Food Additives & Contaminants
Part A - Chemistry, Analysis, Control, Exposure & Risk Assessment

NovaSil clay intervention in Ghanaians at high risk for aflatoxicosis: II. Reduction in biomarkers of aflatoxin exposure in blood and urine

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NovaSil clay intervention in Ghanaians at high risk for aflatoxosis: II. Reduction in biomarkers of aflatoxin exposure in blood and urine

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The efficacy of NovaSil clay (NS) to reduce aflatoxin (AF) biomarkers of exposure was evaluated in 656 blood samples and 624 urine samples collected from study participants during a 3-month phase IIa clinical intervention trial in Ghana. NS was delivered before meals via capsules. Serum AFB\textsubscript{1}–albumin adduct was measured by radioimmunoassay and urinary AFM\textsubscript{1} metabolites were quantified by immunoaffinity-high-performance liquid chromatography (HPLC)-fluorescence methods. Levels of AFB\textsubscript{1}–albumin adduct in serum samples collected at baseline and at 1 month were similar (\(p = 0.2354\) and \(p = 0.3645\), respectively) among the placebo (PL), low dose (LD, 1.5 g NS day\textsuperscript{-1}) and high dose (HD, 3.0 g NS day\textsuperscript{-1}) groups. However, the levels of AFB\textsubscript{1}–albumin adduct at 3 months were significantly decreased in both the LD group (\(p < 0.0001\)) and the HD group (\(p < 0.0001\)) compared with levels in the PL group. Levels of AFM\textsubscript{1} in urine samples collected at baseline and at 1 month were not statistically different among the three study groups. However, a significant decrease (up to 58%) in the median level of AFM\textsubscript{1} in samples collected at 3 months was found in the HD group when compared with the median level in the PL group (\(p < 0.0391\)). In addition, significant effects were found for dose, time, and dose–time interaction with serum AFB\textsubscript{1}–albumin adduct and dose–time interaction with urinary AFM\textsubscript{1} metabolites. The results suggest that capsules containing NS clay can be used to reduce effectively the bioavailability of dietary AF based on a reduction of AF-specific biomarkers.

Introduction

Aflatoxins (AFs), produced predominantly by Aspergillus flavus and Aspergillus parasiticus, represent a group of naturally occurring fungal metabolites (mycotoxins) that have long been recognized as hazardous contaminants of food (Busby and Wogan 1984). Aflatoxin B\textsubscript{1} (AFB\textsubscript{1}) is hepatotoxic and genotoxic, and has been categorized as a known human carcinogen (Group I) (International Agency for Research on Cancer (IARC) 1993, 2002; Wang and Groopman 1999). Acute exposure to AFs via the diet can result in disease (aflatoxosis) and death in humans, as evidenced by numerous reports, including the recent outbreak in Kenya (Azziz-Baumgartner et al. 2005; Lewis et al. 2005). Chronic exposure to AFs is one of the major risk factors in the aetiology of human hepatocellular carcinoma (HCC) in several regions of Africa and South East Asia (Wogan 1992; IARC 1993, 2002). Importantly, AFB\textsubscript{1} has also been shown to be a potent immunotoxid agent in animals and humans (Hinton et al. 2003; Turner et al. 2003; Jiang et al. 2005). Therefore, development and application of practical and highly effective intervention strategies and therapies for aflatoxoses are critical for improving human health, especially in high-risk populations.

Humans and animals (for centuries and on most continents) have been reported to eat clay minerals (geophagy). The reasons for this behaviour are generally ill-defined, but clay eating is usually perceived to be beneficial and safe (Carretero 2002). For example, clay eating by people in close contact with nature is very common, and in many parts of South America and Africa the dietary use of clay is culturally acceptable. NovaSil clay (NS) is a naturally occurring and heat-processed calcium montmorillonite that is commonly used as an anticaking additive in animal feed. Previous research has shown that NS is a selective enterosorbent for AFs when included in the diet at levels up to 0.5% (weight to weight) in animal models (Phillips 1999; Phillips et al. 2002, 2006). NS significantly protected a variety of young animals from...
aflatoxicosis, including chicks (Phillips et al. 1988; Pimpukdee et al. 2004), turkey poults (Kubena et al. 1991), pigs (Lindemann et al. 1993), lambs (Harvey et al. 1991a), and rodents (Mayura et al. 1998). In addition, NS also reduced AF residues in milk from dairy cows (Harvey et al. 1991b) and goats (Smith et al. 1994), as well as biomarkers of AF exposure in rodents (Phillips 1999). Mechanistically, NS decreases the uptake of AF in the gastrointestinal tract, leading to significant reduction in AF bioavailability (to the blood, liver and other organs), and subsequent toxicity (Phillips 1999; Phillips et al. 2002, 2006). Information derived from equilibrium adsorption isotherms and molecular modelling studies has indicated that NS has a preference for AFB1 which contains a planar ketolactone system (Phillips et al. 2002, 2006).

