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Calcium montmorillonite clay reduces AFB₁ and FB₁ biomarkers in rats exposed to single and co-exposures of aflatoxin and fumonisin

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Abstract

Aflatoxins (AFs) and fumonisins (FBs) can co-contaminate foodstuffs and have been associated with hepatocellular and esophageal carcinomas in humans at high risk for exposure. One strategy to reduce exposure (and toxicity) from contaminated foodstuffs is the dietary inclusion of a montmorillonite clay (UPSN) that binds AFs and FBs in the GI tract. In this study, the binding capacity of UPSN was evaluated for AFB₁, FB₁ and a combination thereof in Fischer-344 rats. Rats were pre-treated with different dietary levels of UPSN (0.25 or 2%) for 1 week. Rats were gavaged with a single dose of either 0.125 mg AFB₁ or 25 mg FB₁/kg b.w. and a combination thereof in the presence and absence of an aqueous solution of UPSN. The kinetics of mycotoxin excretion were monitored by analyzing serum AFB₁-albumin, urinary AF (AFM₁), and FB₁ biomarkers over a period of 72 hr. UPSN decreased AFM₁ excretion by 88-97%, indicating highly effective binding. FB₁ excretion was reduced, to a lesser extent, ranging between 45 to 85%. When in combination, both AFB₁ and FB₁ binding occurred, but capacity was decreased by almost half. In the absence of UPSN, the combined AFB₁ and FB₁ treatment decreased the urinary biomarkers by 67 and 45% respectively, but increased levels of AFB₁-albumin, presumably by modulating its cytochrome metabolism. UPSN significantly reduced bioavailability of both AFB₁ and FB₁ when in combination; suggesting that it can be utilized to reduce levels below their respective thresholds for affecting adverse biological effects.

Keywords

aflatoxin; fumonisin; HPLC; toxicity; clay adsorption; biomarkers

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INTRODUCTION

Naturally occurring foodborne carcinogens, such as aflatoxins and fumonisins, are predominantly investigated as independent compounds. However, complex mixtures of mycotoxins occur in the environment and could induce additive, antagonistic, or synergistic interactions when present together (Carpenter et al. 1998). Research has demonstrated that multiple mycotoxins can co-contaminate crops and foods intended for both animal and human consumption (Almeida et al. 2012; Kimanya et al. 2008; Sun et al. 2011). Concomitant exposure to mycotoxins, such as aflatoxin and fumonisin, has been associated with various teratogenic, mutagenic, estrogenic, neurogenic, and immunotoxic effects, as well as growth faltering, cancer and even death in acute incidences (Azziz-Baumgartner et al. 2005; Coulumbe Jr 1994; Gong et al. 2004; Gong et al. 2002; IARC 1993, 2002; Wild and Gong 2010; Williams et al. 2004; Wogan 1992). In particular, foods intended for human consumption in Sub-Saharan Africa have been shown to contain high levels of both aflatoxins and fumonisins (Kimanya et al. 2008; Kpodo et al. 2000; Kumi et al. 2011). Although research investigating the health effects due to co-exposure of these two mycotoxins is limited, several studies have elucidated their additive and synergistic capability. Co-exposure has been shown to increase toxicity in a hydra model (Brown et al. 2012), alter toxic responses in rats and mosquito fish (McKean et al. 2006), lower feed conversion and mean body weight in pigs (Dilkin et al. 2003), and increase the development of hepatocyte nodules in a rodent model (Gelderblom et al. 2002).

Aspergillus flavus and *A. parasiticus* fungi naturally produce four congeners of aflatoxins; AFB₁, AFB₂, AFG₁, and AFG₂. AFB₁ (Fig. 1A) is the most potent carcinogen of the four and is also the most commonly occurring in maize and groundnut crops (CAST 2003). The International Agency for Research on Cancer (IARC) has classified AFB₁ as a Group 1A carcinogen, due to its ability to induce liver cancer in humans. Consumption of contaminated foods has been implicated as the primary route of AFB₁ exposure, resulting in an increased risk for the development of HCC (Qian et al. 1994; Ross et al. 1992; Wang et al. 1996; Yu et al. 1997) and hepatic failure resulting in death (Lewis et al. 2005). Exposure to AFB₁ occurs predominantly in tropical and subtropical regions (including the US) that lie between the latitudes of 40°N and 40°S, where the climate promotes growth of the *Aspergillus* fungi and production of aflatoxins. These regions include Sub-Saharan Africa and Southeast Asia; where liver cancer is most prominent.

