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Characterization and Safety of Uniform Particle Size NovaSil Clay as a Potential Aflatoxin Enterosorbent

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Abstract

NovaSil (NS) clay, a common anti-caking agent in animal feeds, has been shown to adsorb aflatoxins and diminish their bioavailability in multiple animal models. The safety of long-term dietary exposure to NS has also been demonstrated in a 6-month sub-chronic study in rats and in a 3-month intervention in humans highly exposed to aflatoxins. Uniform particle size NovaSil (UPSN) is a refined material derived from parent NS; it contains lower levels of dioxins/furans, and has been selected for a more consistent uniform particle size. Nevertheless, the efficacy and potential safety/toxicity of UPSN for long term-use has not yet been determined. In this research, 4-week-old male and female Sprague Dawley rats were fed rations free of clay (control) and containing UPSN at low dose (0.25%) and high dose (2%) for 13 weeks. AFB₁ sorption characteristics remained the same for both clays. When compared to the control, total body weight gain was unaffected in either sex at the doses tested. No UPSN-dependent differences in relative organ weights or gross appearance were observed. Isolated differences between UPSN groups and the control were observed for some biochemical parameters and selected vitamins and minerals. None of these differences were dose-dependent, and all parameters fell between ranges reported as normal for rats less than 6 month old. The Na/K ratio, Na and vitamin E concentrations were the only parameters that were increased in both males and females in the low dose and high dose UPSN groups. Serum Zn levels in males from the 2% UPSN treatment were lower compared to the control. Serum K levels were lower in the males of UPSN groups than in the control. However, neither Na/K ratio, K, nor Zn values were dose dependent and fell outside ranges reported as normal. These results suggest that dietary inclusion of UPSN at levels as high as 2%

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(w/w) does not result in overt toxicity. Nevertheless, further research on the effects of clays on Na, Zn, K and vitamin E is warranted.

Keywords

Aflatoxin; calcium bentonite; montmorillonite; in vivo; safety; clay

1. Introduction

Ingestion of bentonite clay is a promising approach among dietary intervention strategies to reduce aflatoxin (AF) exposure from food and feed. Aflatoxin B₁ (AFB₁) is a group 1 carcinogen (IARC, 2002) mainly produced by *Aspergillus flavus* and *A. parasiticus* fungi that commonly contaminate maize and peanuts (CAST, 2003). AFB₁ is a potent hepatocarcinogen (Busby and Wogan, 1984; Lopez et al., 2002) with genotoxic (Smela et al., 2001), immunotoxic (Hinton et al., 2003; Turner et al., 2003) and antinutritional (Pimpukdee et al., 2004; Williams et al. 2004) effects. In the U.S., the action level for AFs in foods intended for human consumption has been set at 20 µg kg⁻¹. The EU has set more conservative maximum values, ranging from 0.10 to 15 µg kg⁻¹. These include explicit values for single AFB₁ and total AFs, as well as specific values according to the commodities analyzed.

Montmorillonite has been shown to be the active ingredient in bentonites for AF binding (Kannevischer et al., 2006; Marroquín-Cardona et al., 2009). The proposed mechanism of action is through the adsorption of AFs (mainly onto the interlayer spaces of montmorillonite) in the gastrointestinal tract of animals and humans, thus reducing toxin bioavailability to the blood and organs (Phillips, 1999). Bentonite clays, which are rich in montmorillonite, have been effectively used in dairy cows (Díaz et al., 2004; Harvey et al., 1991; Kutz et al., 2009), goats (Smith et al., 1994) and several other animal species (Harvey et al., 1994; Ledoux et al., 1999; Márquez and Hernandez, 1995; Phillips et al., 1990) to diminish the negative effects of AF exposure. Importantly, findings from a recent clinical intervention study showed that a montmorillonite rich Ca-bentonite (NovaSil-Plus, NS) was effective in reducing AF biomarkers in serum and urine with negligible nutrient interactions in humans naturally exposed to AFs via contaminated foods (Afriyie-Gyawu et al., 2008b; Wang et al., 2008).