No observable adverse effects have been reported in short-term animal studies following the addition of NS to the diet (Phillips 1999; Phillips et al. 2002, 2006). No maternal or foetal toxicity was found in Sprague–Dawley rats ingesting NS at dietary concentrations as high as 2.0% throughout pregnancy (Mayura et al. 1998; Wiles et al. 2004). In addition, no significant changes in trace metal bioavailability were found in a variety of maternal or foetal tissues (Wiles et al. 2004).

In a chronic study, Sprague–Dawley rats treated with 0.25–2.0% NS clay in the diet over a 6-month period did not exhibit dose-dependent adverse effects on body weight gains, feed conversion ratios, relative organ weights, gross and histological appearance of major organs, and haematological and serum biochemistry parameters. In addition, essential nutrient levels including vitamins A and E, iron, and zinc were unaffected (Afriyie-Gyawu et al. 2005).

Given the safety and efficacy of NS in multiple animal models, as well as its low cost, NS inclusion may be beneficial in the diets of humans at high risk for aflatoxicosis especially in developing countries. Initially, a 2-week phase I clinical trial in healthy volunteers showed that daily intake of NS up to 3 g day\(^{-1}\) had no significant adverse effect on human subjects (Wang et al. 2005). Based on the findings from this study, a 3-month randomized, double-blinded, and placebo-controlled phase IIa intervention trial was carried out in 180 Ghanaians who were exposed to AFs from their diet (Afriyie-Gyawu et al. 2007). In this report the efficacy of NS intervention was evaluated by analysing biomarkers in serum and urine samples collected before the study (baseline), at 1 and 3 months of the intervention, and at 1-month post-intervention. Results of this study support the prospect of using NS in the diet of humans to block, or significantly diminish, exposure to AFs and to prevent the adverse effects of AFs in humans consuming AF-contaminated foods.

Materials and methods

Materials

\([^{3}\text{H}]\text{AFB}_1\) was purchased from Moravek Biochemicals (Brea, CA, USA). Standard \(\text{AFB}_1\), \(\text{M}_1\) and radio-immunoassay reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA). Monoclonal antibody 2B11 was kindly provided by Dr G. N. Wogan at MIT. Immunoaffinity columns were purchased from VICAM (Watertown, MA, USA). NS clay was originally obtained from Engelhard Chemical Corporation (Iselin, NJ, USA), and was further examined for potential environmental contaminants including polychlorinated dibenzo-\(p\)-dioxins/furans (PCDDs/PCDFs) and heavy metals to ensure compliance with federal and international standards, as previously described in detail (Wang et al. 2005; Afriyie-Gyawu et al. 2007). NS capsules were prepared at College Pharmacy (Colorado Springs, CO, USA) under sterile conditions according to good manufacturing practices (GMP). All the capsules including the matching placebo were of the same size, shape and colour. All other chemicals and reagents used were obtained commercially at the highest purity available unless otherwise specified.

Study site and population

Six communities from the Ejura-Sekyedumase district (ESD) of the Ashanti Region of Ghana were selected for screening of study subjects. It is worth noting that approximately 76% of the populace in ESD engages predominantly in agriculture. Crops grown in this area mainly include maize, groundnuts, yams, cassava, cotton, and tobacco. AF exposure data and demographic information were established in four of these communities before this study (Jolly et al. 2006).

