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Calcium montmorillonite clay reduces urinary biomarkers of fumonisin B_1 exposure in rats and humans

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Fumonisin B_1 (FB_1) is often a co-contaminant with aflatoxin (AF) in grains and may enhance AF's carcinogenicity by acting as a cancer promoter. Calcium montmorillonite (i.e. NovaSil, NS) is a possible dietary intervention to help decrease chronic aflatoxin exposure where populations are at risk. Previous studies show that an oral dose of NS clay was able to reduce AF exposure in a Ghanaian population. *In vitro* analyses from our laboratory indicated that FB_1 (like aflatoxin) could also be sorbed onto the surfaces of NS. Hence, our objectives were to evaluate the efficacy of NS clay to reduce urinary FB_1 in a rodent model and then in a human population highly exposed to AF. In the rodent model, male Fisher rats were randomly assigned to either FB_1 control, $FB_1 + 2\%$ NS or absolute control group. FB_1 alone or with clay was given as a single dose by gavage. For the human trial, participants received NS (1.5 or 3 g day^{-1}) or placebo (1.5 g day^{-1}) for 3 months. Urines from weeks 8 and 10 were collected from the study participants for analysis. In rats, NS significantly reduced urinary FB_1 biomarker by 20% in 24 h and 50% after 48 h compared to controls. In the humans, 56% of the urine samples analysed ($n = 186$) had detectable levels of FB_1. Median urinary FB_1 levels were significantly ($p < 0.05$) decreased by $>90\%$ in the high dose NS group (3 g day^{-1}) compared to the placebo. This work indicates that our study participants in Ghana were exposed to FB_1 (in addition to AFs) from the diet. Moreover, earlier studies have shown conclusively that NS reduces the bioavailability of AF and the findings from this study suggest that NS clay also reduces the bioavailability FB_1. This is important since AF is a proven dietary risk factor for hepatocellular carcinoma (HCC) in humans and FB_1 is suspected to be a dietary risk factor for HCC and oesophageal cancer in humans.

Keywords: HPLC; toxicology, animal study; clinical study; aflatoxins; fumonisins; mycotoxins

Introduction

Fumonisin B_1 (FB_1) is the most abundant of the naturally occurring fumonisins. FB_1 is produced by *Fusarium* fungi and it has been shown to be hepatotoxic, nephrotoxic and carcinogenic in a number of species (Voss et al. 2002). Epidemiological studies have correlated *Fusarium* spp. and fumonisin contamination of food sources with increased incidences of oesophageal cancer in regions of China and South Africa, neural tube defects along the Texas–Mexico border, and primary liver cancer in China (Chu and Li 1994; Ueno et al. 1997; Marasas et al. 2004; Shephard et al. 2007). However, there are no reports that definitively demonstrate a causative relationship (Stockmann-Juvala and Savolainen 2008). While methods assessing the hazard of fumonisins in foodstuffs exist, few are capable of determining the actual exposure of populations considered to be "at risk" (Shephard et al. 1996, 2007). Changes in sphingolipid ratio due to the inhibitory effect of FB_1 on *de novo* ceramide synthase activity are commonly utilised as biomarkers for FB_1 exposure (Voss et al. 2002; He et al. 2006; Sabourdy et al. 2008). Alterations in the sphinganine:sphingosine ratio have accurately reflected fumonisin exposure in laboratory and farm animals, but have not been shown to be consistent indicators of exposure in human populations consuming fumonisin-contaminated foods (Abnet et al. 2001; Solfrizzo et al. 2004). Metabolic studies in non-human primates and swine have shown that excretion of FB_1 is mainly through the faeces with $\leq 1\%$ urinary excretion (Shephard et al. 1994;

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Fodor et al. 2008). Due to high dietary levels of FB_1 in developing countries, urinary biomarkers have been successfully used to characterise exposure in human populations (Shetty et al. 1998; Turner et al. 1999; Gong et al. 2008; Van der Westhuizen et al. 2011). This biomarker has been applied to evaluate intervention strategies that could reduce exposure to fumonisins (Van der Westhuizen et al. 2011). Our laboratory has previously reported that a Ghanaian population is highly exposed to aflatoxins (AFs) due to the frequent consumption of AF-contaminated foods (Jolly et al. 2006; Phillips et al. 2008; Wang et al. 2008). It has been well-documented that AFs are contributors of immunosuppression, malnutrition and hepatocellular carcinoma (Wogan 1992; Williams et al. 2004; Jiang et al. 2005). Furthermore, *in vitro* and *in vivo* studies have demonstrated that FB_1 can potentiate the effects of AFs (Carlson et al. 2001; IARC 2002; McKean et al. 2006). Kpodo et al. (2000) verified the co-occurrence of *Fusarium* spp. and fumonisins with AFs in maize samples from Ghanaian markets. Hence, it was postulated that participants from our previous study in Ghana, shown to be at high risk for aflatoxicosis, may be co-exposed to fumonisins. To reduce AF exposure, the use of NovaSil (NS) (a dioctahedral smectite clay) as an intervention plan for the enterosorption of the toxin has been shown to be safe and effective in humans (Phillips et al. 2008). Importantly, NS has also been evaluated *in vitro* for FB_1 sorption and found to interact with this mycotoxin at interlayer surfaces at acidic pH (Lemke 2000). A possible mechanism for FB_1 sorption to NS is protonation of the amino group at C2 on the molecule in acidic conditions (i.e. the stomach). The charged FB_1 may be bound through a cationic exchange reaction at negatively charged surfaces of the clay.