Fumonisin B₁ (Fig. 1B) is a congener of the fumonisin toxins produced by *Fusarium verticillioides* and *F. proliferatum* fungi and is the most abundantly produced fumonisin as well as the most toxicologically significant (JECFA 2001). FB₁, like AFB₁, is a common contaminant of maize and has been found in high levels in Sub-Saharan Africa, Central America, and Southeast Asia. Exposure to FB₁ has been implicated in various disease outcomes, illustrating the diverse biological effects that this toxin is capable of eliciting. Fumonisin has been linked to esophageal cancer, liver tumors, immune stimulation and suppression, neural-tube defects, nephrotoxicity, and other abnormalities (Chu and Li 1994; Grenier et al. 2011; Marasas et al. 2004; Voss et al. 2002). A recent study indicated that FB₁ is also associated with stunting in infants (Kimanya et al. 2010). FB₁ exhibits hepato – and

nephrocarcinogenic effects in rats and is classified as a Group 2B carcinogen, i.e. possibly carcinogenic to humans (IARC 1993, 2002). FB₁ is mainly regarded as a liver cancer promoter (Gelderblom et al. 1988), and has been shown to have synergistic interactions with AFB₁ in two-stage cancer initiation/promotion liver models utilizing rainbow trout and rats (Carlson et al. 2001; Gelderblom et al. 2002).

Since AFB₁ and FB₁ commonly co-contaminate foods, any therapeutic approach that could mitigate both mycotoxins would be highly attractive and more cost-effective than a combination of approaches. In particular, populations most at risk for exposure to both mycotoxins also suffer from food insecurity and poor economic conditions. Thus, a remediation strategy for such communities must function to reclaim contaminated foods in their entirety and cause minimal interference to daily life. In this study, a refined calcium montmorillonite clay, uniform particle size NovaSil (UPSN), is investigated for its potential to simultaneously and adequately sorb both toxins *in vivo*. UPSN is from the family of smectites that has been shown to sorb AFB₁ *in vitro* and in numerous animal and human models, thereby decreasing biomarkers of exposure in the urine and blood and protecting animals from toxic endpoints (Beaver et al. 1990; Colvin et al. 1989; Edrington et al. 1996; Phillips et al. 1988; Pimpukdee et al. 2004). Recently, *in vitro* analyses indicated that UPSN efficaciously bound FB₁ as well as mixtures of AFB₁ and FB₁ (Brown et al. 2012). This same study found that 1.4% UPSN adequately protected *Hydra vulgaris* organisms from AFB₁ and FB₁ co-exposure.

In the current study, our objectives were 1) to determine whether a dual protection would be feasible in a mammalian gastrointestinal system and 2) to assess the difference in UPSN efficacy when a mixture of AFB₁ and FB₁ is present, as opposed to a single toxin exposure. To investigate the effect that UPSN would have on AFB₁ and FB₁ bioavailability, well-established biomarkers of exposure to both toxins, i.e. urinary AFM₁, FB₁, and serum AFB₁-albumin adducts, were utilized (Cai et al. 2007; Gan et al. 1988; Groopman et al. 1994; Shephard et al. 2007). Levels of each biomarker were analyzed following exposure to a single treatment of AFB₁, FB₁, or a combination of both toxins with and without UPSN treatment.

MATERIALS AND METHODS

Reagents

High Performance Liquid Chromatography (HPLC) grade methanol and acetonitrile, as well as reagent grade hydrochloric acid, formic acid, sodium phosphate monobasic, sodium borate, sodium cyanide, dibasic potassium phosphate, and pH buffers (4.0, 7.0, and 10.0) were purchased from VWR (Atlanta, GA). *O*-phthaldialdehyde (OPA), AFM₁, and FB₁ analytical standards were purchased from Sigma-Aldrich (Saint Louis, MO). FB₁ specified for animal treatment was purchased from the PROMEC Unit of the South African Medical Research Council (Tygerberg, South Africa). UPSN was obtained from Texas Enterosorbents (Bastrop, TX). Ultrapure deionized water (18.2 MΩ) was used in all experiments.