Study design and procedures

The trial was initiated in September 2005 and completed in April 2006. The overall study design followed the guidelines for a randomized, double-blinded, and placebo-controlled Phase II clinical trial as previously described (Afriyie-Gyawu et al. 2007). Study protocol was approved by the Institutional Review Boards at Texas A&M University and Noguchi Memorial Institute for Medical Research in Ghana. Figure 1 shows the overall study design and sample collection procedure. Briefly, 180 subjects were recruited from a total of 507 screened volunteers who met the following criteria: signed informed consent; serum \(\text{AFB}_1\)-albumin adduct levels >0.5 pmol \(\text{AFB}_1\) mg\(^{-1}\) albumin; age 18–58 years; healthy status based on physical examination results, haematological parameters, liver and renal function indicators, no history of chronic disease(s), no
use of prescribed medications for chronic or acute illness; and non-pregnant and/or non-breastfeeding for females. These participants were randomly assigned to one of three groups: high-dose (HD), low-dose (LD), and the placebo (PL) and took two capsules containing either 0.5 g NS, 0.25 g NS, or 0.25 g placebo three times per day (before meals and with at least 100 ml of water). This implies that participants in the HD and LD groups received a total amount of 3.0 and 1.5 g NS day⁻¹, respectively, over a period of 3 months. It must be noted that residents from these communities normally have three meals per day, which is why dosing was three times a day. Dose selection was based on the efficacy and safety of NS demonstrated in previous animal studies (Phillips 1999; Phillips et al. 2002, 2006) and dosimetry data from a short-term human study in the USA (Wang et al. 2005). Blood and urine samples were collected from each study participant at the beginning of the study (baseline), at 1 and 3 months of intervention, and at 1-month following completion of the trial. Serum, plasma and blood cells were immediately separated and stored at −20°C. Morning urine samples were collected, measured for volume, and 50-ml aliquots were stored at −20°C. Aliquots of each sample were shipped frozen to Texas A&M University and Texas Tech University for biomarker analysis. The laboratory personnel who performed the analyses were blinded to sample sources.

**Determination of serum AFB₁–albumin adduct level**

Serum AFB₁–albumin adduct was measured by a quantitative RIA procedure (Wang et al. 1996). Briefly, serum samples were concentrated and resuspended in phosphate-buffered saline (PBS). Serum albumin was quantified by a bromocresol purple dye binding method (Sigma), and the amount of total protein was determined using the Bradford procedure (Pierce Biotechnology, Rockport, IL, USA). Subsequently, total protein was digested with Pronase (Calbiochem, La Jolla, CA, USA) and the digests were extracted with acetone. AFB₁–albumin adduct was quantified with the RIA procedure (Wang et al. 1996) in duplicate serum protein digests each containing 2 mg protein. Pooled normal human serum standards (Sigma) were used to determine non-specific inhibition in the assay. A non-linear regression method (Gange et al. 1996) was used to establish the standard curve for the radioimmunoassay. Concentrations of AFB₁–albumin adduct were expressed as the amount of AFB₁ mg⁻¹ albumin. The detection limit of the assay was 0.01 pmol mg⁻¹ albumin.

**Determination of AFM₁ in urine**

AFM₁ levels in urine samples were analysed with immunoaffinity column purification followed by HPLC-fluorescence detection described by Groopman et al. (1992a), with the modifications of Sarr et al. (1995) and Wang et al. (1999). Briefly, each of the urine samples (5.0 ml) was adjusted to an acidic pH with 0.5 ml of 1.0 M ammonium formate (pH 4.5), and the volume was increased to 10 ml with water and vortexed. The sample was then loaded on a 1 ml preparative Aflatest P immunoaffinity column (VicamP, Watertown, MA, USA) at a flow rate of approximately 0.3 ml min⁻¹ as described previously (Wang et al. 1999). After washing, the purified AF fraction was eluted with 80% methanol and dried under N₂ for analysis using a Waters HPLC system (Waters Corporation, Milford, MA, USA) with fluorescence detection capabilities. A 250 × 4.6 mm LiCrospher RP-18 endcapped column with a pore size of 100 Å and a particle size of 5 μm (Alltech Associates, Deerfield, IL, USA) was used to resolve AF metabolites. The mobile phase consisted of 22% ethanol in water which was buffered with 20 mM ammonium formate (pH 3.0). Chromatographic separation of AFs was achieved by isocratic elution of the mobile phase for 20 min. Samples were injected (100 μl) on the column and the elution rate was 1.0 ml min⁻¹. The AFM₁ peak was detected at a retention time of approximately 15.4 min. The limit of detection for this method was 0.5 pg ml⁻¹ of urine for AFM₁. Creatinine concentrations in the urine samples were measured at the Laboratory of St Joseph’s Regional Health Center using an auto-analyser. Urinary concentrations of AFM₁ were expressed as pg mg⁻¹ creatinine in order to correct for variations in urine dilution among individual samples.

