Peanut CRSP 2012 Final Report for Project TAM149

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1. FINAL SUMMARY

A. STATEMENT OF OVERALL GOAL:

Our overall goal for TAM149 was to characterize the efficacy of a uniform particle size NovaSil clay (UPSN) in populations at high risk for aflatoxicosis in Ghana.

B. SIGNIFICANT TECHNICAL ACHIEVEMENTS:

Aflatoxin (AF) is a mycotoxin produced by the fungi *Aspergillus flavus* and *A. parasiticus,* which often contaminate cereal grains and oilseed crops around the world. It is estimated that 4.5 billion of the world's population is exposed to AFs. Enhanced exposure is most often seen in countries within the tropics such as Sub-Saharan Africa and Southeast Asia where high temperatures and drought promotes growth of the *Aspergillus* fungi in staple crops such as corn, peanuts and rice. Food storage practices, inadequate regulations for mycotoxin contamination, food insecurity and economic burdens make these populations at high risk for life-long exposure to these harmful toxins. It is well-established that aflatoxin B_1 (AFB_1) is a potent naturally-occurring hepatocellular carcinogen in humans and animals. AFB_1 is classified as a Group 1 carcinogen in humans by the International Agency for Research on Cancer. In areas where hepatitis B virus (HBV) is endemic, such as sub-Saharan Africa, it is important to note that aflatoxin and HBV act synergistically. In HBV-infected people, an increased incidence of HCC may be associated with enhanced hepatocyte mutations and aflatoxin-DNA damage in the presence of continuous liver regeneration. Several animal studies have shown that, while AFs are carcinogenic, they are also immunosuppressive and cause growth faltering/stunting. These and other harmful effects seen in agricultural animals have led to increased concern for the health status of humans who are highly and frequently exposed to AFs in developing countries.

Over the duration of our Peanut CRSP project, work at our study site in the Ejura-Sekyedumase district of Ghana has proven that this community is at high risk for AF exposure. Serum and urinary biomarkers (i.e. AFB_1-albumin adduct and AFM_1) were assessed in multiple epidemiological studies and in clinical intervention trials at this site in the Northern Ashanti Region of Ghana. The AFB_1-albumin marker in serum is indicative of exposure over a number of weeks to months, whereas, the AFM_1 marker in urine is predictive of recent AF exposure over a period of days. In our initial work, 182 people from Ejura were screened for participation in a clinical intervention trial. Of those, 100% had detectable levels of AFB_1-albumin adduct in the serum, while 98% had detectable levels of AFM_1 in the urine suggesting a critical need for strategies to reduce aflatoxin exposures in this community.

In further work at Texas A&M University, parent NovaSil clay was refined by fractionation to make a product with uniform particle size for inclusion as a food additive (UPSN). UPSN was processed to contain the highest percentage of its particles within the size range of 45-100 µm resulting in less quartz and large particles that might influence palatability. Our work in animals and humans has demonstrated that NovaSil and UPSN clays does not significantly interfere with the utilization of important vitamins and essential minerals. Our studies indicated that UPSN clay has a high binding affinity and capacity for aflatoxins similar to parent NS clay. Structurally, calcium montmorillonite clays are composed of dioctahedral smectites that contain interlayer regions held open by divalent cations and water. The high cation exchange capacity of UPSN allows for substitutions within the clay octahedral layers. These cation substitutions result in a net negative charge on the clay platelets, resulting in electrostatic interactions with positively charged molecules. Aflatoxin binding to UPSN clay is tight and thought to occur mainly on interlayer surfaces. The reaction is exothermic in the forward direction and thermodynamically favored with an estimated enthalpy (heat of sorption) between -40 and -50 kJ/mol.

In recent work, we studied the palatability of UPSN included as a powder in regular foods consumed in Ghana and its ability to reduce urinary AFM1 biomarkers of exposure. Study participants were recruited from five communities in the Ejura-Sekyedumase district of the Ashanti Region of Ghana. Socio-demographic data for these communities was established previously. All recruited participants were between 21-70 years of age. Consent was sought following a community meeting with study personnel. Consent documents were translated and explained to participants in private rooms and signed by each individual participant before initiation