Animal housing, diet, and treatments

Five-week old male Fischer-344 rats were purchased from Harlan (Houston, TX) and allowed to acclimate for one week before being randomly divided into ten treatment groups consisting of six animals per group (Table 1). Briefly, the treatment groups included an absolute control, positive controls (dosed with AFB₁, FB₁, or a mixture of the two), and groups treated with a one-time toxin dose in addition to UPSN clay (0.25 or 2% w/w feed consumption). These concentrations were chosen based on previous single toxin animal and human studies completed by our laboratory, in which 0.25% and 2% significantly reduced aflatoxin and fumonisin biomarkers, respectively (Phillips et al. 2002; Pimpukdee et al. 2004; Robinson et al. 2012). Control animals (no UPSN) were maintained on a nutritionally complete powdered feed (Teklad rodent diet 8604, Harlan) and water *ad libitum*. Animals that were placed in the 0.25 or 2% UPSN treatment groups received Teklad 8604 powdered feed homogeneously mixed with UPSN at the respective percentages and water *ad libitum*. Animal maintenance, husbandry, and treatment protocols involving AFB₁ and FB₁ exposures were reviewed and approved by the Institutional Animal Care and Use Committee at Texas A&M University. All rats were housed in a climate-controlled environment (22–25°C, 50% humidity) that was artificially illuminated (12-hr dark/light cycle) and free from chemical contamination. Rats were inspected daily for general appearance, behavioral changes, and signs of morbidity or mortality, although none were observed. Following the one week acclimation period animals weighing 131.2 ± 15.0 g were gavaged with 0.125 mg AFB₁/kg b.w. and/or 25 mg FB₁/kg b.w. These levels were minimal doses required to provide sufficient levels of urinary AFM₁ and FB₁ for quantitation (Robinson et al. 2012; Sarr et al. 1995). Aqueous gavage solutions administered to UPSN treatment groups also contained UPSN at an amount equivalent to one day's intake. UPSN and toxins were mixed immediately before the gavage procedure to simulate the addition of UPSN to mycotoxin-contaminated foods just prior to human consumption. Based on an average daily feed consumption of 20 g the doses were as follows: the 0.25% group was gavaged with 0.05 g and the 2% groups were given 0.4 g UPSN, respectively. Following gavage, animals were housed individually in metabolic cages (Nalgene™) for 72 hr and urine was collected at 12, 24, 36, 48, and 72 hr. Samples were stored at –20°C for AFM₁ and FB₁ biomarker analyses. Animals were anesthetized via isoflurane gas administration 72 hr following gavage, then blood samples were collected via heart stick. Euthanasia was accomplished by exsanguination. Serum samples were stored at –20°C until AFB₁-albumin analysis. Liver and kidney weights were also recorded and expressed as a percentage of the total body weight.

Analysis of urinary AFM₁

AFM₁ concentrations in the urine were analyzed using immunoaffinity column purification using methods previously described (Groopman et al. 1992; Sarr et al. 1995; Wang et al. 1999). Each urine sample (1 ml) was adjusted to an acidic pH with 1.0 M ammonium formate (pH 4.5) and the volume increased to 10 ml with water. Samples were then passed through a VICAM™ AflaTest WB immunoaffinity column (VICAM, Watertown, MA) at a rate of 1 ml/min. Following two separate washing steps, AFM₁ was eluted with 2 ml of 80% methanol. Samples were dried under nitrogen gas and re-constituted with 100 µl of 20 mM

ammonium formate and 100 μ l of 80% methanol. A Waters HPLC system (Waters Corporation, Milford, MA) with fluorescence detection (excitation 365 nm and emission 425 nm) was used to separate sample constituents and isolate the AFM₁ metabolite. Chromatographic separation was achieved by isocratic elution of the mobile phase (22% ethanol: 78% 20 mM ammonium formate in water) on a 250 X 4.6 mm LiCrospher RP-18 end-capped column with pore size 100 Å and 5 μ m particle size (Discovery Sciences, Deerfield, IL). Quantification of AFM₁ was based on peak area and retention time as compared to external standards run between every 5 samples. The limit of detection for urinary AFM₁ using this method was 4.8 pg. Creatinine analysis was performed on all urine samples at the Texas Veterinary Medical Diagnostic Laboratory (College Station, TX) to adjust for variations in urine dilution.

Analysis of urinary FB₁

Following centrifugation, a 300 μ l aliquot of supernatant was loaded into a pre-equilibrated VICAMTM FumoniTest immunoaffinity column and passed through by gravity. After washing with 10 ml of PBS, FB₁ was eluted three times with 0.5 ml of 20% methanol in 10 mM hydrochloric acid directly onto a pre-conditioned Waters Oasis HLB cartridge (3 cc/60 mg, 30 μ m particle size). The HLB cartridge was sequentially washed with 2 ml water and 2 ml 30% aqueous methanol, and then eluted three times with 0.6 ml of 2% formic acid in methanol. Eluents were evaporated to dryness under nitrogen gas while incubated at 35°C. Dry residues were reconstituted with 150 μ l of 50% aqueous methanol.

HPLC-fluorescence analysis was carried out on an Agilent 1100 liquid chromatography system (Agilent Technologies, Santa Clara, CA). FB₁ molecules were derivatized with ophthalaldehyde (OPA) prior to fluorescence detection (Xu et al. 2010). To avoid degradation of the derivatives, an on-line automatic injector thoroughly mixed 20 μ l of OPA reagent with the sample for one minute immediately prior to each injection. Chromatographic separations were performed on a Luna C18 (2) column (Phenomenex Torrance, CA; 5 μ m particle size, 250 $\times$ 4.6 mm), maintained at 35°C. Molecules were resolved with a linear gradient starting from 0.1 M sodium phosphate monobasic (pH 3.4): methanol (35/65, v/v) to 0.1 M sodium phosphate monobasic (pH 3.4): methanol (20/80, v/v) over 13 min, with a flow rate of 1.0 ml/min and injection volume of 100 μ l. The OPA derivative was monitored at an emission wavelength of 440 nm and excitation wavelength of 330 nm. The limit of detection for this method was 20 pg.

