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Title

Intervention trial with calcium montmorillonite clay in a south Texas population exposed to aflatoxin.

Permalink

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Journal

Food additives & contaminants. Part A, Chemistry, analysis, control, exposure & risk assessment, 33(8)

ISSN

1944-0049

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Publication Date

2016-08-01

DOI

10.1080/19440049.2016.1198498

Peer reviewed

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To cite this article: Brad H. Pollock, Sarah Elmore, Amelia Romoser, Lili Tang, Min-su Kang, Kathy Xue, Marisa Rodriguez, Nicole A. Dierschke, Holly G. Hayes, H. Andrew Hansen, Fernando Guerra, Jia-Sheng Wang & Timothy Phillips (2016) Intervention trial with calcium montmorillonite clay in a south Texas population exposed to aflatoxin, Food Additives & Contaminants: Part A, 33:8, 1346-1354, DOI: [10.1080/19440049.2016.1198498](https://doi.org/10.1080/19440049.2016.1198498)

To link to this article: <http://dx.doi.org/10.1080/19440049.2016.1198498>

Accepted author version posted online: 20 Jun 2016.
Published online: 28 Jul 2016.

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Intervention trial with calcium montmorillonite clay in a south Texas population exposed to aflatoxin

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ABSTRACT

South Texas currently has the highest incidence of hepatocellular carcinoma (HCC) in the United States, a disease that disproportionately affects Latino populations in the region. Aflatoxin B₁ (AFB₁) is a potent liver carcinogen that has been shown to be present in a variety of foods in the United States, including corn and corn products. Importantly, it is a dietary risk factor contributing to a higher incidence of HCC in populations frequently consuming AFB₁-contaminated diets. In a randomised double-blind placebo controlled trial, we evaluated the effects of a 3-month administration of ACCS100 (refined calcium montmorillonite clay) on serum AFB₁-lysine adduct (AFB-Lys) level and serum biochemistry in 234 healthy men and women residing in Bexar and Medina counties, Texas. Participants recruited from 2012 to 2014 received either a placebo, 1.5 g or 3 g ACCS100 each day for 3 months, and no treatment during the fourth month. Adverse event rates were similar across treatment groups and no significant differences were observed for serum biochemistry and haematology parameters. Differences in levels of AFB-Lys at 1, 3 and 4 months were compared between placebo and active treatment groups. Although serum AFB-Lys levels were decreased by month 3 for both treatment groups, the low dose was the only treatment that was significant ($p = 0.0005$). In conclusion, the observed effect in the low-dose treatment group suggests that the use of ACCS100 may be a viable strategy to reduce dietary AFB₁ bioavailability during aflatoxin outbreaks and potentially in populations chronically exposed to this carcinogen.

ARTICLE HISTORY

Received 5 April 2016
Accepted 2 June 2016

KEYWORDS

Aflatoxin; biomarkers; AFB₁-lysine adduct; calcium montmorillonite clay; clinical intervention trial; enterosorption

Introduction

Aflatoxins (AFs), fungal metabolites produced by *Aspergillus flavus* and *A. parasiticus*, represent a group of naturally occurring mycotoxins that have long been recognised as hazardous food contaminants. Importantly, AFs are heat stable, survive a variety of food-processing procedures, and occur as contaminants of important commodities (particularly those derived from corn and peanuts). Aflatoxin B₁ (AFB₁), the most toxic of the naturally occurring AFs, is a potent mutagen and has been shown to disrupt genes involved in carcinogenesis and tumour suppression (McMahon et al. 1986, 1990; Aguilar et al. 1993). It reacts *in vivo* with DNA to give trans-8,9-dihydro-8-(N7-quanyl)-9-hydroxy-AFB₁ as the primary AFB₁-DNA adduct (Essigmann et al. 1977; Martin & Garner 1977). Along with hepatitis viral infection, it has been implicated as a major risk factor in the aetiology of human hepatocellular carcinoma (HCC)

(IARC 2002) and end-stage liver disease (Kuniholm et al. 2008).

Although chronic exposure to AFs is one of the major risk factors of HCC in many developing regions of the world, such as Southeast Asia and Sub-Saharan Africa (Turner et al. 2002; Kensler et al. 2003), the US food supply is highly regulated and typically presents less risk of exposure (Phillips et al. 1994; Brown et al. 1999). However, there is a potential for increased exposure in individuals consuming diets that can be relatively high in foods prone to contamination, such as corn and corn-based products (e.g., cornmeal, corn tortillas, etc.). It has been estimated that there will be 35,660 new cases and 24,550 deaths in the United States due to HCC in 2015 (Siegel et al. 2015). The state of Texas reports the highest HCC mortality in the United States and end-stage liver disease mortality is significantly higher in Latino populations (Perez et al. 2004). Specifically, South Texas Latinos

have the highest HCC rates in the country, which are three to four times higher than those of non-Latino whites (Ramirez et al. 2014). Although the causative factors for this disparity are not well delineated and may be attributed to a variety of factors including hepatitis infection, exposure to environmental and dietary carcinogens are also potential risk factors in this population.