**Statistical analysis**

All of the data generated were stored in an Excel database and analysed with SAS software.
version 9.3 (SAS Institute, Inc., Cary, NC, USA). Median, mean, standard deviations (SD) and detectable range were calculated for concentrations of AFB₁–albumin adduct, and AFM₁ and the values were expressed as median and mean ± SD unless otherwise stated. To assess the efficacy of NS intervention, the statistical evaluation focused on the comparisons among different treatment levels and different time points. To the AFB₁–albumin data that were normally distributed, two-factorial ANOVA and Bonferroni procedures were used to compare significant differences between means of different treatment arms and times. To the AFM₁ data that were not normally distributed the Kruskal–Wallis test or Wilcoxon rank-sum test were used to compare the differences among different treatment groups and different time points. To evaluate the effect of dose and time interactions on NS treatment, a non-parametric mixed-effect model was applied as previously described (Brunner et al. 2002). A p-value < 0.05 (two-tailed) was considered significant.

Results

Sample collection over the study period
A total of 180 subjects were recruited for this intervention trial with NS and treatment was initiated with 177 subjects. The overall adherence among the participants and sample availability for biomarker analyses were satisfactory. A total of 162 subjects (91.5%) completed the 3-month trial. Detailed information about the number of samples collected at each time point for analyses of AFB₁–albumin adduct in serum and AFM₁ levels in urine are listed in Table 1.

Modulation of serum AFB₁–albumin adduct levels
A total of 656 serum samples collected over a 4-month study period were analysed for AFB₁–albumin adduct. Average levels (mean ± SD) and the range of serum AFB₁–albumin adduct in the three study groups, placebo (PL), 1.5 g NS (LD), and 3 g NS (HD), at different time points are shown in Table 2. The distributions of overall AFB₁–albumin adduct levels in these three groups throughout the study duration are shown in Figures 2 and 3. High AFB₁–albumin adduct levels were observed in baseline samples, and there were no significant differences among groups (p = 0.2354). No statistically significant differences were observed in AFB₁–albumin adduct levels among the three study groups at 1 month after the NS intervention (p = 0.3645). However, statistically significant decreases in AFB₁–albumin adduct levels were observed at 3 months after the intervention in both the LD and HD groups (p < 0.0001) as compared with the PL group. No statistically significant differences in AFB₁–albumin adduct levels were found among the three groups at 4 months, which was 1 month post-intervention. As shown in Figure 3, significant decreases in adduct levels were seen in all three treatment groups over the 4-month study period, showing a significant time effect on the AFB₁–albumin adduct levels. However, the pattern of time effect was different between the NS treated groups and the placebo group. For the PL group, the reduction rates

Table 1. Sample information for each collection time and each study group in NovaSil (NS) intervention study.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Serum</th>
<th>Baseline</th>
<th>1 month</th>
<th>3 months</th>
<th>4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>55</td>
<td>56</td>
<td>54</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>NS 1.5 g</td>
<td>57</td>
<td>56</td>
<td>52</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>NS 3.0 g</td>
<td>59</td>
<td>57</td>
<td>53</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>53</td>
<td>52</td>
<td>55</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>NS 1.5 g</td>
<td>53</td>
<td>53</td>
<td>51</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>NS 3.0 g</td>
<td>53</td>
<td>52</td>
<td>53</td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

*Valid sample numbers for final statistical evaluation.