Analysis of serum AFB₁-albumin adduct

Serum from all AFB₁ and AFB₁/FB₁ treatment animals was assessed for AFB₁-albumin levels 72 hr following gavage. The methodology to complete this analysis was adopted as previously reported (Qian et al. 2010; Qian et al. 2013). Briefly, 150 μ L of serum was digested with pronase (pronase:total protein, 1:4, w:w) for 3 h at 37°C. The digest was further purified by a solid phase cartridge, evaporated, reconstituted, and injected into an Agilent 1200 HPLC system for quantification by fluorescence signal. Excitation and emission wavelengths were 405 and 470 nm, respectively. The limit of detection was 0.4 pg/mg albumin. Serum AFB₁-albumin adduct concentrations were adjusted by total serum albumin content.

Statistical analysis

Serum levels of AFB₁-albumin adduct and urinary AFM₁ and FB₁ are expressed as mean $\pm$ standard deviation (SD). Data was not normally distributed and was therefore log transformed for statistical analysis. Comparisons of these parameters between treated groups and the control group were conducted using one-way analysis of variance (ANOVA) followed by a Dunnett's test to compare each UPSN group with its respective positive control. Differences between the UPSN treated groups were analyzed via a Tukey's test. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Organ and body weights

No significant differences in body weight, body weight gain, or target organ weights were observed during the study. At the conclusion of the experiment (72 hr), animals weighed 145.3 ± 14.5 g. Table 2 shows the somatic index for each organ by treatment group.

AFM₁ analysis from AFB₁ treated groups

Mean urinary AFM₁ was calculated based on six rats per treatment group. Absolute control animals receiving an equal volume (0.75 ml) gavage of ultra-pure water did not have detectable AFM₁ metabolites in the urine at any of the collection time points. Excretion of AFM₁ peaked between 12 and 24 hours following AFB₁ treatment. Five out of six rats were completely void of AFM₁ by the 36 hr timepoint (Figure 2A). The positive control group displayed the highest average concentration of AFM₁ (201 ng/mg creatinine) at the 12 hr time point. Comparisons of AFM₁ levels between UPSN treatment groups and positive controls showed a marked decrease in overall excretion of AFM₁ metabolites. UPSN treatment reduced AFM₁ biomarkers in a dose-dependent manner with the largest reduction observed in the 2% treatment group. UPSN at 0.25% reduced AFM₁ excretion by 88%, 94%, and 85% at 12, 24, and 36 hr time points, respectively (Figure 2B). AFM₁ levels in the 2% UPSN treatment group were reduced by 97%, 99%, and 58% at 12, 24, and 36 hr, respectively. The decrease in AFM₁ observed in both of the UPSN treatment groups was highly significant ($p < 0.001$) at 12 and 24 hr from the positive controls. The 0.25% UPSN group was also significantly different from the 2% UPSN group ($p = 0.0215$).

AFM₁ levels in AFB₁ /FB₁ treatment groups

AFM₁ excretion patterns were different between AFB₁ treatment groups and AFB₁/FB₁ mixture groups. AFM₁ excretion by the co-exposure group peaked at 12 hrs and was almost undetectable by 24 hrs. The highest average AFM₁ concentration in the urine was 118 ng/mg creatinine at 12 hr in animals dosed with the AFB₁/FB₁ mixture (Figure 2C). This value is approximately 2 times lower than the average concentration in animals dosed with AFB₁ alone. There was considerable variation observed in the overall excretion of AFM₁ in the AFB₁/FB₁ treated positive control group (SD of 117,002). Therefore, although the average AFM₁ output at 12 hr was almost 2 times higher for the AFB₁ treated group alone the two groups were not significantly different from each other. UPSN treatment dose-dependently reduced AFM₁ excretion in animals exposed to the AFB₁/FB₁ mixture.

However, the percent reductions observed in the mixture groups were lower than those in the AFB₁ control groups. Compared to AFB₁/FB₁ positive controls, the 0.25% UPSN group exhibited AFM₁ decreases of 66% and 32% at 12 and 24 hr, respectively. AFM₁ levels were non-detectable in any groups treated with UPSN by the 36 hr timepoint. AFM₁ excretion levels observed during UPSN inclusion were significantly different from the AFB₁/FB₁ control group for the 2% UPSN only at the 12 hr time point ($p=0.0039$). The 0.25% UPSN group excreted levels significantly higher than the 2% UPSN group ($p=0.0286$) at 12 hr. AFM₁ excretion in the 0.25% UPSN group was not significant at this time point most likely due to the high standard deviation observed in the AFB₁/FB₁ control group. Although the UPSN treated groups had lower AFM₁ output at 24 hr than the control group, the difference was not statistically significant.

