Texas.

Table 1

Descriptive characteristics and HBV/HCV serology in Bexar County study participants (n = 184)

Characteristic	n (%)
Sex	
Male	49 (26.6)
Female	135 (73.4)
Ethnic Group	
Hispanic	179 (97.3)
African American	2 (1.1)
Native American	3 (1.6)
Age (years)	
18-29	24 (13.0)
30-39	37 (20.1)
40-49	34 (18.5)
50-59	34 (18.5)
60-69	36 (19.6)
> 70	19 (10.3)
Smoking Status	
Smoker[a]	31 (16.8)
Non-smoker	153 (83.2)
Hepatitis Status	
HBsAg+[b]	0 (0.0)
Anti-HCV+[c]	13 (7.1)

[a] Tobacco smoking status based on participant questionnaire.

[b] Hepatitis B virus surface antigen.

[c] Antibodies to hepatitis C virus.

Table 2
Food consumption in study population by distribution of aflatoxin M_1 biomarker in urine

Amount of Food Consumed[a]		Aflatoxin M_1[n (%)][b]	
		Detectable	Non-detectable
Corn	< ½ cup (1 ear)	2 (10.5)	28 (18.9)
	≥ ½ cup (1 ear)	17 (89.5)	120 (81.1)
Corn tortillas	< 1 tortilla	2 (11.8)	2 (1.4)
	≥ 1 tortilla	15 (88.2)*	142 (98.6)
Corn bread/muffins	< 1 piece (muffin)	1 (12.5)	12 (13.0)
	≥ 1 piece (muffin)	7 (87.5)	80 (87.0)
Corn chips	< 1 cup (10 chips)	5 (26.3)	44 (30.3)
	≥1 cup (10 chips)	14 (73.7)	101 (69.7)
Rice	< ½ cup	0 (0.0)	31 (19.9)
	≥ ½ cup	18 (100.0)*	125 (80.1)
Peanut butter	< 1 tablespoon	4 (25.0)	17 (19.3)
	≥ 1 tablespoon	12 (75.0)	71 (80.7)
Nuts	< ¼ cup	2 (12.5)	54 (39.7)
	≥ ¼ cup	14 (87.5)*	82 (60.3)

[a] Amount of food consumed at each time of consumption.

[b] Numbers within subgroups differ slightly from the total number of samples analyzed for AFM_1 due to missing responses.

* $p \leq 0.05$ in comparison of distribution in AFM_1-detectable and AFM_1-non-detectable groups in Fisher exact test.