Previously, we have shown that AF exposure in a predominantly Latino community in San Antonio, Texas, may be a contributing factor in the significantly increased incidence of HCC in the region, where levels of AF in blood and urine correlated with consumption of corn tortillas and rice (Johnson et al. 2010). Since it can be difficult to avoid staple dietary components (and potential exposure to AF), intervention therapies to alleviate AF-induced liver disease and cancer in such populations are high priorities. To address this need, the efficacy of a refined calcium montmorillonite clay (ACCS100) intervention, as measured by AF biomarkers of exposure, was evaluated in 640 serum samples collected from 234 study participants during a 3-month randomised phase II intervention trial in Bexar and Medina counties, Texas. Biomarker levels were evaluated prior to the beginning of the study (baseline), at 1 and 3 months, and 1 month post-intervention.

Materials and methods

Recruitment and eligibility

The study was approved by the institutional review boards at the University of Texas Health Science

Center in San Antonio (UTHSCSA) and Texas A&M University, and by the Protocol Review Committee of the Cancer Therapy and Research Center at UTHSCSA (*Clinicaltrials.gov* Identifier: NCT01677195) and by the Cancer Therapy and Research Center Protocol Review Committee. After obtaining written informed consent (from September 2012 to May 2014), 380 participants were screened from a variety of public and residential sites in Bexar County and adjacent Medina County, Texas. These sites contain most of the population of the metropolitan area of San Antonio, Texas. Many of the participants were not fluent in English. To address this, our recruitment team was bilingual and sensitive to the cultural dimensions of the study population. Individuals with a history of uncontrolled chronic disease and women who were pregnant or breastfeeding were not allowed to participate. Eligibility for the randomised trial portion of this study was restricted to the 234 voluntary recruited participants (54 men and 180 women, ages 18–77) with AFB₁–lysine adduct (AFB-Lys) ≥ 1.0 pg mg⁻¹ albumin. Socio-demographic and general health information was collected via a questionnaire administered at baseline including medical history and diet. Anthropometric data including age, race/ethnicity, height, weight, and vital signs, were collected during the study. A schedule of procedures and tests performed is shown in Figure 1. An independent data and safety monitoring committee (DSMC) oversaw the study protocol and procedures, and reviewed the trial data at least biannually.

Figure 1. Summary of the study procedures.

ACCS100 dosing and treatment schedule

This clinical study was conducted under US FDA IND #114005. The test article was produced by Premier Research Laboratories (PRLabs, Austin, TX, USA) and provided at no cost by Texas EnteroSorbents Inc. (TxESI, Bastrop, TX, USA). This material was examined for various environmental contaminants, including dioxins and heavy metals, to ensure compliance with federal and international standards. Metal and dioxin analyses of ACCS100 were reported to be well under the tolerable daily intake or provisional tolerable daily intake set forth by the WHO and JECFA (Marroquin-Cardona et al. 2011). ACCS100 was sterilised by gamma-radiation (Sterigenics, Fort Worth, TX, USA) to prevent any possible bacterial or viral contamination before trial initiation. The clinical intervention was a randomised, double-blind, placebo-controlled trial in which participants were randomly allocated to three groups (stratified on gender): high dose, low dose and placebo. The high-dose group received two 500 mg ACCS100 capsules and the low-dose group received two capsules containing 250 mg ACCS100 and 250 mg calcium carbonate placebo each, whereas the placebo group received the same size and colour capsules containing 500 mg calcium carbonate. Two capsules were consumed before each meal, three times a day. Doses were derived based on previous titrations with parent clay (NovaSil) *in vivo*. More specifically, the high dose of ACCS100 (3 g) represented an inclusion rate of only 0.25% (w/w) in the diet which was equal to a minimum effective dose (MED) required for efficacy in earlier work in animals (Phillips et al. 2002; Pimpukdee et al. 2004). Participant study medication compliance using pill count was recorded during each visit.