Because of their natural origin, clays may contain numerous contaminants including some metals, dioxins and furans. For instance, in 1997, the FDA banned the use of “ball clay”, a kaolinite rich material, in animal feed due to the high levels of dioxins (FDA, 1997). Some bentonites contained significantly lower levels of these contaminants (Afriyie-Gyawu et al. 2008a); all proposed AF binders should be rigorously tested for the presence of dioxins/furans and other potentially hazardous substances. Uniform particle size NovaSil clay (UPSN) is a refined Ca-bentonite that has been selected to minimize the content of particles > 100 µm. This size selection is expected to diminish batch to batch differences. Expected low levels of contaminants and a more uniform particle size make UPSN a candidate for potential use as an AF sorbent in humans, upon verification of its safety and efficacy. Hence, the objectives of this study were to investigate the mineralogy and AF sorption characteristics of the refined UPSN to compare with parent NS, and to determine the safety of UPSN ingestion in a 3-month rodent study.

2. Experimental

For all experiments, ultrapure deionized water ($\text{DI-H}_2\text{O}$ $18\text{M}\Omega\text{ cm}^{-1}$) was prepared by processing distilled water through a PURELAB Ultra (ELGA Woodridge, IL). All reagents used in the sample preparation for mineralogical analyses were analytical grade. AFB_1 from *Aspergillus flavus* was purchased from Sigma Aldrich. UPSN clay was obtained from Texas Enterosorbents, Inc. (Bastrop, TX), and NS was obtained from Engelhard Chemical Corporation (Iselin, NJ). Levels of chlorinated dibenzo-p-dioxin/furan (CDDs/CDFs) in UPSN clay were measured by Columbia Analytical Services, Inc. (Houston, TX). Procedures followed the U.S. Environmental Protection Agency (USEPA) methods for sample preparation, clean-up and analysis with high resolution capillary column gas chromatography/high resolution mass spectrometry (USEPA Method 1613B). The analysis included the 17 USEPA priority compounds. Dioxin and furan levels in NS were previously reported by Afriyie-Gyawu et al. (2008a). UPSN and NS samples were sent to Columbia Analytical Services (Kelso, Washington) for total recoverable element analysis of As, Ba, Cd, Co, Cr, Mn, Mo, Ni, Pb, Sr and Zn. As pretreatment, samples were digested with nitric acid and hydrogen peroxide according to USEPA method 3050B. Inductively coupled plasma-mass spectrometry (ICP-MS) was used for As, Cd, Co, Cr, Mo, Ni, Pb and Zn following USEPA method 200.8, while ICP-atomic emission spectrometry was used for Ba, Mn and Sr as reported in USEPA method 6010B. For Hg analysis, cold vapor atomic absorption was used in accordance with USEPA method 7471A.

2.1 Mineralogical analyses

Mineralogical analyses included an initial sample evaluation to determine moisture content and to assess the presence of carbonate minerals, sulfides, manganese oxides and evaporites. Most of the mineralogical analyses were performed following standard procedures previously reported (Soukup et al., 2008), with minor modifications. The presence of calcite (CaCO_3) was checked by using 1M hydrochloric acid (HCl). Presence of oxidable or reducible components was evaluated using hydrogen peroxide (30% H_2O_2). Presence of ferromagnetic minerals was assessed by using a magnetic stir bar. Occurrence of water soluble minerals was qualitatively estimated by measurement of electrical conductivity (EC) and acetone precipitation. For EC measurement, 10 g of each sample were weighed into 250 mL plastic bottles. The ratio of deionized water: solid used was 5:1. Samples were shaken for 30 min on a rotary shaker at room temperature and then centrifuged at 2000 rpm for 10 min. After centrifugation, the supernatants were used to measure the EC and pH sequentially. Two mL of the supernatants were mixed with the same volume of acetone to evaluate the presence of sulfates.

Other analyses included size fractionation, X-ray diffraction (XRD), Fourier transform infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), transmission electron microscopy (TEM), cation exchange capacity (CEC) and extractable bases determination with ammonium acetate (NH_4OAc) and DI-water. The CEC determination was done following a slightly modified procedure reported by the U.S. Salinity Laboratory Staff (1969), while the extractable bases using NH_4OAc were determined with a method described by the U.S. Soil Survey Staff (1996); both analyses were done by the Soil Characterization Laboratory from the Soil and Crop Sciences Department at Texas A&M University using a mechanically controlled variable rate leaching device as reported by Holmgren et al. (1977). Additionally, extractable bases using DI-water were determined by the Soil, Water and Forage Testing Laboratory at Texas A&M University, using a saturated paste extract method based on Rhoades and Clark (1978). An amount of 100 g of sample was used for this analysis. For NS, most of the mineralogical probes were performed, except for SEM and TEM.