Table 2. Levels of serum AFB₁–albumin adducts at different time points in every group with various NovaSil (NS) treatments.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Baseline</th>
<th>1 month</th>
<th>3 months</th>
<th>4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>1.49 ± 0.38 (0.96–2.93)</td>
<td>1.25 ± 0.34 (0.81–2.53)</td>
<td>1.20 ± 0.22 (0.84–1.83)</td>
<td>1.14 ± 0.18 (0.82–1.74)</td>
</tr>
<tr>
<td>NS 1.5 g</td>
<td>1.56 ± 0.32 (0.99–2.50)</td>
<td>1.21 ± 0.22 (0.87–1.99)</td>
<td>0.89 ± 0.16** (0.55–1.21)</td>
<td>1.10 ± 0.18 (0.76–1.55)</td>
</tr>
<tr>
<td>NS 3.0 g</td>
<td>1.51 ± 0.32 (0.96–2.58)</td>
<td>1.17 ± 0.24 (0.62–1.91)</td>
<td>0.90 ± 0.16** (0.49–1.25)</td>
<td>1.12 ± 0.18 (0.87–1.56)</td>
</tr>
</tbody>
</table>

*Data are mean ± SD (range).
**p < 0.001 as compared with the placebo.
of AFB1–albumin adduct at 1 and 3 months after the intervention were 16.1 and 19.9%, respectively. For the LD and HD groups, the reduction rates of AFB1–albumin adduct levels were 22.3 and 22.4% at 1 month after the intervention and were 42.8 and 40.2% at 3 months after the intervention, respectively. There were no consistent changes in the PL group between 3 and 4 months; however, levels of serum AFB1–albumin adduct increased significantly in the two NS-treated groups and were back to levels comparable with those of the placebo group at 4 months. Non-parametric mixed-effect model analysis further showed significant effects of dose, time, and dose–time interaction for reducing serum AFB1–albumin adduct levels, and this reduction was attributable to the NS intervention (Table 3). In addition, no significant gender difference was found among the study groups.

![Figure 2](image_url)

**Figure 2.** Dose effects of NovaSil intervention on serum AFB1–albumin adduct levels over the study duration. The box plots show distributions of AFB1–albumin adduct levels in each group at each time point. The box values ranged from 25 to 75 percentiles of the total samples, the line within it indicating the median value. The bars on both sides of a box represent values ranging from 5 to 25 and from 75 to 95 percentiles, respectively.
Modulation of urinary AFM₁ level

A total of 624 urine samples over the 4-month study period were analysed for AFM₁. About 87% (541/624) of the samples had detectable AFM₁ and no significant difference was found in detection rate (frequency) among three study groups. Average levels and the range of AFM₁ in three study groups at different time points are presented in Table 4. The distribution of

![Box plots showing the effect of NovaSil intervention on serum AFB₁–albumin adduct levels over the study duration.](image)
urinary AFM$_1$ levels in these three groups throughout the study duration is shown in Figures 4 and 5. Since the AFM$_1$ data is highly skewed, non-parametric analysis was applied for all statistical evaluations. There were no significant differences in median AFM$_1$ levels among the three study groups at baseline ($p = 0.2485$). No significant differences were found in median AFM$_1$ levels among the three groups at 1 month after the NS intervention ($p = 0.3342$). However, statistically significant decreases in median AFM$_1$ levels were observed at 3 months after the NS intervention ($p = 0.0445$). While the median AFM$_1$ level was comparable between the PL group and the LD group ($p = 0.3951$), a reduction rate of 58.7% in the median AFM$_1$ level was also found between the HD group and the PL group ($p = 0.0219$). Significant differences in median AFM$_1$ levels ($p = 0.0024$) were also found among the three study groups 1 month post-intervention, which was mainly due to higher AFM$_1$ levels in the LD group. As shown in Figure 5, significant decreases in AFM$_1$ levels were seen in the HD group over the 4-month study period, showing a significant time effect ($p = 0.009$). Although a significant time effect was also noticed in the PL group ($p = 0.002$), levels of AFM$_1$ were highly variable, as shown by higher median levels at baseline and 3 months compared with the lower median levels at 1 and 4 months. No significant time effect for AFM$_1$ levels was found in the LD group over the 4-month study period ($p = 0.3277$). Non-parametric mixed-effect model analysis also showed a significant dose–time interaction delineating a reduction in urinary AFM$_1$ levels, which was attributable to the NS intervention (Table 3). In addition, no significant gender difference was found among the study groups.