of the study. Previous research from our laboratory in humans and animals has shown 3 g/day of NovaSil clay to be the minimal effective dose for reducing symptoms of AF exposure. This dose represents approximately 0.25% UPSN (w/w) of the total amount of food consumed daily by the average adult Ghanaian. Participants were selected evenly between the five communities (10 from each) and randomly assigned into one of two treatment groups. A local caterer prepared a breakfast and dinner meal for all participants daily. Participants were responsible for any snacks consumed and their lunch meals. Trained study monitors mixed each participant's treatment into their respective food before consumption. Each study participant received 1.5 g of placebo (calcium carbonate) or UPSN in their breakfast meal and their dinner meal. Breakfast meals consisted of a corn-based porridge called "koko" and the dinner meals were a common soup (i.e. peanut soup, lamb lite soup) and corn dough called "banku". Treatment group 1 consumed placebo (3 g/day) in their foods for five days followed by a two-day washout period and consumption of 3 g/day of UPSN for an additional five days. Treatment group 2 consumed UPSN (3 g/day) for the first five days followed by a two-day washout period and an additional five days of placebo treatment (3 g/day). The crossover study design allowed for a smaller number of participants and each participant was used as their own control during data analysis to account for inter-individual variations in aflatoxin metabolism. Urine samples were collected at baseline, daily during treatments and at day 20. Overnight urine samples were collected daily, and 15 ml aliquots were stored at -20°C. Samples were transported cold to Noguchi Memorial Institute for Medical Research for biomarker analysis. Laboratory employees were blinded to treatment groups during analysis. Following consumption of each meal, participants were asked to rate the food based on four criteria; 1) overall taste, 2) texture, 3) aroma and 4) would they eat the food again. The first three criteria had the following rating options: poor, unacceptable, acceptable or good while the fourth criterion was rated yes or no. Questionnaires were given to participants in English or translated to the local language by study monitors.

For analysis, urine samples were centrifuged at 2300 rpm, and 5.0 ml of supernatant was collected, acidified with 0.5 ml of 1.0M ammonium formate (pH 4.5) and diluted with water to a total volume of 10.0 ml. Samples were then loaded onto a 3 ml preparative Aflatest® WB immunoaffinity column (VICAM, Watertown, MA, USA) at a flow rate of 1 ml/min. Following washing of the column the AF fraction was eluted from the column with 2 ml of 80% methanol, dried under N_2 and re-suspended in 200 µl of a 1:1 solution of methanol:20mM ammonium formate. Samples were analyzed using a Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) with fluorescence detection capabilities. A 250 x 4.6 mm LiCrospher RP-18 column with pore size 100 Å and particle size 5 µm (Alltech Associates, Deerfield, IL, USA) was used to resolve AF metabolites. The mobile phase consisted of 22% ethanol buffered with 20 mM ammonium formate (pH 3.0) in water. Isocratic elution of the mobile phase for 20 min at a rate of 1 ml/min allowed for proper chromatographic separation. External AFM1 standards were prepared weekly and injected following every set of 5-sample injections. The limit of detection for this method was 0.5 pg/ml of urine for AFM1. Urinary AFM1 concentrations were expressed as pg/mg creatinine to correct for variations in urine dilution among samples. Creatinine concentrations were measured by a Selectra E auto-analyzer. All statistical analysis was run on JMP 9 software (SAS Institute, Cary, NC, USA). AFM1 data was not normally distributed; thus the data was analyzed with a nonparametric test (Kruskal-Wallis). However, all data was also analyzed under parametric conditions (ANOVA) following a log transformation of the data. Both parametric and nonparametric analyses were used to compare groups by days and by treatment arms. A p-value <0.05 (two-tailed) was considered significant. Statistical significance was not changed between parametric and nonparametric testing. Data was analyzed with participants acting as their own controls over two different time periods and with AFM1 levels being compared between participants during a common time period. Data was also grouped by treatment for days 1-5 and grouped separately for days 8-12 and analyzed by ANOVA. Questionnaire data was analyzed categorically with a chi-square test by treatments.