Adverse effects monitoring

Based on the existing scientific literature describing consumption of dioctahedral smectite clays (including calcium montmorillonite) in adults and children, no severe toxicity was expected as a result of ACCS100 treatment. Adverse effects were carefully monitored throughout the trial. Daily diary worksheets and symptom checklists were provided to study participants as assessment tools for adverse events monitoring and were completed two times daily after ingestion of each treatment dose. Adverse events are described as percentages of the total numbers of adverse event reports out of the total numbers of completed daily diary worksheets per treatment group. In the event of an adverse treatment effect or unrelated condition, medical treatment was available

to participants at no cost to the participant. Adverse events (AEs) and symptoms were graded according to the following criteria:

- Grade 1: Mild; asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated.
- Grade 2: Moderate; minimal, local or non-invasive intervention indicated; limiting age-appropriate instrumental activities of daily life.
- Grade 3: Severe or medically significant but not immediately life-threatening; hospitalisation or prolongation of hospitalisation indicated; disabling; limiting self-care.
- Grade 4: Life threatening consequences; urgent intervention indicated.
- Grade 5: Death related AE

Any participant experiencing a severe symptom was advised to seek immediate medical attention. Any symptoms that were attributed to the ACCS100 treatment by the study physicians on the DSMC would result in immediate discontinuation of treatment for that subject.

Blood and urine collection and processing

Blood was collected from participants during screening (visit 1) at the community recruitment sites. Subsequent blood and urine specimens were obtained at baseline (visit 2), at weeks 4 (visit 3), 12 (visit 4), and 16 (visit 5) (Figure 1) conducted at UTHSCSA or community recruitment sites. A portion of the blood was centrifuged within 1 h of collection to separate serum, and stored at -80°C , then shipped to the University of Georgia for AFB-Lys analysis. Aliquots were also sent to LabCorp (San Antonio, TX, USA) for complete blood count with differential and comprehensive metabolic profile. Premenopausal women provided urine for hCG pregnancy tests at visits 1–3. Additionally, these participants were dispensed a pregnancy test at visit 3 with instructions to test at 8 weeks (between visits 3 and 4).

Serum AFB-Lys analysis

Detection and quantification of serum AFB-Lys was performed as previously reported (Wang et al. 1996; Qian et al. 2010). In brief, serum samples were digested with Pronase to release mono-AFB-Lys. The digests were loaded onto an Oasis Max cartridge (Waters, Milford, MA, USA) which was sequentially washed to concentrate and purify the adduct. The adduct was eluted with 2% formic acid in methanol. The eluents were evaporated to dryness and reconstituted with 10% methanol prior to

HPLC analysis (Agilent 1200). Chromatographic separation was performed on an Agilent C18 column (5 μ m particle size, 250 $\times$ 4.6 mm) and the mobile phase consisted of 20 mM ammonium phosphate monobasic (pH 7.2) and methanol in a linear gradient. The concentrations of AFB-Lys were monitored at 405 nm (ex) and 470 nm (em). Authentic AFB-Lys standard was spiked into normal human serum (Sigma-Aldrich) for generating a standard curve and for quality control. AFB-Lys levels were adjusted by serum albumin concentration and expressed as the amount of AFB-Lys in pg mg⁻¹ albumin. The LOD was 0.4 pg mg⁻¹ albumin and the average recovery with various spiked AFB-Lys concentrations was 92%.

Statistical considerations

All efficacy analyses were conducted using an intent-to-treat approach for randomised subjects who received and took at least one dose of the test article (placebo or ACCS100). Sample size was determined based on a comparison of quantitative reduction from baseline to 3-month adduct levels of at least 20% in the high-dose arm compared with 0% in the placebo arm with 80% power and a two-sided $\alpha = 0.05$.

The statistical analysis for the intervention data was comprised of four steps: a comparison of serum AFB-Lys levels among treatment arms at the baseline prior to ACCS100 administration; an evaluation of the overall effects of ACCS100 on serum AFB-Lys levels; an evaluation for effects underlying the baseline values adjustment, and analyses of each time point for treatment groups. Response variables that were not normally distributed were logarithmically transformed to improve normality. To evaluate the overall treatment effects, log mixed-effect models for serum AFB-Lys were constructed. The models included the intercept, indicators for treatment group, time, and a treatment $\times$ time interaction term as fixed effect terms. Then, individual-level intercept and time variables were included as random effects. The final mixed model was fitted using PROC MIXED in SAS software (Brown & Prescott 2009). Parameters of the mixed model were estimated using the maximum likelihood estimation method. Akaike's information criteria (AIC) and the Bayesian information criteria (BIC), where smaller values for both are considered more preferable, were used as measures of the relative qualities of particular models. Both AIC and BIC dealt with the trade-off between the goodness of fit of the model and the complexity of the model, and thus provided valid means for model selection (Egner et al. 2014). The separate analyses at different time points were conducted using the

Wilcoxon rank-sum test. Hypothesis tests were two-tailed and assumed an $\alpha = 0.05$. All analyses were conducted in SAS 9.4 (SAS Institute, Cary, NC, USA).