Thus, the main objectives of this study were to: (1) determine the efficacy of NS to reduce urinary FB_1 in Fischer 344 rats; (2) assess exposure to fumonisins in a Ghanaian population highly exposed to AFs using the FB_1 urinary biomarker; and (3) assess the efficacy of NS to reduce fumonisin exposure in this population. Importantly, strategies that reduce co-exposures to AFs and fumonisins are highly desirable for populations at risk for both agents.

Materials and methods

Chemicals

FB_1, *o*-phthalaldehyde (OPA), methanol, 2-mercaptoethanol, sodium phosphate dibasic and phosphoric acid were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Naphthalene 2,3-dicarboxaldehyde (NDA), potassium cyanide (KCN), sodium tetraborate (STB), phosphate-buffered saline (PBS) and potassium hydroxide (KOH) were also obtained from Sigma. Fumonitest WB columns were purchased from VICAM (Watertown, MA, USA). All of the experiments were done using filtered and deionised water (18.2 $M\Omega \cdot cm$) (Millipore, Milford, MA, USA), and all other chemicals and reagents were purchased commercially at the highest degree of purity available.

Preparation of FB_1 standard and derivatisation solutions

FB_1 standard solution ($1\,mg\,ml^{-1}$) was prepared in 50:50 (v/v) methanol/water. NDA and OPA solutions were made daily and stored at 4 °C in the dark. NDA was prepared as described by He et al. (2005) and OPA was prepared according to Cai et al. (2007). These solutions were prepared according to the above mentioned methods, except during the fluorescence comparison analysis in which equal molar concentrations of NDA and OPA were used. For the experiments comparing sensitivity of the derivatising agents, excitation and emission parameters were set at 335 and 440 nm for OPA and 252 and 483 nm for NDA.

Rodent model experimental design

A total of 18 male Fisher 344 rats (5-week-old male, 110–130 g body weight, bw) were purchased from Harlan (Houston, TX, USA) and maintained on nutritionally complete powdered feed (Teklad rodent diet 8604; Harlan, Madison, WI, USA) and water *ad libitum*. The rats were randomised into three equal groups: (A) Absolute control; (B) FB_1 control; and (C) FB_1 plus 2% (w/w) NS inclusion in the feed. The dietary NS clay concentration was based on the highest level previously determined to be safe in a chronic rodent study (Afriyie-Gyawu et al. 2005). After a brief acclimation period (1 week), the rats were gavaged and placed in metabolism cages (1 rat per cage) for 8 days and daily urine samples were collected. Groups B and C were administered $3.75\,mg\,FB_1$ per rat ($25\,mg\,kg^{-1}$ bw based on an average of 150 g bw) via aqueous gavage. The gavage solution for group C also contained 2% NS (the daily amount inclusion based on an average of 20 g feed consumed per day). Group C also received dietary NS (2% wet weight, ww) during the acclimation week and the week after gavage. All rats were housed in a climate-controlled environment (temperature 22–25°C) that was artificially illuminated (12-h dark/light cycle) and free from chemical contamination. Rats were inspected daily for general appearance, behavioural changes and signs of morbidity and mortality. Body weights were measured prior to acclimation, prior to gavage, and every 2 days during sample collection.

Human study site and population

Stored urine samples were available from our previous Phase IIa clinical trial assessing the safety and efficacy of NovaSil for reducing AF exposure in a Ghanaian population (Afriyie-Gyawu et al. 2008; Phillips et al. 2008). Volunteers were recruited from six communities within the Ejura-Sekyedumase district in the Ashanti Region of Ghana. Biomarker data regarding exposure to fumonisins was unknown for this area; however, maize samples from neighbouring districts in Ghana had been shown to contain fumonisins (Kpodo et al. 2000). To our knowledge, this is the first study to measure FB_1 in a human population in Ghana. A total of 177 volunteers (male and female) were selected to participate in the NS intervention study based on predetermined inclusion criteria. Participants met the following conditions: signed consent form, healthy status based on physical examination, age 18–58 years, intake of corn and/or groundnut-based foods at least four times per week, blood AFB_1-albumin adduct levels >0.5 pmol AFB_1 mg^{-1} albumin, no history of chronic disease(s), no use of prescribed medications, non-pregnant and non-breastfeeding females, normal ranges of haematological parameters and normal liver and renal function indicators (blood and urine parameters). The FB_1 levels in food consumed by our study participants were not measured and hence could not be correlated with urinary excretion patterns.

Human study design and protocol

The study protocol was approved by the Institutional Review Boards at Texas A&M University and Noguchi Memorial Institute for Medical Research in Ghana for Ethical Clearance. All participants were provided written informed consent, as well as an oral explanation prior to beginning the study. The overall study design adhered to guidelines set for a randomised, double-blind, placebo controlled Phase IIa clinical trial (Sangare-Tigori et al. 2006). Participants were randomly divided into one of three groups: High dose (NS 3.0 g day^{-1}), low dose (NS 1.5 g day^{-1}) or placebo control (1.5 g day^{-1}; microcrystalline cellulose) for a period of 3 months. Trained study monitors collected blood and urine samples (morning urine) from each participant at multiple time points. Aliquots of the urines were stored separately in polypropylene tubes and shipped frozen to Texas A&M University where they were stored at $-80^{\circ}C$ prior to biomarker analysis. Samples collected at weeks 8 and 10 from all three treatment groups (186 samples) were used in this preliminary study to assess fumonisin exposure and intervention efficacy. Baseline urine samples were not available for analysis.