**Discussion**

Safety and efficacy are the two most important criteria for assessing potentially therapeutic and/or clinical intervention agents. The safety (and dosimetry) of NS has been well documented in animal and human studies (Phillips 1999; Phillips et al. 2002, 2006; Wiles et al. 2004; Afriyie-Gyawu et al. 2005; Wang et al. 2005), including this 3-month trial in Ghana (Afriyie-Gyawu et al. 2007). The main objective of this study was to determine efficacy of NS for the reduction of AF exposure in humans. The ability of NS to sorb AF preferentially in the stomach and intestines resulting in decreased AF bioavailability and toxicity has been clearly demonstrated in various animal models (Phillips et al. 1988, 2002, 2006; Harvey et al. 1991a; Kubena et al. 1991; Lindemann et al. 1993; Mayura et al. 1998; Phillips 1999; Pimpukdee et al. 2004). Results from this study confirm our work in animals and show that administration of NS for 3 months significantly reduced serum levels of AFB$_1$–albumin adduct and urinary AFM$_1$ levels in human subjects. To our knowledge this is the first study to explore the efficacy and health benefits of dietary inclusion of NS clay by monitoring AF-specific biomarkers in a human population at risk for aflatoxicosis.

AF-specific biomarkers currently used in human and animal studies include AFB$_1$ metabolites and AFB$_1$ macromolecular adduct, i.e. AFM$_1$ in urine and AFB$_1$–albumin adduct in serum (Groupman et al. 1994, 1996a; Wang et al. 2001a).

Table 3. Non-parametric mixed-effects model analysis.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Serum AFB$_1$–albumin adducts</th>
<th>Urinary AFM$_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose</td>
<td>7.890 ($p = 0.0004$)*</td>
<td>2.234 ($p = 0.1072$)</td>
</tr>
<tr>
<td>Time</td>
<td>179.330 ($p = 0.0000$)</td>
<td>5.764 ($p = 0.0007$)</td>
</tr>
<tr>
<td>Dose*Time</td>
<td>13.992 ($p = 0.0000$)</td>
<td>2.143 ($p = 0.0495$)</td>
</tr>
</tbody>
</table>

*Statistic box-approximation value ($p$-value).

Table 4. Levels of urinary AFM$_1$ at different time points in every group with various NovaSil (NS) treatments.

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Baseline</th>
<th>1 month</th>
<th>3 months</th>
<th>4 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placebo</td>
<td>53.42</td>
<td>24.58</td>
<td>52.38</td>
<td>17.32</td>
</tr>
<tr>
<td></td>
<td>644.22 ± 2026.53</td>
<td>94.71 ± 160.13</td>
<td>181.26 ± 675.90</td>
<td>56.84 ± 110.14</td>
</tr>
<tr>
<td></td>
<td>(0.89–13297.67)</td>
<td>(1.66–798.11)</td>
<td>(2.02–5006.34)</td>
<td>(0.04–529.41)</td>
</tr>
<tr>
<td>NS 1.5 g</td>
<td>45.54</td>
<td>34.19</td>
<td>51.17</td>
<td>32.87</td>
</tr>
<tr>
<td></td>
<td>183.58 ± 334.96</td>
<td>202.06 ± 639.73</td>
<td>307.08 ± 1248.35</td>
<td>358.59 ± 1594.00</td>
</tr>
<tr>
<td></td>
<td>(0.66–1547.39)</td>
<td>(0.46–4338.52)</td>
<td>(1.59–8878.78)</td>
<td>(2.87–10510.81)</td>
</tr>
<tr>
<td>NS 3.0 g</td>
<td>60.27</td>
<td>20.99</td>
<td>21.61**</td>
<td>12.22</td>
</tr>
<tr>
<td></td>
<td>256.30 ± 615.17</td>
<td>175.09 ± 818.41</td>
<td>67.31 ± 102.54</td>
<td>70.39 ± 155.68</td>
</tr>
<tr>
<td></td>
<td>(0.69–3901.90)</td>
<td>(0.70–5882.71)</td>
<td>(0.80–411.68)</td>
<td>(0.39–873.72)</td>
</tr>
</tbody>
</table>

Data are median, mean ± SD (detectable range).