Statistical analysis of all other data was conducted using JMP 10 software (SAS Institute). Analysis of variance and Tukey's tests were conducted on all demographic, haematological, and biochemical parameters for comparisons among treatment groups. Adverse events data was analysed using a χ^2 -test. A two-sided p -value ≤ 0.05 was considered statistically significant.

Results

Sample collection and demographics

A total of 380 participants were screened in the Bexar and Medina counties region, including urban and rural locations. Demographic characteristics of the study population and anthropometric information, including age, weight, height, blood pressure and pulse are presented in Table 1. The three treatment groups were similar in terms of gender, age, height, and ethnicity. Body weight, systolic blood pressure, diastolic blood pressure, and pulse were not significantly affected after 3 months of ACCS100 treatment and were not different among treatment groups. Of the 380 participants, 234 subjects had detectable serum adduct (234/380, 61.5%) with levels > 1 pg mg⁻¹ albumin and were randomised to a high-dose group ($n = 71$), low-dose group ($n = 83$), or placebo group ($n = 80$). The overall

Table 1. Demographic distribution (% (n) or mean $\pm$ SD) of enrolled participants by treatment.

	Treatment group		
	Placebo	Low dose	High dose
Participants who completed the trial	52	51	44
<i>Gender</i>			
Male (%)	10 (19)	10 (20)	14 (32)
Female (%)	42 (81)	41 (80)	30 (68)
Age (years)	42.2 $\pm$ 13.6	39.2 $\pm$ 13.9	41.9 $\pm$ 14.2
<i>Ethnicity</i>			
Hispanic (%)	35 (67)	34 (67)	32 (73)
Non-Hispanic (%)	17 (33)	17 (33)	12 (27)
<i>Body weight (kg)</i>			
Baseline	83.9 $\pm$ 24.9	85.8 $\pm$ 21.3	78.9 $\pm$ 14.7
Post-treatment	83.6 $\pm$ 24.5	86.2 $\pm$ 21.8	79.9 $\pm$ 14.9
Height (m)	1.6 $\pm$ 0.1	1.6 $\pm$ 0.1	1.6 $\pm$ 0.1
<i>Systolic blood pressure (mmHg)</i>			
Baseline	130 $\pm$ 19	129 $\pm$ 17	129 $\pm$ 17
Post-treatment	125 $\pm$ 15	126 $\pm$ 19	127 $\pm$ 18
<i>Diastolic blood pressure (mmHg)</i>			
Baseline	80 $\pm$ 11	80 $\pm$ 11	80 $\pm$ 10
Post-treatment	79 $\pm$ 10	79 $\pm$ 10	78 $\pm$ 11
<i>Pulse (BPM)</i>			
Baseline	71 $\pm$ 11	75 $\pm$ 11	74 $\pm$ 13
Post-treatment	74 $\pm$ 9	77 $\pm$ 11	74 $\pm$ 13

Table 2. Participant adherence and completion of treatment regimen.

	Treatment group			Overall
	Placebo	Low dose	High dose	
<i>Participants</i>				
Randomised	80	83	71	234
Completed (3 months of treatment)	52	51	44	147
Completion (%)	65.0	61.4	62.0	62.8
<i>Treatment regimen</i>				
Capsules to be ingested	27216	27888	23688	78792
Capsules missed ^a	4504	3967	3236	11707
Capsules taken ^a	22712	23921	20452	67085
Total reported adherence (%)	83.5	85.8 ^b	86.3 ^b	85.1

Notes: ^aReported at visits 3 and 4.^b $p < 0.05$ compared with the placebo group.

study completion was similar between treatment groups (Table 2). A total of 147 subjects (62.8%) completed the 3-month trial. The study regimen had an overall adherence (number of times capsules were taken) of 85.1%. However, the high- and low-dose groups had significantly better adherence than the placebo group ($p < 0.0001$ for both treatments). Detailed information about medication adherence is listed in Table 2.

Trial efficacy

Out of 640 samples collected from trial participants over 4 months, all had detectable levels of the serum AFB-Lys (> 0.4 pg mg⁻¹ albumin). In Table 3, mean values for AFB-Lys are shown for the treatment groups at baseline (0 weeks), the first follow-up (4 weeks), the second follow-up (12 weeks), and 4 weeks after discontinuation (16 weeks). There were no significant differences in mean serum AFB-Lys level at the baseline among treatment groups (4.26, 4.09 and 3.77 pg mg⁻¹ albumin for the placebo, low- and high-dose group, respectively). Overall, log transformed levels decreased for all groups over time ($p = 0.0448$, data not shown). Following baseline adjustment of serum AFB-Lys values across the treatment arms, it was determined by nonparametric analysis that the low-dose group was significantly decreased at 1 month compared with placebo ($p < 0.003$). Levels of AFB-Lys at 1, 3 and 4 months in the active treatment groups were

Table 3. Change in aflatoxin AFB-Lys (pg mg⁻¹ albumin) over time.