FB_1 urine analysis

Frozen urine samples were thawed at room temperature and centrifuged at 500 g for 5 min. Portions (10 ml) of the urine samples were passed through Fumonitest WB columns (VICAM, Watertown, MA, USA) at a flow rate of approximately 1 ml min^{-1}). Columns were washed with 6 ml phosphate-buffered saline and 6 ml of water before elution with 2 ml of 100% methanol. Samples were dried at 50 °C under nitrogen gas and reconstituted in 150 µl of 50:50 (v/v) methanol/water. Samples were derivatised with NDA as previously described by Cho et al. (2002). Briefly, aliquots of NDA solution (300 µl) were added to samples which were vortexed and heated (50 °C) for 10 min. Analyte extracts were injected (200 µl) onto a 250×4.6 mm C_{18} LUNA column, 5 µm particle size (Phenomenex, Torrance, CA, USA) with mobile phase consisting of 77:23 (v/v) methanol/sodium phosphate dibasic (0.1 M, pH 3.35) at a flow-rate of 1 ml min^{-1}. FB_1 analysis was performed using a Waters HPLC System equipped with an auto-injector (model 717A) and fluorescence detector (model 2475). Excitation and emission parameters were set at 252 and 483 nm for detection of NDA-derivatised samples. Creatinine concentrations in animal urine samples were measured at the Texas Veterinary Medical Diagnostic Laboratory in College Station (TX, USA). Creatinine concentrations in human urine samples were measured at St. Joseph's Regional Health Center Laboratory in Bryan (TX, USA). The identity of the urine metabolite was confirmed via MALDI-TOF on a Kratos Kompact Probe MALDI-MS instrument (Kratos Analytical). A saturated solution of alpha-cyano-4-hydroxycinnamic acid (in methanol) was used to facilitate ionisation of the sample as previously described by Neupert et al. (2009).

Statistics

Data generated from HPLC analyses were transferred into an Excel database for management. Mean, median, confidence interval (CI) and range were calculated for concentrations of FB_1. To show the effect of NS ingestion on FB_1 levels, statistical evaluation focused on the comparisons among different treatment groups in the rodent model and human population. Two time points were utilised to further evaluate the efficacy of the NS sorbent in the human clinical intervention trial. For statistical analysis, non-detectable samples = LOD/2. The Kruskal–Wallis and Wilcoxon rank-sum non-parametric tests were used to compare the differences among and between treatment groups. A p-value ≤ 0.05 (two-tailed) was considered significant. Statistical analyses were done using SPSS software version 15.0 (SPSS Inc., Chicago, IL, USA).

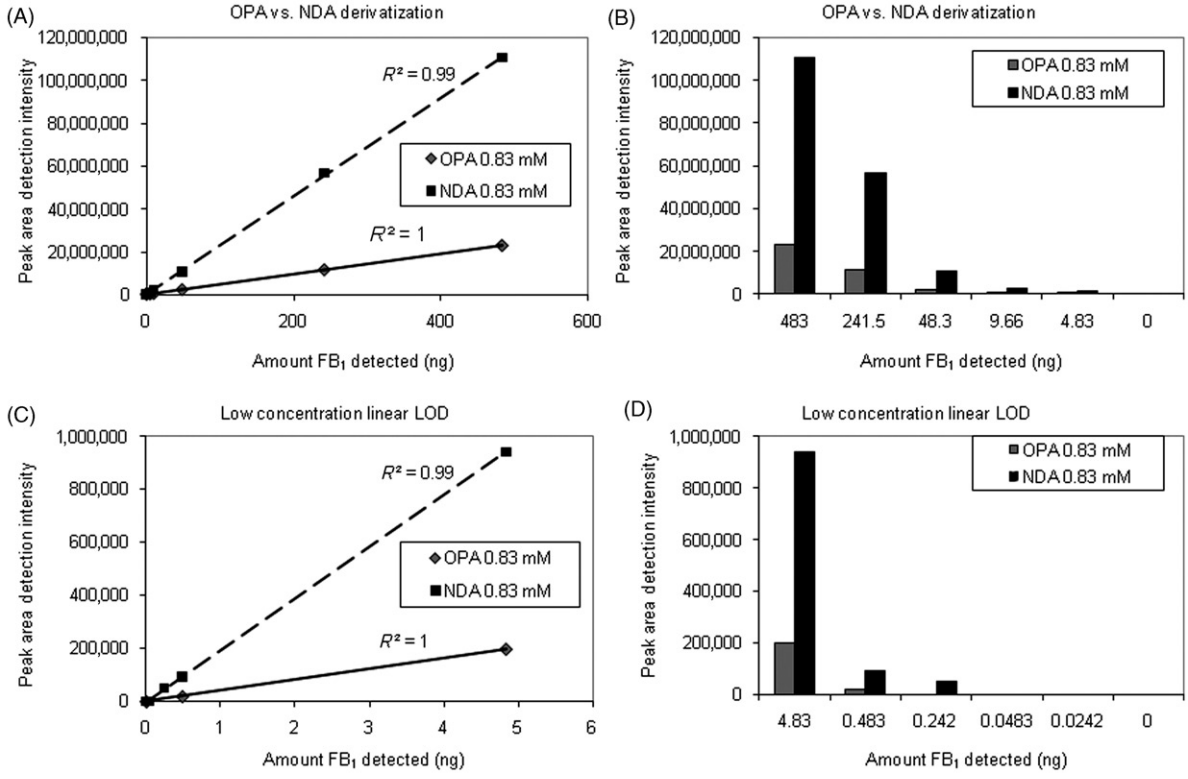

Figure 1. Comparison of FB_1 with OPA derivatisation vs. NDA derivatisation. Both OPA and NDA derivatisation products were linear within a range of 483–4.83 ng (A and B). The relative intensity of NDA derivatisation was 2–5 times higher than that of OPA (concentration dependent) allowing for a lower limit of detection (0.242 ng) while retaining linearity (C and D).