*p < 0.05 as compared with the placebo.
The AFB1–albumin adduct, compared with urinary AF metabolites, serves as a very important biomarker since its longer in vivo half-life may reflect integrated exposures over longer time periods (Wang et al. 1996, 2001a; Wild et al. 1996). From a practical perspective pertinent to epidemiological studies the measurement of serum AFB1–albumin adduct offers a rapid and facile approach that can be used to screen very large numbers of people, e.g. the present study where we screened 507 people (Afriyie-Gyawu et al. 2007) and other intervention studies (Kensler et al. 1998a, b; Huang et al. 2004). The AFB1–albumin adduct is also the most reliable molecular biomarker for studying human exposures to AFs (Groopman and Kensler 1993; Wild and Turner 2002). Highly significant associations between AFB1–albumin adduct levels and AFB1 intakes were found in human populations from several regions of the world (Gan et al. 1988; Wild et al. 1990; Wang et al. 2001b; Turner et al. 2007). Furthermore, about 2% of the ingested AFB1 is reported to be covalently bound to serum albumin, a value very similar to that observed when rats were administered AFB1 (Wild et al. 1996). Using various analytical techniques, AFB1–albumin adduct was detectable in almost 100% of sera from adults and in 12–100% of sera from children in China and various

Figure 4. Dose effects of NovaSil intervention on urinary AFM1 levels over the study duration. The box plots show distributions of AFM1 levels in each group at each time point. The box values ranged from 25 to 75 percentiles of the total samples, the line within it indicating the median value. The bars on both sides of a box represent values ranging from 5 to 25 and from 75 to 95 percentiles, respectively.
African countries (Gan et al. 1988; Wild et al. 1990, 1992a; Wang et al. 1996, 2001a, b; Sun et al. 2002; Tao et al. 2005). In addition to studying AF exposure, AFB$_1$–albumin adduct has been used as a biological response indicator of acute and chronic aflatoxicosis in Africa (Azziz-Baumgartner et al. 2005), risk of HCC in Taiwan (Wang et al. 1996; Lunn et al. 1997), China (McGlynn et al. 1995; Kuang et al. 1996; Groopman et al. 1996b; Wang et al. 2001a), and Africa (Wild et al. 2000), and infectious disease-linked immune suppression (Turner et al. 2003; Jiang et al. 2005). Moreover, AFB$_1$–albumin adduct has been regularly used as the surrogate efficacy biomarker of AF exposure for assessment of different therapeutic/intervention agents and techniques in human intervention trials (Kensler et al. 1998a, b; Tang et al. 2003; Turner et al. 2005).

In this study high levels of serum AFB$_1$–albumin adduct were observed in the participants at baseline before NS intervention (1.52 ± 0.34 pmol mg$^{-1}$ albumin; range = 0.96–2.93 pmol mg$^{-1}$ albumin). These levels were higher than those reported from The Gambia (Wild et al. 1992a), Benin (Gong et al. 2003; Egal et al. 2005) and the UK (Turner et al. 1998), and were comparable with levels found in populations at high-risk for liver cancer in China (Wang et al. 1996,
Therefore, the study participants in Ghana represented a population at high risk for AF exposure. In this study we found that daily NS capsule administration produced significant dose and time effects in the reduction of serum AFB$_1$–albumin adduct (Table 3). A significant (>40% reduction in AFB$_1$–albumin adduct levels) was observed at 3 months in both NS-treated (LD and HD) groups compared with the PL group (Table 2 and Figure 2). Although decreases in this biomarker level were also observed 1 month after the intervention both NS treatment groups, no significant differences were found due to the decreased adduct level in the PL group (Figure 3). The delay in adduct reduction seen in this study was similar to a previous chemoprevention trial with Oltipraz in Qidong, China (Kensler et al. 1998b), in which a significant reduction of serum AFB$_1$–albumin adduct levels was observed only after the fifth week of treatment. This delay is probably attributable to the long half-life of albumin, which is estimated to be approximately 3 weeks in normal and healthy people. The recovery of AFB$_1$–albumin adduct levels in serum following withdrawal of NS treatment was expected and is also consistent with the half-life of AFB$_1$–albumin. Importantly, the AFB$_1$ bound to albumin (AFB$_1$–albumin adduct) may be stable for years (Skipper and Tannenbaum 1990; Wang et al. 2001b). Similar findings were reported with Oltipraz, where AFB$_1$–albumin adduct levels were detected until albumin turnover had passed three half-lives (Kensler et al. 1997). The significant time effect observed in this study, including the decrease in the levels of the biomarker in the PL group, is also consistent with previous findings in the Oltipraz trial (Kensler et al. 1998b) and a recent intervention study employing community education as the strategy (Turner et al. 2005). In the current study AFB$_1$–albumin adduct levels in both the LD and HD groups were reduced during NS treatment and were similar to the PL group level at the fourth month, 1 month after the intervention, which further confirmed the efficacy of NS in reducing AFB$_1$ exposure.