Week	Placebo	Low dose	High dose
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
0	4.26 $\pm$ 3.15	4.09 $\pm$ 2.25	3.77 $\pm$ 2.52
4	3.06 $\pm$ 1.66	2.62 $\pm$ 1.08	3.00 $\pm$ 1.30
12	3.22 $\pm$ 1.85	2.71 $\pm$ 1.35	2.87 $\pm$ 1.53
16	3.03 $\pm$ 1.77	2.99 $\pm$ 1.26	2.74 $\pm$ 1.44

compared with placebo. Although AFB-Lys levels were decreased by month 3 for both treatment groups, the low dose was the only treatment that was significant ($p = 0.0005$) (Figure 2).

The results of the mix model regression analysis showed a significant association between AFB-Lys level and time ($p < 0.001$), but not for the treatment group $\times$ time interaction term ($p = 0.99$). The model-adjusted means are shown in Figure 3 for the three treatment groups over time.

Adverse events and serum biochemistry

Participants were encouraged to report all potential adverse effects to the study monitors in person, or by phone at any time. A variety of AEs were reported throughout this study for all treatment groups, including the placebo and both doses of ACCS100. Most of the AEs that were reported were graded as 'mild' (grade 1) and were gastrointestinal in nature, including indigestion/heartburn, nausea, constipation, diarrhoea, flatulence, abdominal discomfort, and bloating (data not shown). For grade 1 symptoms, a significant difference was observed between the placebo group and the low-dose ($p = 0.0014$) and high-dose ($p = 0.0044$) groups. However, most of these events were reported by only one to three participants in the study. Importantly, all AEs reported were determined to be unrelated to the study agent; no participants were hospitalised throughout the trial, and no grade 5 events occurred.

Haematological and blood serum analysis (Table 4) revealed no significant differences or dose-dependent effects in any of the parameters tested at visit 1 (baseline) or visit 4. Protein values decreased after treatment for all groups ($p = 0.003$), including the placebo group, but remained within normal levels. Analysis of serum chloride levels indicated a significant difference between treatment arms ($p = 0.03$). Upon further post hoc analysis, the values in the placebo group were not significantly different from either the low- or high-dose ACCS100 groups at visit 1 or 4.

Discussion

AFB₁ has been implicated as a major factor in the aetiology of HCC (IARC 1993, 2002) and end-stage liver disease (Kuniholm et al. 2008). The incidence of HCC in the United States has steadily increased over time (El-Serag & Mason 1999), with the state of Texas reporting the highest mortality rate in the country (Devesa et al. 1999). In South Texas, the incidence of HCC for Latinos is considerably higher than the

Figure 2. Box and whisker plot showing the distribution of aflatoxin B₁-lysine adduct (pg mg⁻¹ albumin) over time for each of the treatment groups. The upper, middle and lower horizontal lines of the box represent the 75th, 50th and 25th percentile, respectively. Whiskers represent the 95% confidence interval and the extreme values are shown as dots.

general population (Ramirez et al. 2012). Multiple factors may be attributed to higher incidence including diet, environment, lifestyle, hepatitis B or C infection status, and possibly genetic susceptibility. Additionally, as climate warms and weather patterns become less predictable, countries such as the United States may become more vulnerable to environmental contaminants.

Due to increased temperatures and droughts which encourage *Aspergillus* growth, good agricultural practices may not be sufficient to prevent AF contamination in the food supply; it has been postulated that contamination may become widespread in areas such as the US Midwest that were previously unaffected (Cotty & Jaime-Garcia 2007). Generally, AF exposure

Figure 3. Log mixed-effect regression model showing estimated aflatoxin AFB-Lys (pg mg⁻¹ albumin) levels for each treatment group over time.