FB₁ analysis from FB₁ treated animals

Urine samples were also analyzed for FB₁ with HPLC-coupled fluorescence detection. Mean FB₁ levels were calculated from six animals per treatment group. Absolute control animals did not have detectable levels of FB₁ present in their urine at any time point assessed. As expected, the highest levels of FB₁ excretion occurred 12 hr post-gavage and decreased rapidly, (Figure 3A). At 12 hr, mean urinary excretion for animals dosed with FB₁ alone was 737 ± 110 ng/mg creatinine. Addition of UPSN clay at the 2% w/w level significantly decreased the amount of FB₁ excreted at 12, 24, and 36 hr compared to the positive control group ($p<0.0001$). The 2% UPSN group also excreted significantly lower FB₁ than the 0.25% UPSN group at the 12, 24, and 36 hr time points ($p=0.0013$, $p<0.0001$, and $p=0.0127$, respectively). However, the 0.25% UPSN group was not significantly different from the positive control group at any of the time points except 36 and 72 hr ($p=0.0141$ and 0.0284 , respectively). Although not significant at 12 and 24 hr, 0.25% UPSN decreased overall excretion of FB₁ as well. Mean levels of FB₁ excreted by the 0.25% and 2% UPSN groups at 12 hr were 407 ± 168 and 109 ± 136 ng/mg creatinine, respectively, and 258 ± 139 and 12 ± 72 ng/mg creatinine at 24 hr. Overall reduction of the FB₁ biomarker for the 0.25% UPSN inclusion group was 45% at 12 hr and 55% at 24 hr, compared to the positive control group. UPSN inclusion at the 2% level reduced urinary FB₁ by 85% and 98% at 12 and 24 hr (Figure 3B).

FB₁ analysis from AFB₁ /FB₁ co-treatment

Mean urinary levels of FB₁ were 572 ± 260 and 94 ± 62 ng/mg creatinine at 12 and 24 hr post-gavage, respectively, in the AFB₁/FB₁ co-treated positive control samples. A dose-dependent reduction in urinary FB₁ was observed for the UPSN treated AFB₁/FB₁ groups, with the 2% UPSN group exhibiting the largest difference from the positive control group (Figure 3C). The FB₁ biomarker in the 0.25% UPSN group was reduced by 28% and 39% at 12 and 24 hr after gavage and by 51% and 59% for the 2% UPSN group, respectively (Figure 3D). Reduction in FB₁ was significant in the 2% UPSN group at 12 ($p=0.0177$), 24 ($p=0.0284$), and 72 hr ($p<0.0001$) time points compared to the positive controls. The reduction observed in the 0.25% UPSN group was not statistically significant except at the 72 hr time point ($p=0.0369$).

Area under the curve calculations

Figure 4 shows the differences in kinetics for total AFM₁ and FB₁ excretion in the urine by calculations of area under the curve (AUC) for each treatment group. Based on AUC, approximately 75% of the total AFM₁ excreted was detected within the first 24 hr after AFB₁ exposure in the positive control group. The 0.25% UPSN group excreted the equivalent of 9% of the total AFM₁ that was excreted by the AFB₁ treated positive control group (Figure 4A). Based on the total AUC, the 2% UPSN group excreted the equivalent of 2% of the amount of AFM₁ excreted by the AFB₁ treated positive control, respectively. The mean excretion of AFM₁ biomarkers was significantly different ($p=0.0002$) between animals dosed with AFB₁ alone and animals receiving both AFB₁ and FB₁. The AUC value for AFM₁ excretion in the AFB₁/FB₁ control animals was approximately three times smaller than the AUC for the AFB₁ control animals (Figure 4A). Based on AUC values, the 0.25% and 2% UPSN groups excreted the equivalent of 37% and 7% of the AFM₁ excreted by the AFB₁/FB₁ positive controls.

Approximately 71% of the total FB₁ excreted by the FB₁ treated positive control group was excreted within the first 24 hr after gavage. Based on the AUC calculations, the 0.25% UPSN group excreted the equivalent of 52% of the total FB₁ excreted by the FB₁ treated control group. The 2% UPSN group excreted the equivalent of 16% of the total FB₁ excreted by the FB₁ treated control group (Figure 4B). Similar to the change in kinetics of AFM₁ excretion after AFB₁/FB₁ co-exposure, total FB₁ excreted in AFB₁/FB₁ combination groups was significantly lower than total FB₁ excreted in the FB₁ treated controls ($p=0.0377$). Total AUC values indicated a 45% reduction in the amount of FB₁ excreted by the AFB₁/FB₁ combination group compared to the positive control group. Based on the AUC calculations, the 0.25% and 2% UPSN groups excreted the equivalent of 64 and 43% of the total FB₁ excreted by the AFB₁/FB₁ positive controls (Figure 4B). UPSN treatment was less effective in reducing AFM₁ and FB₁ urinary biomarkers after AFB₁/FB₁ co-exposures than after exposure to either AFB₁ or FB₁ alone.