Results

Comparison of OPA versus NDA derivatisation sensitivity

Detection of both OPA and NDA derivatised FB_1 was linear (0–480 ng), with NDA having a stable lower LOD (0.242 ng) (Figure 1A–D). The detected relative intensity of NDA derivatised FB_1 was two to five times the intensity of OPA derivatised FB_1 (Figure 1A, B). Thus, NDA derivatisation was used and favoured for analysis of urine samples in both the rodent model and human study.

NovaSil efficacy in the rodent model

Urines collected at 24, 48 and 72 h post-gavage were analysed for FB_1. Most of the FB_1 dosed was eliminated in urine within 72 h (Figure 2). At 24 h, the mean total urinary FB_1 excretion for rats in group B was 864.7 ng FB_1 ml^{-1} urine (194.1 ng FB_1 mg^{-1} creatinine). After 48 h, excretion of the biomarker was reduced to 183.1 ng FB_1 ml^{-1} urine (9.4 ng FB_1 mg^{-1} creatinine). Rats in group C showed a mean total urinary excretion of 625.7 ng FB_1 ml^{-1} urine (158.2 ng FB_1 mg^{-1} creatinine) at 24 h post-gavage. After 48 h, the FB_1 excretion was reduced to 83.3 ng FB_1 ml^{-1} urine (4.4 ng FB_1 mg^{-1} creatinine). The urines from animals in group A were used to verify the lack of urinary FB_1 (Table 1). There was a statistically significant difference ($p < 0.05$) in the median (Mdn) levels of urinary FB_1 when all three treatment groups were compared. At 24 h post-gavage, median value of urinary FB_1 was significantly reduced by 34% in the NS treatment group (Mdn = 590.4 ng ml^{-1} urine) compared to the FB_1 control group (Mdn = 892.4 ng ml^{-1} urine) ($p \leq 0.05$). When standardised with creatinine, urinary FB_1 was still significantly reduced by 20%. At 48 h post-gavage, there was a significant reduction of 50% in urinary FB_1 in the NS treatment group when compared to the FB_1 control group.

NovaSil efficacy in the human study

A total of 186 urine samples from study participants at week 8 and week 10 were analysed for the presence of FB_1. Mean and median levels of FB_1 are presented in Table 2. When placebo groups were compared, 60.9% of week 8 (14/23) and 72.1% of week 10 (31/43), samples were found to be positive for the presence of FB_1 with a median urinary FB_1 concentration of 2.00 and 2.91 ng mg^{-1} creatinine, respectively. Comparison of the week 8 high and low dose NS treatment groups to placebo revealed a decreased percentage of

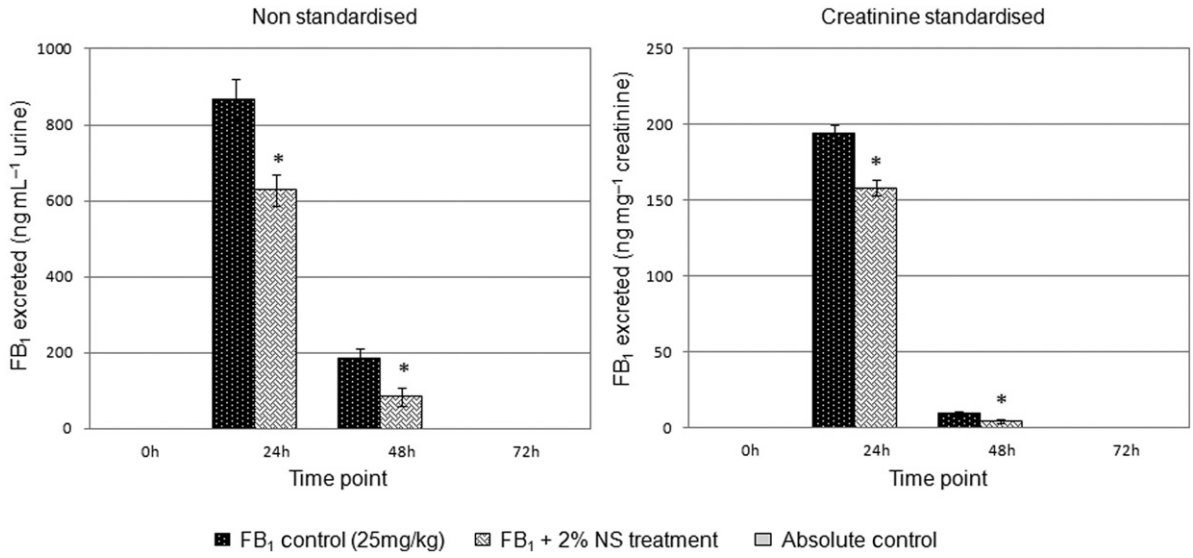

Figure 2. NovaSil reduction of total excreted and creatinine standardised urinary FB_1 in Fischer 344 rats. Data represents the cycle of urinary FB_1 excretion following 25 mg kg^{-1} dose by gavage. Urinary FB_1 was reduced by 27% in the NS treated group. This reduction was statistically significant ($p \leq 0.05$) when NS treated and non-treated groups were compared using the Kruskal–Wallis and Wilcoxon rank-sum test. Data represents the means and standard error. Non-detectable levels of FB_1 were documented for the control group at all time points.