levels in many parts of the developing world are well above those observed in the United States, where the food supply is commonly regulated. As a result, the US public is less aware of AF contamination and its associated adverse health effects. Importantly, we observed a prevalence of AF exposure approximately three times higher than reported in our previous survey in Bexar County (Johnson et al. 2010) and serum AFB-Lys levels comparable to those reported in parts of Africa. For example, in a cross-sectional study on human AF exposure in Kenya, the median level for the various regions sampled was 1.78 pg mg⁻¹ albumin (Yard et al. 2013) compared with the average baseline level of 4.04 pg mg⁻¹ albumin reported here. Notably, one Kenyan district sampled in 2007 reported median serum AF level of 11.9 pg mg⁻¹ albumin (range: 6.58–19.5) (Yard et al. 2013). In contrast, baseline levels from a similar 3-month study conducted in Ghana (1.52 pmol mg⁻¹ albumin or 474.2 pg mg⁻¹ albumin) were almost 100 times higher than those observed in Texas (0.013 pmol mg⁻¹ albumin or 4.04 pg mg⁻¹ albumin). Due to the fact that AF exposure trends seem to be increasing in Texas and agriculture in the United States may be vulnerable based on unpredictable climate change, there is a potential need to educate and mitigate exposures to AFs in the United States.

As a result of the previously reported AF exposure in a Texas population, a 3-month double-blind clinical trial was initiated to investigate the safety and efficacy ACCS100 clay from September 2012 to May 2014. Although dose-dependent reductions were not

Table 4. Haematological analysis and serum biochemistry analysis.