AFB₁-albumin analysis

Analysis of AFB₁-albumin adducts in the serum was conducted following euthanasia (72 hr after gavage). Means and standard deviations were calculated based on six animals per group. Compared to positive control groups, both UPSN concentrations significantly reduced AFB₁-albumin adduct levels (all p -values <0.0001). UPSN concentration of 0.25% was also significantly different ($p<0.0001$) compared to the 2% group (Figure 5A). The mean AFB₁-albumin concentration was 632 pg/mg albumin for the positive control group (AFB₁ treatment alone), whereas serum from the 0.25% and 2% UPSN groups contained 101 and 36 pg/mg albumin, respectively. The percent reduction in AFB₁-albumin levels by UPSN treatment group are shown in Figure 5B. Animals that were gavaged with a combination of AFB₁/FB₁ had significantly higher AFB₁-albumin levels ($p=0.0061$) than the AFB₁-treated controls. However, UPSN clay was still capable of significantly decreasing the AFB₁-albumin biomarker in a dose-dependent manner (Figure 5A). In the AFB₁/FB₁ combination group, the mean AFB₁-albumin level was 1,112 pg/mg albumin. The adduct concentrations in the 0.25% and 2% UPSN groups co-exposed to AFB₁/FB₁ were significantly different from each other (242 and 37 pg/mg albumin, respectively).

DISCUSSION

Previous isothermal data from our laboratory indicated that NovaSil clay (a calcium montmorillonite) can tightly sorb AFB₁ and reduce the bioavailability of AFB₁ in the gastrointestinal tract of animals and humans (Phillips et al. 1995; Phillips et al. 2008). Importantly, clay treatment resulted in lower levels of well-established short and long-term biomarkers of aflatoxin exposure in the urine and serum, i.e. AFM₁ and AFB₁-albumin adducts (Wang et al. 2008). Recent studies demonstrated that NovaSil clay also reduced biomarker levels of FB₁ in rats and humans (Robinson et al. 2012). A uniform particle size NovaSil clay (UPSN) was produced to maintain purity and consistency from batch to batch for use in human populations. In preliminary work, UPSN was shown to retain the binding properties of the parent product (NovaSil) (Marroquin-Cardona et al. 2011). Binding analysis performed with heat-collapsed UPSN suggests that AFB₁ and FB₁ binding is saturable and occurs largely within the interlayer regions of the clay (Brown et al. 2012; Grant and Phillips 1998) providing preliminary evidence that both mycotoxins may compete for the same binding sites on surfaces of UPSN.

In the present study, inclusion of UPSN montmorillonite clay during aflatoxin exposure decreased the excretion of AFM₁ in the urine and the production of the AFB₁-albumin adduct in the serum of rats. The overall excretion of AFM₁ was similar to that reported in a study utilizing Fischer-344 rats in which the majority of AFM₁ excreted in the urine occurred within the first 12 hours following gavage of AFB₁ (Sarr et al. 1995). A study utilizing dogs demonstrated that calcium montmorillonite treatment, via food inclusion, following AFB₁ gavage could lower urinary excretion of AFM₁ by as much as 65% (Bingham et al. 2004). Species differences in absorption, metabolism, length of digestion, clay dose dissemination, and treatment regimen could all possibly account for the differences in efficacy seen between these studies. While dogs will consume an entirety of a meal in one sitting, this is not typically the case in rodents. Thus, it was deemed necessary to include UPSN in the gavage solution for the rodent model to ensure adequate opportunity for UPSN to interact with AFB₁, thereby preventing adsorption into the vascular system. The difference in binding efficacy obtained in the current study could be ascribed to the route of exposure (i.e., gavage vs. dietary) and other physiological conditions prevailing in the gut of rodents.

Although extensive research focusing on the binding capacity of calcium montmorillonite for aflatoxin has been conducted, it is only recently that its sorption capability for fumonisin has been addressed. Robinson et al. (2012) demonstrated a significant decrease in fumonisin biomarkers of exposure following treatment with clay in both rats and humans. In the rodent model given 2% clay, FB₁ excretion in the urine was reduced by 20% at 24 hr post-gavage and 50% at 48 hr post-gavage (Robinson et al. 2012). In the current study, the reduction of FB₁ biomarkers following inclusion of 2% UPSN was higher (97%) after 24 hr. The protocols for these two studies were similar; however, in this study a refined form of calcium montmorillonite (UPSN) was used with a narrower range of particle size (45-100 µm) compared to the parent clay utilized in the Robinson et al. study. This difference could influence binding affinity and the reduction of urinary metabolites. Isothermal analysis has

yet to be conducted to verify this hypothesis, therefore binding affinity could still be considered a factor in the increased reduction of urinary metabolites.