Table 1. Reduction in FB_1 levels with NovaSil treatment in Fischer 344 rats.

	Absolute control	FB_1 control	$FB_1 + 2\%$ NS treatment
Urine collection 24 h post-gavage			
% detectable FB_1 samples	0 ($n=6$)	100 ($n=6$)	100 ($n=6$)
Mean FB_1 (95% CI)	N.D.	864.68 (719–1010.14)	625.77 (554.17–696.83)
Median FB_1 (ng ml^{-1} urine)	N.D.	892.35	590.41*
Mean FB_1 (95% CI)	N.D.	194.07 (161.55–226.59)	158.17 (138.21–178.14)
Median FB_1 (ng mg^{-1} creatinine)	N.D.	191.95	154.32*
Urine collection 48 h post-gavage			
% detectable FB_1 samples	0 ($n=6$)	100 ($n=6$)	100 ($n=6$)
Mean FB_1 (95% CI)	N.D.	183.07 (110.16–255.98)	83.29 (18.40–148.19)
Median FB_1 (ng ml^{-1} urine)	N.D.	183.65	91.92*
Mean FB_1 (95% CI)	N.D.	9.38 (6.03–12.72)	4.42 (1.00–7.85)
Median FB_1 (ng mg^{-1} creatinine)	N.D.	9.91	4.95*

Notes: Data represent the mean, confidence interval (CI) and median from each treatment group.
* Significantly different from FB_1 control group, $p \leq 0.05$ (Wilcoxon rank-sum test).
N.D., not detected.

participants with detectable urinary FB_1 (39.1 and 45.5%, respectively). A similar numerical reduction was noted when week 10 high and low dose treatment groups were compared to placebo with 54.3 and 52.5% being positive for FB_1, respectively. Median FB_1 levels for the high dose treatment group were found to be significantly lower than the placebo group at week 8 ($p \leq 0.05$). Median levels for the low dose group were reduced but not found to be significant. Similarly, week 10 median urinary FB_1 levels from both, high and low dose groups were significantly reduced ($p \leq 0.05$) when compared to placebo (Figures 3 and 4; Table 2).

MALDI-TOF analysis

MALDI-TOF mass spectrometry (MTMS) analysis of parent ($M + H^+$), NDA derivatised, and a NDA derivatised + sodium ion ($M + Na^+$) FB_1 confirmed a mass/charge of 723.0, 906.5, and 928.3, respectively (Figure 5A–C). MTMS analysis of FB_1 peaks collected from HPLC analysis of randomly selected positive urine samples verified the presence and identity of FB_1, with peaks at 907.4 ($M + H^+$) and 928.5 ($M + Na^+$) (Figure 5C). The lack of parent peak (~722.8) in MTMS analysed samples may provide an indirect indicator of the efficiency of the NDA derivatisation reaction.

Table 2. Reduction in FB_1 levels with NovaSil treatment in the Ghanaian population.

	NovaSil treatment		Control
	High dose ($3.0\,g\,day^{-1}$)	Low dose ($1.5\,g\,day^{-1}$)	Placebo[a] ($1.5\,g\,day^{-1}$)
Week 8 urinary FB_1 analysis			
% detectable FB_1 samples	39.1 (9/23)	45.5 (10/22)	60.9 (14/23)
Mean FB_1 (95% CI)	4.05 (0.99–7.10)	5.61 (1.74–9.48)	9.36 (4.51–14.22)
Median FB_1 ($ng\,ml^{-1}$ urine)	0.19*	0.19	5.45
Mean FB_1 (95% CI)	1.07 (0.22–1.91)	5.61 (0.81–10.42)	7.12 (1.69–12.52)
Median FB_1 ($ng\,mg^{-1}$ creatinine)	0.20*	0.20	2.00
Week 10 urinary FB_1 analysis			
% detectable FB_1 samples	54.3 (19/35)	52.5 (21/40)	72.1 (31/43)
Mean FB_1 (95% CI)	0.81 (0.53–1.09)	0.73 (0.37–1.08)	5.03 (3.76–6.30)
Median FB_1 ($ng\,ml^{-1}$ urine)	0.44*	0.21*	5.45
Mean FB_1 (95% CI)	1.02 (0.45–1.59)	1.69 (0.51–2.87)	6.29 (3.75–8.84)
Median FB_1 ($ng\,mg^{-1}$ creatinine)	0.28*	0.20*	2.91

Notes: Data represent the mean, confidence interval (CI) and median from each treatment group.
*Significantly different from placebo group, $p \leq 0.05$ (Wilcoxon rank-sum test).
[a]Placebo = microcrystalline cellulose.

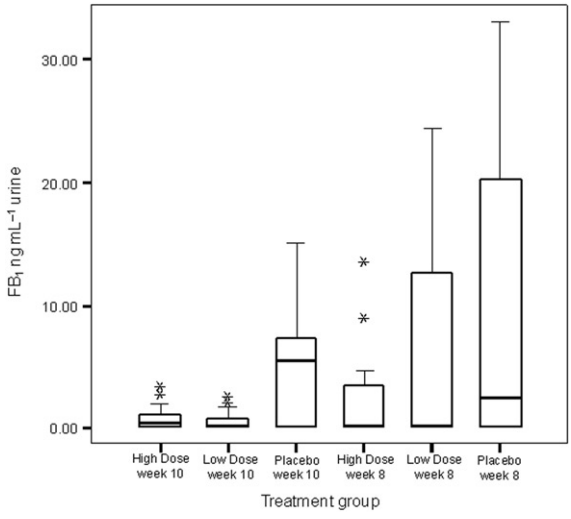

Figure 3. Distribution of urinary FB_1 between treatment groups at weeks 8 and 10. Data represents the distribution of FB_1 ($ng\,FB_1\,ml^{-1}$ urine) in the various treatment groups based on a NovaSil intervention in Ghana. There was a statistically significant difference among treatments ($p \leq 0.05$). There was a significant difference between the median FB_1 levels in the NS treated groups vs. the placebo group ($p \leq 0.05$). There was not a significant difference between median FB_1 levels between the high and low dose groups ($p = 0.566$).