	Haematology						Reference range
	Placebo		Low dose		High dose		
	Visit 1	Visit 4	Visit 1	Visit 4	Visit 1	Visit 4	
WBC (×10E3 ×l ⁻¹)	7.0 ± 1.5	6.9 ± 1.5	7.1 ± 1.9	7.0 ± 2.0	6.7 ± 1.9	6.5 ± 1.5	3.4–10.8
RBC (×10E6 ×l ⁻¹)	4.7 ± 0.4	4.6 ± 0.4	4.7 ± 0.3	4.6 ± 0.3	4.6 ± 0.4	4.6 ± 0.4	3.8–5.8
Haemoglobin (g dl ⁻¹)	14.2 ± 4.1	13.7 ± 1.1	13.6 ± 1.2	13.4 ± 1.2	13.8 ± 1.5	13.8 ± 1.4	11.1–17.7
Haematocrit (%)	39.9 ± 5.8	40.8 ± 2.9	40.5 ± 3.0	40.2 ± 2.8	41.3 ± 3.8	41.1 ± 3.7	34.0–46.6
MCV (fL)	87.6 ± 4.7	88.1 ± 4.3	87.0 ± 4.9	87.0 ± 4.9	89.2 ± 4.9	88.8 ± 4.2	79.0–97.0
MCH (pg)	29.4 ± 1.9	30.7 ± 8.8	29.1 ± 2.1	29.1 ± 2.2	29.8 ± 2.0	29.6 ± 2.3	26.6–33.0
MCHC (g dl ⁻¹)	33.6 ± 1.0	33.5 ± 1.1	33.5 ± 1.0	33.4 ± 1.1	33.4 ± 1.1	33.6 ± 0.9	31.5–35.7
RDW (%)	13.9 ± 0.9	14.0 ± 0.8	14.2 ± 0.9	14.3 ± 1.0	13.9 ± 0.8	14.1 ± 1.4	12.3–15.4
Platelets (×10E3 ×l ⁻¹)	270.1 ± 59.4	268.6 ± 53.0	277.6 ± 79.6	277.6 ± 81.1	276.6 ± 59.4	261.8 ± 59.2	150–379
Neutrophils (%)	58.4 ± 8.3	59.5 ± 8.6	59.0 ± 9.3	59.9 ± 7.3	56.4 ± 9.1	56.8 ± 8.6	40–74
Lymphocytes (%)	31.6 ± 7.3	31.1 ± 7.7	31.6 ± 7.2	31.2 ± 6.3	33.1 ± 8.3	33.1 ± 7.9	14–46
Monocytes (%)	7.1 ± 2.0	6.6 ± 2.0	6.4 ± 1.9	6.3 ± 1.7	6.9 ± 2.1	6.5 ± 2.0	4–12
Eosinophils (%)	2.4 ± 1.3	2.3 ± 1.5	2.2 ± 2.4	2.2 ± 1.7	2.9 ± 2.1	3.0 ± 1.6	0–5
Basophils (%)	0.3 ± 0.5	0.4 ± 0.5	0.4 ± 0.5	0.5 ± 0.5	0.6 ± 0.8	0.6 ± 0.5	0–3
Neutrophils, abs (×10E3)	4.1 ± 1.2	4.1 ± 1.2	4.3 ± 1.5	4.2 ± 1.6	3.8 ± 1.5	3.7 ± 1.1	1.4–7.0
Lymphocytes, abs (×10E3)	2.3 ± 0.7	2.1 ± 0.7	2.2 ± 0.7	2.1 ± 0.6	2.2 ± 0.7	2.1 ± 0.6	0.7–3.1
Monocytes, abs (×10E3)	0.5 ± 0.1	0.5 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.4 ± 0.2	0.1–0.9
Eosinophils, abs (×10E3)	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.1	0.2 ± 0.1	0.0–0.4
Basophils, abs (×10E3)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.0–0.2
Immature Grans (%)	0.0 ± 0.0	0.0 ± 0.1	0.3 ± 2.0	0.0 ± 0.1	0.2 ± 0.6	0.0 ± 0.0	0–1
Immature grans, abs (×10E3)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0–0.1
Serum biochemistry							
Glucose, serum (mg dl ⁻¹)	93.5 ± 28.6	98.7 ± 35.7	92.9 ± 13.8	94.5 ± 22.9	100.8 ± 33.4	103.8 ± 44.9	65–139
BUN (mg dl ⁻¹)	13.2 ± 3.7	13.2 ± 3.3	12.9 ± 4.4	12.8 ± 3.5	16.0 ± 16.3	14.0 ± 4.7	5–27
Creatinine, serum (mg dl ⁻¹)	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.8 ± 0.2	0.57–1.27
eGFR if non-African American (ml min ⁻¹ /1.73 ²)	100.2 ± 18.3	98.8 ± 17.2	122.2 ± 143.9	100.2 ± 24.1	100.0 ± 14.1	104.3 ± 16.0	>59
eGFR if African American (ml min ⁻¹ /1.73 ²)	115.7 ± 20.9	114.1 ± 19.8	117.9 ± 23.1	117.1 ± 23.5	115.5 ± 16.4	120.2 ± 18.5	>59
BUN/creatinine ratio	17.1 ± 5.1	17.3 ± 4.7	17.0 ± 4.9	17.0 ± 4.7	17.5 ± 5.1	18.9 ± 5.4	9–23
Sodium, serum (mmol l ⁻¹)	139.4 ± 2.2	139.0 ± 2.3	139.3 ± 2.3	138.9 ± 2.0	139.1 ± 2.7	138.5 ± 2.9	134–144
Potassium, serum (mmol l ⁻¹)	4.1 ± 0.4	4.1 ± 0.4	4.1 ± 0.3	4.1 ± 0.4	4.2 ± 0.4	4.2 ± 0.3	3.5–5.2
Chloride, serum (mmol l ⁻¹)	102.1 ± 2.2	101.0 ± 2.2	102.0 ± 2.0	102.3 ± 1.8	101.2 ± 2.5	101.2 ± 2.7	97–108
Carbon dioxide, total (mmol l ⁻¹)	22.4 ± 1.9	23.0 ± 2.1	22.0 ± 1.8	22.2 ± 2.1	22.8 ± 2.8	22.5 ± 2.3	18–29
Calcium, serum (mmol l ⁻¹)	9.4 ± 0.3	9.4 ± 0.4	9.4 ± 0.3	9.3 ± 0.4	9.5 ± 0.4	9.4 ± 0.4	8.7–10.2
Protein, total, serum (g dl ⁻¹)	7.0 ± 0.4	7.0 ± 0.4	7.1 ± 0.4	6.9 ± 0.4	7.2 ± 0.4	7.0 ± 0.5	6.0–8.5
Albumin, serum (g dl ⁻¹)	4.5 ± 0.7	4.4 ± 0.3	4.4 ± 0.2	4.3 ± 0.3	4.5 ± 0.3	4.3 ± 0.3	3.2–4.6
Globulin, Total (g dl ⁻¹)	2.6 ± 0.4	2.6 ± 0.4	2.7 ± 0.4	2.6 ± 0.3	2.7 ± 0.4	2.6 ± 0.4	1.5–4.5
A/G ratio	1.7 ± 0.3	2.0 ± 2.0	1.7 ± 0.3	1.7 ± 0.3	1.7 ± 0.4	1.7 ± 0.3	0.7–2.0
Bilirubin, total (mg dl ⁻¹)	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.4 ± 0.3	0.4 ± 0.2	0.4 ± 0.2	0.0–1.2
Alkaline phosphatase, S (IU l ⁻¹)	78.7 ± 26.3	75.7 ± 26.8	73.4 ± 17.7	69.2 ± 14.2	78.5 ± 23.1	77.0 ± 24.0	39–142
AST (SGOT) (IU l ⁻¹)	22.8 ± 10.1	22.8 ± 9.8	20.0 ± 5.9	20.6 ± 8.0	23.1 ± 10.1	21.8 ± 8.2	0–40
ALT (SGPT) (IU l ⁻¹)	26.7 ± 20.5	26.9 ± 26.3	21.9 ± 11.0	23.2 ± 15.6	23.9 ± 14.5	24.6 ± 15.2	0–44
Bilirubin, direct (mg dl ⁻¹)	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.0–0.4

observed, a significant reduction rate of 36% at 1 month and 33% at 3 months of intervention was reported in the low-dose treatment group. Based on this reduction, it is possible that an ACCS100 dose as low as 1.5 g/day could significantly protect against AF-derived adverse health effects. Furthermore, we found that the ACCS100 was well tolerated in that there were few high-grade AEs and none of these were deemed to be attributable to the test article.