A total of 50 participants were recruited for this intervention trial with 25 randomly placed into one of two treatment groups; 1) placebo days 1-5 and UPSN days 8-12, and 2) UPSN days 1-5 and placebo days 8-12. Groups had roughly the same number of males and females and similar age ranges. The overall compliance among study participants and sample availability for biomarker analysis was satisfactory with a total of 46 participants included in data analysis. The four participants who were excluded from the study either missed two urine collections or treatment doses in a row. A total of 534 urine samples were collected and analyzed for AFM1 over the course of the 20-day study. All samples analyzed had detectable AFM1, and no significant difference was found at baseline levels between the groups (p=0.8737). The crossover in treatments for the groups with a switch in AFM1 levels occurring by day 9. There was no significant difference between placebo and UPSN treatment for group 1 (p=0.1782), however there was a significant difference between placebo and UPSN treatment for group 2 (p<0.0001). Due to dietary intake and possible aflatoxin intake differences between the legs of the study, the data was also analyzed by comparing groups during the same time period (i.e., group 1 vs. group 2 during days 1-5 and days 8-12). Average AFM1 values were significantly decreased when placebo treatment was compared to the UPSN between groups during both days 1-5 (p=0.0011) and days 8-12 (p=0.0072). There was no significant difference between groups when both were on UPSN treatment (p=0.0804) or placebo treatment (p=0.2546). Median urinary AFM1 had a 45.67% reduction with UPSN treatment during days 1-5 and a 55.21% reduction during days 8-12. Also, there was a significant difference between groups at day 20 (p= 0.0098) (1 week following completion of treatment). There were no age or gender differences found with AFM1 excretion between the study groups. Participants never deemed any food products as unacceptable or poor during the study and all participants said that they would eat the food again. Both placebo and UPSN treated foods received a higher percentage of "good" ratings than "acceptable" ratings. Pearson chi-squared tests were used to analyze the difference

between "good" and "acceptable" ratings within each group by placebo or UPSN treatment. There were no significant differences between placebo and UPSN when rating for taste, aroma or texture. During the 10 days of treatment there were two incidents of constipation reported that lasted 24 and 12 hr. while one participant complained of diarrhea that lasted for 12 hr. However, these were isolated events that occurred while the respective participants were taking placebo treatment and were not associated with UPSN consumption. The young of all species are the most susceptible to aflatoxins. Results from a survey at our study site in Ejura indicated that children may be highly exposed to aflatoxin from maize and groundnuts that are commonly used in nutritional supplements such as "weanimix." Besides their harmful effects on the liver and immune system, aflatoxins have also been reported to cause growth faltering in young animals and humans. Based on our extensive previous work confirming that NS/UPSN was safe and palatable, we recently finalized a 2-week dosimetry study in children at Ejura. The analysis of the data from this study is ongoing.

NOTE: Ethical clearance and institutional review board approval for the 2-week crossover in adults and the 2-week children's study in Ejura were obtained from both Texas A&M University (IRB 2009-0412; IRB 2011-0684) and the Noguchi Memorial Institute for Medical Research in Ghana (IRB 005/08-09; IRB 043/11-12).

C. SIGNIFICANT ISSUES/CHALLENGES:

Transport of samples from our study site in Ghana to our laboratory in Accra
Transport of samples from our remote study site in Ghana to our laboratory at TAMU

D. CAPACITY DEVELOPMENT:

Due to sample handling and transport challenges in Ghana, we established an analytical laboratory at the Noguchi Memorial Institute for Medical Research with the capacity to accurately detect aflatoxin and fumonisin biomarkers in biological fluids. This new capacity has adequately addressed the sample transport issue and has greatly enhanced our capabilities for future field research in Ghana.

E. HUMAN CAPACITY/TRAINING:

Alicia Marroquín-Cardona; female; Mexico; PhD, Texas A&M University (Phillips)
Natalie Johnson; female; USA; PhD, Texas A&M University (Phillips)
Nikki Mitchell; female; USA; PhD candidate, Texas A&M University (Phillips)
Abraham Robinson; male; USA; DVM, PhD, Texas A&M University (Phillips)
Justice Kumi; male; Ghana; BS, University of Ghana (Ankrah)

F. SHORT-TERM TRAINING:

US, Biomarker Training at TAMU, 1 male from Ghana
US, Biomarker Training at TAMU, 1 male from Ghana

G. PUBLICATIONS:

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2. IMPORTANCE AND FINAL SUMMARY

A variety of strategies for reducing AF contamination in food and feed have been reported. These include the use of competitive fungal species, establishing drought resistant crops, food processing and sorting and improved storage processes. However, none of these methods are suitable as therapy to alleviate acute aflatoxicosis and reduce lethality from high aflatoxin exposures. Toxin enterosorbent intervention with UPSN clay has the capability to rapidly impact exposures and rescue individuals (animals and humans) suffering from acute and sub-acute aflatoxicosis.