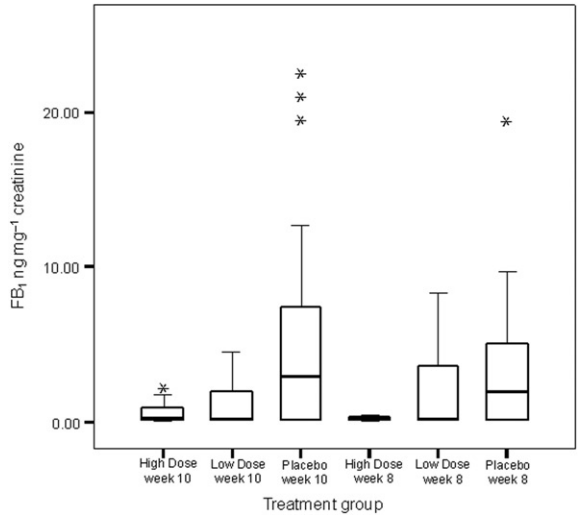

Figure 4. Distribution of creatinine standardised urinary FB_1 between treatment groups at weeks 8 and 10. Data represents the distribution of FB_1 ($ng\,FB_1\,mg^{-1}$ creatinine) in the various treatment groups based on a NovaSil intervention in Ghana. There was a statistically significant difference among treatments ($p \leq 0.05$). There was a significant difference between the median FB_1 levels in the NS treated groups vs. the placebo group ($p \leq 0.05$). There was not a significant difference between median FB_1 levels in the high and low dose groups ($p = 0.955$).

Discussion

Human exposure to mycotoxins has received increased attention, and recent studies in West Africa have confirmed the co-occurrence of AFs and fumonisins in maize-based foodstuffs found in Cote d'Ivoire and Ghana (Kpodo et al. 2000; Sangare-Tigori et al. 2006). While surveys have demonstrated that maize-based products are often co-contaminated with diverse mycotoxins; there is a paucity of published data measuring human exposure to fumonisins and AFs. Use of urinary FB_1 as a biomarker of exposure in humans was first proposed by Shetty and Bhat (1998). Turner et al. (1999) reported that analysis of urinary