Interestingly, a decreasing trend in AFB-Lys levels over time was observed in all three groups. This suggests that subjects may have adjusted their intake of foods associated with AF exposure. Since adduct levels never returned to baseline after a month of no treatment, a behavioural change within the study population possibly occurred, thus resulting in the observed trend. The baseline assessment included a dietary questionnaire that focused on foods that are associated with

AF exposure (unpublished data). No significant differences between treatment groups and placebo were observed in the dietary questionnaire results and therefore not accounted for in the efficacy analyses. The detailed outcomes of food consumption data from the questionnaire in relationship to baseline AFB-Lys levels are under evaluation and will be published separately. However, introduction to the dietary survey may have altered subject behaviour by decreasing consumption of specific foods associated with AF exposure. This behaviour is largely unavoidable and has been observed in other clinical trials because investigators are required to explain detailed study protocols, potential risks and benefits to study participants. Moreover, it is a common practice and a well-known ethical standard for human subject studies.

The absence of a dose-dependent trend in AFB-Lys levels may have occurred for four reasons including: (1)

overall lower AF exposures in the United States (making it difficult to detect substantial reductions over the course of the study); (2) limitations in recruitment methods for large communities; (3) uneven distribution of participants at randomisation and completion of the study; and (4) suboptimal subject adherence. Since AF contamination is a dynamic process that can change from year to year and exposure levels vary by the amount of contaminated food consumed, a rolling enrolment process could have allowed for clustering within the treatment groups. However, rolling enrolment is the most common practice for large community-based intervention studies and the randomisation procedure we used produced an even distribution of the adduct levels at the baseline in the three treatment groups. Twelve more participants were randomised into the low dose group (83) compared with the high dose group (71). The uneven distribution translated to a greater number of completers in the high dose (51) compared with only 44 participants completing the study in the low-dose group. Although this difference was not statistically significant, it could have affected dose-dependent trends. Self-reported adherence rate for the current study was satisfactory, but it may not have translated to actual compliance. Deviation from the study protocol could explain why we did not observe a dose-dependent response in the high-dose group.

Based on the fact that ACCS100 was well-tolerated in the majority of participants and no significant changes in serum haematology or biochemistry were detected in any treatment group, we postulate that this AF-reduction strategy is safe for a period of 3 months in this population. This supports the findings from our similar clinical trial in Ghana (Wang et al. 2008). Moreover, extensive clinical intervention trials in both animals and humans treated with a MED of calcium montmorillonite clays (HSCAS, NS, UPSN, ACCS100) have demonstrated significant AF biomarker reductions in serum and urine samples ranging from 33% to 78% in humans, or an average of 55.5% (Phillips et al. 2008; Mitchell et al. 2013, 2014). Importantly, this reduction in metabolites and adducts of AF is consistent with other AF interventions and potential therapies (Groopman et al. 2008). Even though biomarker reduction is not complete, significant protection from the adverse effects of AF in numerous animal models has been reported (Phillips 1999; Johnson et al. 2014). This suggests that reduced biomarker levels in humans and significant protection from the adverse effects of AF in animals may occur with low inclusion rates of calcium montmorillonite clay in the diet.

Long-term use of ACCS100 and similar clays could be a viable strategy for populations chronically exposed to this carcinogen. However, since AF exposure levels can vary and are greatest during periods of drought,

clay enterosorption therapy may be even more beneficial during emergency outbreaks of aflatoxicosis. Studies are warranted to establish the dosimetry of ACCS100 in humans and to further investigate its use as a toxin enterosorbent for emergency treatment.

Acknowledgements

The authors thank Texas Enterosorbents Inc. (TxESI) and Premier Research Labs (PRLabs) for providing and packaging the test articles utilised in this study.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the National Institute on Minority Health and Health Disparities [grant number NIH RO1 MD005819: T. Phillips, J.-S. Wang and B. Pollock], and the National Cancer Institute [grant number NIH P30 CA054174: I. Thompson]. The NIH had no involvement in the conduct of the research and/or preparation of the article.

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