AFM$_1$ is a metabolite of AFB$_1$ that is prevalent in urine (Zhu et al. 1987) and milk (Zarba et al. 1992), and its formation from parent AFB$_1$ is catalysed mainly by hepatic CYP1A2 in humans (Coulombe 1993; Wild and Turner 2002). The excretion of AFM$_1$ in urine represents recent AFB$_1$ exposure (i.e. within 24–48 h). Thus, AFM$_1$ levels in urine are used as a short-term biomarker of AFB$_1$ exposure (Groopman et al. 1994; Wang et al. 1999). Both serum AFB$_1$–albumin adduct and urinary AFM$_1$ have been extensively characterized and validated as biomarkers for AFB$_1$ exposure in many human populations. Levels of serum AFB$_1$–albumin adduct and AFM$_1$ excreted in human urine have shown significant correlation with dietary intake of AFs (Zhu et al. 1987; Gan et al. 1988; Groopman et al. 1992b; Wild et al. 1992b; Egal et al. 2005; Jolly et al. 2006) and with the risk of human HCC (Ross et al. 1992; Qian et al. 1994; Kuang et al. 1996; Wang et al. 1996). Concurrent with reductions in serum AFB$_1$–albumin adduct levels at 3 months after the intervention, urinary AFM$_1$ levels were also significantly reduced in the HD group as compared with the other treatment arms in this study (Table 4 and Figure 4). A reduction rate of up to 58.7% in the median AFM$_1$ level found in the HD treatment group is comparable with the reduction rate of 55% in the median AFB$_1$-N$_7$-guanine level after 3 months’ intervention with 100-mg chlorophyllin (Egger et al. 2001). The AFB$_1$-N$_7$-guanine product is also a short-term biomarker like AFM$_1$. In this study no significant effect in urinary AFM$_1$ levels was observed in the LD group, which is potentially due to considerable intra- and inter-individual variations in the measurement of a short-term biomarker. Significant time effect of AFM$_1$ levels was also observed in all study groups, including the PL group, which may reflect variations in daily dietary AF exposure levels. Variations of urinary AFM$_1$ levels were also found in previous screening studies in a similar population in Ghana (Jolly et al. 2006) as well as other populations (Wang et al. 2001b). A very wide range of AFM$_1$ levels (from undetectable to 13.3 ng mg$^{-1}$ creatinine) was observed in our study participants. This may be attributed to seasonal changes in food contamination in the region, as well as genotypic or phenotypic variations in AF-metabolizing enzymes and individual susceptibility. Nevertheless, significant dose–time interaction effects (Table 3) associated with reduced urinary AFM$_1$ levels in this study confirmed the efficacy of NS administered in capsules.

In summary, the results of this study suggest that intervention with NS clay can effectively reduce AF exposure from contaminated diets, as represented by AF-specific biomarkers in blood and urine, i.e. AFB$_1$–albumin adduct and AFM$_1$, respectively. Long-term (phase IIb or III) studies will ultimately be required to evaluate further the efficacy of NS intervention as an enterosorbent therapy for acute AF exposure and for the prevention of chronic AF-induced disease when included in the diet of populations at high risk for aflatoxicosis. Eventually, NS may be delivered like iodine (through its inclusion in salt) or as an additive in common foods such as peanut butter. Economically feasible and culturally acceptable approaches to NS clay intervention and therapy are necessary to sustain this strategy, particularly in developing countries and populations at high risk for aflatoxicosis. Before this development, agencies such as the Joint Committee of Food and Agricultural Organization/World Health Organization (FAO/WHO), US Agency for International Development (USAID), US Department of Agriculture (USDA), Centers for Disease Control
(CDC), and non-governmental organizations (NGOs) must recognize the importance of exposure to AFs from food and its impact on infectious disease and malnutrition. Importantly, young children in these countries are highly susceptible to the effects of AFs and cannot avoid exposure. Delivery of NS clay in foods and beverages contaminated with AFs will require strong partnerships and global alliances committed to solving this problem and improving public health.

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