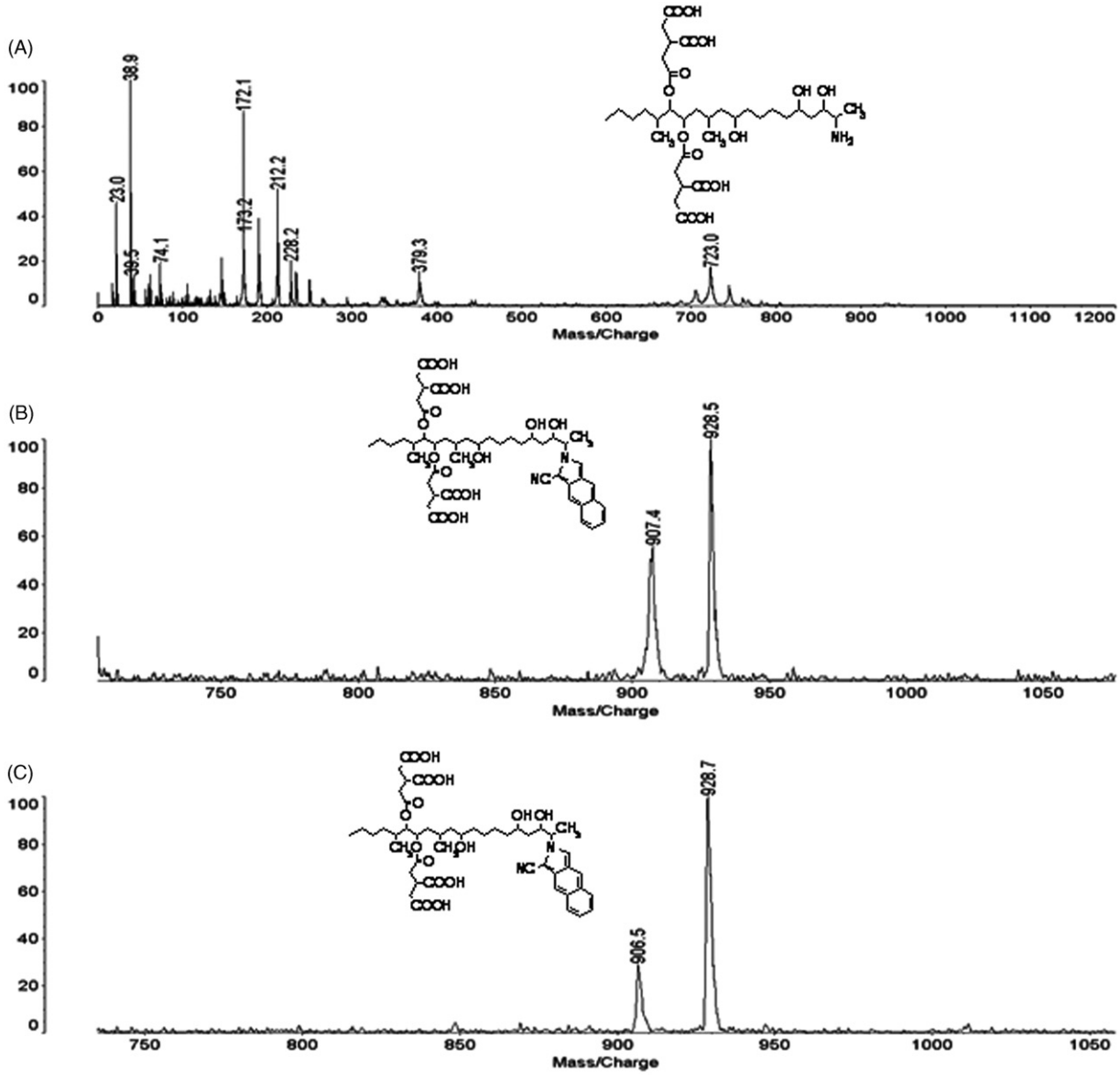

Figure 5. MALDI-TOF mass spectra (MS). MS are based on relative intensity (y-axis) of depicted peaks (highest peak = 100%). (A) MS of underivatised standard FB_1 in water (723 m/z). (B) MS of NDA derivatised FB_1 from spiked urine sample (~906.1 m/z) and NDA derivative + Na ion (~928 m/z). (C) MS of a representative FB_1 positive participant urine sample verifying presence of FB_1.

FB_1 held significant promise as there was a need for a reliable biomarker of exposure to fumonisins. A recent study by Gong et al. (2008) inferred a dose–response relationship between consumption of tortillas containing fumonisins and human urinary FB_1. Data from the present study verified that urinary FB_1 biomarker could be quantified using a rodent model following a single dose exposure. Furthermore, data from our human study in Ghana supports the work by Gong et al. (2008) in Mexico, which suggests that urinary FB_1 is a sensitive biomarker of fumonisin exposure. The decision to use urinary FB_1 as the diagnostic marker of NS efficacy was critical to our *in vivo* analyses. In our rodent study, all animals dosed with FB_1 displayed the urinary FB_1 biomarker within 24 h, regardless of treatment group. Levels of urinary FB_1 were considerably reduced 48 h after gavage. By 72 h, urinary FB_1 was not detectable. This 48-h window is similar to other urinary biomarkers of mycotoxin exposure (Wang et al. 2008). NS clay has been shown to sorb both aflatoxin and FB_1 *in vitro,* and can decrease the bioavailability and exposure to aflatoxins in humans and animals. Thus, it is possible that NS may also confer protection against FB_1 where dual-contamination of the diet is common. It is then proposed that NS was able to bind FB_1 either at the time of gavage or during the gastrointestinal passage. However, other possibilities may exist. For instance, clays similar to NS have been previously used as mucosa protectants (Droy-Lefaix and Tateo 2006) and

NS may have reduced the urine levels of this mycotoxin by limiting or delaying the contact of FB_1 with the intestinal cell barrier. Our study has two important limitations. One of them is that faecal levels of FB_1 were not measured and this would have been a better verification of our proposed mechanism of action of NS onto FB_1. Another limitation is that our study design does not allow to completely determine if FB_1 was bound to gavaged NS or if dietary NS was responsible for the reduction of FB_1 in urine. Nonetheless, in our rodent model, NovaSil (either by gavage or dietary) was able to significantly reduce urinary FB_1 by 20% at 24 h and by 50% at 48 h post-gavage. These data suggest that NS may be a suitable enterosorbent for the reduction of fumonisin exposure, confirming previous reports in our laboratory (Lemke 2000).

In our Ghanaian study, 60.9 and 72.1% of analysed samples from the placebo group contained detectable levels of urinary FB_1, compared to 75% detected in a Mexican population consuming a high intake of maize-based tortillas (Gong et al. 2008). The range of FB_1 in urine samples from our study varied from non-detectable to 33.12 ng FB_1 ml^{-1} urine, higher than values reported by Gong et al. (2008) (non-detectable to 9.31 ng FB_1 ml^{-1} urine). The week 8 and 10 mean FB_1 levels measured in participants of the placebo group in our study (9.36 and 5.03 ng FB_1 ml^{-1} urine, respectively) were higher than mean urinary FB_1 concentration reported in Mexico (0.147 ng FB_1 ml^{-1} urine) (Gong et al. 2008). Based on ~1% urinary excretion, an estimated metabolic rate of 1500 ml urine day^{-1} and the average size of an adult (70 kg), it can be estimated that the exposure to FB_1 at week 8 was 20.06 μg kg^{-1} bw day^{-1} and 10.78 μg kg^{-1} bw day^{-1} at week 10 in our placebo groups (Shephard et al. 1994; Fodor et al. 2008). More importantly, recent studies in a South African population consuming corn naturally contaminated with FBs have estimated an average of 0.075% urinary excretion rate of FB_1 (Van der Westhuizen et al. 2011). If this is the case, our Ghanaian population, may have been exposed to 10 times more than what we have previously estimated. These values are higher than the FB_1 levels that could be extrapolated from the Mexican study (0.368 μg kg^{-1} bw day^{-1}) (Gong et al. 2008). These differences between Mexican and Ghanaian population biomarker levels could be attributed to variations in maize storage, processing, and/or consumption. A market survey in Ghana detected levels of FB_1 between 70 and 2621 (mean 608.8) μg kg^{-1} in maize intended for human consumption (Jolly et al. 2006). Based on an average maize intake of 369.5 g day^{-1} (MacIntyre et al. 2002), a 1% carryover of FB_1 from the diet, and an average urinary excretion (1500 ml), Ghanaians from our site could potentially excrete urinary FB_1 levels between 0.172 to 6.46 ng ml^{-1} urine. Actual mean biomarker levels measured in the placebo group of the present study were similar to this estimate, with measured values of 9.36 and 5.03 ng FB_1 ml^{-1} urine in the week 8 and week 10 groups, respectively.