The present study verifies the results reported for other rodent based metabolic studies indicating that treatment with UPSN can reduce the excretion of AFM₁ and FB₁ in the urine following exposure to the mycotoxins separately. However, AFB₁ and FB₁ can co-occur under natural conditions (Almeida et al. 2012; Kimanya et al. 2008; Sun et al. 2011) which could enhance risk due to possible synergistic and/or additive effects. The mixture of AFB₁ and FB₁ resulted in a slightly lowered efficacy of UPSN in reducing these mycotoxins. Based on AUC calculations, the 2% UPSN group reduced the total amount of AFM₁ excreted by 98% in the AFB₁ treated group alone, and 93% in the AFB₁/FB₁ treated group. AUC values indicated that 2% UPSN decreased total fumonisin excretion by 84% in the FB₁ treated group and only reduced by 57% in the AFB₁/FB₁ mixture. The reduced efficacy of UPSN binding observed in the mixture groups could be an indicator of competition for binding sites on the clay. It is evident from this data that although UPSN can bind both AFB₁ and FB₁, it will preferentially bind AFB₁ during co-exposures. This hypothesis is further supported by the fact that there were more molecules of fumonisin (3.46×10^{-5} mole/kg) available for binding than molecules of aflatoxin (4.01×10^{-7} mole/kg). However, when kinetic excretion data represented in Fig. 2 and 3 is reassessed on a molar basis, trends in excretion between single and co-exposures remain consistent.

Apart from the effect of UPSN, the amount of AFM₁ and FB₁ excretion in the urine was significantly lower when the toxins were dosed together than when either was dosed alone in control groups. While it is known that male rats are susceptible to renal damage and potential urinary output changes following repetitive FB₁ exposure (JECFA, 2001), a one-time dose of 25 mg/kg b.w. is not likely to alter renal function. This conclusion is based upon the fact that male Fischer 344 rats gavaged with up to 23 mg/kg b.w. for 14 consecutive days exhibited no evidence of renal damage (Gelderblom et al., 1994). Since it is known that AFB₁ is more toxic than FB₁, the relative reduction in their absorption from the gut should also be considered. In addition, it is known that AFB₁ is absorbed at a high rate, whilst FB₁ is poorly absorbed ranging from 1 to 6% in non-ruminants (Gan et al. 1988; Martinez-Larranaga et al. 1999; Scholl et al. 1996). Studies, utilizing trans-epithelial electrical potential suggest that mycotoxins modulate the Na⁺ co-transport involved in sugar and amino acid transport carrier systems (Grenier and Appelgate 2013). It is not known whether the uptake of AFB₁ and FB₁ also co-interact via these mechanisms, although the current study implies that they countered each other's' absorption from the gut. The modulating role of FB₁ on lipid metabolism with the disruption of sphingolipid, phospholipid, cholesterol, and fatty metabolism has been reported, resulting in toxic effects including an increase in oxidative damage to different cellular constituents including the cellular membrane (Abel and Gelderblom 1998; Gelderblom et al. 2001; Riley et al. 2001). These different adverse biological parameters are likely to disrupt membrane integrity, leading to intestinal toxicity (Bouhet and Oswald 2007) and potentially altered absorption.

Although the urinary biomarkers were reduced when AFB₁ and FB₁ were co-treated, the blood AFB₁-albumin biomarker was increased in the presence of FB₁. Multiple pathways of detoxification and activation occur during metabolism of AFB₁ involving many different

cytochrome P-450 enzymes (CYP-450). In particular, the AFM₁ and AFB₁-albumin products are considered to be involved in two separate pathways (detoxification and activation, respectively) but can be products of the same enzymes (Massey et al. 1995). In rats, the formation of AFM₁ is catalyzed predominantly by CYP1A1 and 1A2, while the production of the AFB₁ 8,9-epoxide occurs through catalysis with CYP1A2, CYP2A3, CYP2B7, CYP3A3, and CYP3A4 with the latter isoform having the highest affinity for AFB₁ (Massey et al. 1995). Higher levels of the AFB₁-albumin adduct suggest increased production of the most toxic and carcinogenic metabolite, AFB₁ 8,9-epoxide in the presence of FB₁. In earlier work, it was shown that FB₁ induced the activity of CYP1A1, 3A1 and 4A1 in rats following a 6 day intraperitoneal treatment (Martinez-Larranaga et al. 1996). This induction may be responsible for the higher level of the AFB₁-albumin adduct observed in this study. Glutathione S-transferases (GSTs) also play a major role in detoxification of the AFB₁-epoxide in rats, through conjugation with glutathione (GSH). Therefore, a decrease in GST activity and cellular GSH levels could also negatively affect the amount of the AFB-alb biomarker in serum as well. Thus, the carcinogenic potency of AFB₁ is likely to increase in the presence of FB₁. However, recent work has indicated that FB₁ treatment results in an increase in intracellular GSH levels (Domijan et al. 2011; Rumora et al. 2007). More studies are warranted to support this conclusion and the consequences of toxin combinations in the diet.