In the previous clinical trial in Ghana, significant decreases in median urinary aflatoxin M_1 levels were observed at 12 weeks after NS intervention ($p = 0.0445$) (Wang et al. 2008). Since samples that were collected at 12 weeks were depleted for aflatoxin biomarker analysis, we utilised samples at weeks 8 and 10, to assess the effect of NS treatment on urinary FB_1 levels. With NS, the week 8 samples had mean values of 4.05 and 5.61 ng ml^{-1} urine in the high and low dose treatment groups, respectively, compared with 9.36 ng ml^{-1} in the placebo group. At week 10, samples had lower mean values of 0.81 and 0.73 ng FB_1 ml^{-1} urine in the high and low dose treatment groups, respectively, versus 5.03 ng FB_1 ml^{-1} urine in the placebo group. While the lower levels of FB_1 measured in the participants of low dose and high dose NS are consistent with the effects of NS in reduction of FB_1 bioavailability, the possibility that the groups (placebo, low dose NS and high dose NS) were consuming different levels of FB_1 in the diets, represents a valid question. Nonetheless, the diets ingested by participants were likely to be uniform throughout the study period since the sources of maize tend to be constrained community wide. Furthermore, the effect of NS or placebo on FB_1 urinary levels was observed at two different time points.

When evaluating the means between the placebo (humans) or FB_1 control group (rats) and NS treated groups the reduction in urinary biomarker levels due to NS was more evident in the human study (a 49% reduction in week 8 and an 85% reduction in week 10) than in the rat study (a 19% reduction after 24 h and a 50% reduction after 48 h). This disparity between the mean reductions in FB_1 excretion (rats vs. humans) is not unexpected. In the clinical trial, Ghanaians were chronically exposed to FB_1 through the diet and treatment with NS (by capsules) occurred for a period of 3 months, whereas, in the rat study, group C was given FB_1 as a onetime dose and NS treatment only lasted for 2 weeks. Additionally, it is known that gastrointestinal transit times vary according to size and weight of different species. Thus, it is possible that, in the humans, NS and FB_1 had a longer time to interact resulting in a larger reduction in FB_1 excretion. Nevertheless, in the rat and human studies, NS significantly reduced the excretion of FB_1 biomarker in the urine. Further studies are warranted to establish whether a time-related effect exists allowing NS treatment to exert more effect at later time points. Based on the 10-week mean urine values in the NS treated groups, exposure could be estimated at 1.72 and 1.54 μg kg^{-1} bw day^{-1} for the high and low dose participants, respectively. Importantly, following consumption with NS, participant exposure to FB_1 was

reduced below the JECFA provisional maximum tolerable daily intake for FB_1 of $2\,\mu g\,kg^{-1}\,bw\,day^{-1}$ (JECFA 2001). Based on our previous research, NS clay has displayed: (1) favourable thermodynamic characteristics of aflatoxin sorption; (2) tolerable levels of priority metals, dioxins/furans and other hazardous contaminants; (3) safety and efficacy in multiple animal species and humans; (4) safety and efficacy in long-term animal studies; and (5) negligible interactions with vitamins, iron and zinc and other micronutrients. Additionally, recent work in our laboratory has shown that non-framework micronutrients associated with NS (i.e. Sr^{2+}) are bioavailable suggesting that NS has the potential to act as a supplement for these micronutrients as well as a mycotoxin enterosorbent in humans and animals (Afriyie-Gyawu et al. 2008). Further work is warranted to delineate the specific molecular mechanism(s) and surface chemistry involved in the sorption process of FB_1 onto NS and to confirm the efficacy and safety of NS clay as a multifunctional intervention for aflatoxins and fumonisins in animals and humans.

Mycotoxins have been linked to death and disease, with an estimated 4.5 billion people facing uncontrolled exposure in developing countries (Williams et al. 2004). The mycotoxin problem to public health is longstanding, unavoidable, and seemingly inextricable. Frequent and concurrent exposure to aflatoxins and fumonisins may enhance/synergise the toxic, carcinogenic, and teratogenic effects of these foodborne contaminants. This study is the first to demonstrate the efficacy of an enterosorbent intervention for FB_1 exposure using fumonisin biomarkers in human urine. Importantly, it also illustrates that a susceptible population in Ghana co-exposed to aflatoxins and fumonisins may be protected from the adverse effects of these mycotoxins using NS clay. As an intervention, capsules or other dose forms of NS could be used in clinical practice for ''acute emergencies'' and dietary practice for ''chronic exposures.'' Additional studies are planned to confirm these findings in long-term clinical intervention trials in Ghana and to look at the nutritional implications of NS supplementation of the diet. Clay-based enterosorbent strategies represent a novel approach to the problem in populations that are at high risk for mycotoxicosis. A similar clinical intervention with NS in a population at risk for hepatocellular carcinoma (HCC) in the USA is ongoing, and will examine dietary and environmental risk factors for HCC including aflatoxins and fumonisins and the effect of NS intervention.

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