The results from this study provide evidence for an economical and sustainable intervention to reduce exposure to both aflatoxin and fumonisin from contaminated diets. Interesting interactive effects were noticed related to the combined treatment of the mycotoxins in the absence of UPSN, resulting in a 45% and 67% reduction in FB₁ and AFB₁ adsorption. The metabolic conversion of AFB₁ could also be altered resulting in an increased conversion into the 8,9 epoxide and AFM₁ presumably due to the selective induction of CYP isoforms by FB₁. While more research is warranted to find an optimal inclusion level for UPSN, the prospect of utilizing this clay as a binder for both toxins is promising. This could facilitate important applications to selectively reduce levels below the carcinogenic thresholds for initiation and promotion of hepatocellular carcinoma.

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Figure 1.
(A) Aflatoxin B₁ and (B) fumonisin B₁.

Figure 2.

Mean excretion pattern of AFM₁. (A) 0.125 mg AFB₁ per kg body weight-treated groups with 0, 0.25 and 2% UPSN. (B) Table of percent reduction between the AFB₁-treated positive control group (no clay) and each AFB₁/UPSN group at time points 12, 24 and 36 h after gavage. (C) AFB₁/FB₁ mixture groups with 0, 0.25 and 2% UPSN. (D) Table of percent reduction between the AFB₁/FB₁-treated positive control group (no clay) and each AFB₁/FB₁/UPSN group at time points 12, 24 and 36 h after gavage.

Figure 3.

Mean excretion pattern of FB₁. (A) 25 mg FB₁ per kg body weight-treated groups with 0, 0.25 and 2% UPSN. (B) Table of percent reduction between FB₁-treated positive control group (no clay) and each FB₁/UPSN group at time points 12, 36, and 24 h after gavage. (C) AFB₁/FB₁ mixture groups with 0, 0.25 and 2% UPSN. (D) Table of percent reduction between the AFB₁/FB₁-treated positive control group (no clay) and each AFB₁/FB₁/UPSN group at time points 12, 36, and 24 h after gavage.

Figure 4.

(A) Area under the curve (AUC) for pharmacokinetic excretion of AFM₁ in urine. The black bars are indicative of total AUC for rats treated with 0.125 mg AFB₁ per kg body weight and 0, 0.25 or 2% UPSN. Gray bars are indicative of total AUC for rats treated with the AFB₁/FB₁ mixture and 0, 0.25 or 2% UPSN. (B) AUC for pharmacokinetic excretion of FB₁ in urine. Black bars are indicative of total AUC for rats treated with 25 mg FB₁ per kg body weight and 0, 0.25 or 2% UPSN. Gray bars are indicative of total AUC for rats treated with the AFB₁/FB₁ mixture and 0, 0.25 or 2% UPSN. * Indicates a significant difference between the single toxin treatment and the mixture treatment with the same inclusion percent of UPSN ($P < 0.01$).

Figure 5.

(A) Mean serum AFB₁-albumin levels for the AFB₁ and AFB₁/FB₁-treated groups.

*Indicates a significant difference between the positive control group and UPSN-treated groups for the respective toxin treatments ($P < 0.01$). (B) Table of percent reduction between the positive control group (no clay) and each UPSN group.

Table 1

Study treatment groups

Treatment groups	0% UPSN	0.25% UPSN	2.0% UPSN
0.125 mg AFB ₁ /kg b.w.	6	6	6
25 mg FB ₁ /kg b.w.	6	6	6
25 mg FB ₁ /kg b.w. + 0.125 mg AFB ₁ /kg b.w.	6	6	6
Absolute Control	6	0	0

Table 2

Percent weight of target organs by total body weight

Treatment Group	Somatic Index	
	Liver	Kidney
Absolute Control	4.86 ± 0.45	0.48 ± 0.04
AFB ₁	4.76 ± 0.16	0.48 ± 0.01
FB ₁	4.85 ± 0.17	0.48 ± 0.03
AFB ₁ /FB ₁	4.77 ± 0.20	0.43 ± 0.02
AFB ₁ + 0.25% UPSN	4.94 ± 0.21	0.48 ± 0.05
AFB ₁ + 2% UPSN	4.74 ± 0.16	0.48 ± 0.02
FB ₁ + 0.25% UPSN	4.89 ± 0.20	0.48 ± 0.03
FB ₁ + 2% UPSN	4.54 ± 0.20	0.44 ± 0.02
AFB ₁ /FB ₁ + 0.25% UPSN	4.65 ± 0.15	0.42 ± 0.01
AFB ₁ /FB ₁ + 2% UPSN	4.59 ± 0.20	0.44 ± 0.02