Before size fractionation, cementing and flocculating materials were removed using sodium acetate buffer (pH 5). Organic matter (OM) was removed using 30% H₂O₂. Samples were then dispersed with 50 mL of pH 10 sodium carbonate (Na₂CO₃) solution. The sand fraction (> 53 µm) was separated using a 53 µm sieve. The clay fraction (< 2 µm) was separated from the silt fraction (2–53 µm) by centrifugation using pH 10 Na₂CO₃ as dispersant. Sand and silts weights were recorded after drying at 105 °C overnight. Clay suspensions were flocculated with sodium chloride (NaCl) and then dialyzed until EC measurements were close to the values of DI-water (< 2 µS cm⁻¹). Air dry fractionation of clays was done using an Octagon 200 sieve shaker (Endecotts). Percentage of particles > 100 µm, 100–45 µm and < 45 µm were calculated. Additionally, fractionation using water (without dispersant) was achieved using the hydrometer method according to Milford (1997).

XRD patterns were recorded on a Bruker D8 ADVANCE X-ray diffractometer. Sand and silt fractions were ground in a mortar, passed through a 140 mesh (105 µm) sieve and mounted as powder to collect the XRD patterns. Magnesium and potassium saturated clay suspensions were air dried on glass discs to obtain oriented films for XRD analysis. Mineral identification was determined using the EVA Bruker (Madison, WI) computer software. Infrared patterns were recorded in a Spectrum 100 FTIR (Perkin Elmer, Inc.) using the diffuse reflectance infrared Fourier transform (DRIFT) method and the attenuated total reflectance (ATR) method. For the DRIFT method, 0.005 g of sample was placed in a holder, using KBr as background material. For the ATR method, 1 mg of the clay fraction was mixed with 300 mg of KBr under a lamp to maintain dryness. Clay samples and KBr were mixed in a steel capsule for 30 seconds and then placed in a holder for reading.

Silt samples of UPSN were examined in a JEOL 6400 scanning electron microscope with energy dispersive X-ray (EDS) recording capability. For TEM analysis, clay samples from UPSN were mounted on a holey carbon grid, and images were recorded on a JEOL 2010 microscope with EDS capability.

2.2 AFB₁ sorption analyses

Isotherms were performed based on methods reported by Grant and Phillips (1998) and previously described in detail by Marroquín-Cardona et al. (2009). Isotherms were conducted in triplicate at pH 2 and 6.5. Computer-generated equilibrium isotherms were extrapolated from additive data and fit to the Langmuir model (based on r²-values and randomness of the residuals). The parameters of Q_{max} (mol AFB₁ kg⁻¹ additive) and K_d were estimated to delineate the maximum sorption to the surface and the affinity of the sorption interaction.

2.3 Rodent study experimental design

Authorization for the study was obtained by Texas A& M University, according to the animal use protocol (AUP) 2008–39. Sprague Dawley®TM (SD) rats were obtained from Harlan (Houston, TX). Animals in each group came from different litters to assure independence of samples. Four-week-old male (100–124 g) and female (75–99 g) rats were maintained on rodent feed 8604 Teklad Harlan (Madison, WI) meal form and water *ad libitum*. After a brief acclimation period (5 days), the rats (1 rat per cage) were allocated to either the control group (basal rodent diet) or one of the two treatment groups; 0.25% and 2% w/w inclusion of UPSN, with basal diet respectively. The corresponding groups consisted of 10 female and 10 male rats each. Dietary clay contents were based on the minimum effective dose (0.25% w/w) of NovaSil clay used in human studies (Afriyie-Gyawu et al. 2008b), and the highest level allowed (2% w/w) by the FDA as generally recognized as safe (GRAS) additive (Electronic Code of Federal Regulations, 2010). Animals were housed in a climate controlled environment (temperature 22–25°C),

artificially illuminated (12 hr dark/12 hr light), and free from chemical contamination. Animals were inspected daily for general appearance, behavior, and signs of morbidity and mortality. Body weights were measured at baseline and every three days throughout the course of the study. Feed consumption was also recorded every three days. In total, body weight and feed consumption were recorded at 30 and 29 time points, respectively. After 13 weeks, final body weights were recorded, and blood was drawn via cardiac puncture under isoflurane anesthesia. Following euthanasia, organs and tissues of interest were removed and evaluated for gross abnormalities. Wet weights of liver, kidneys, heart, lungs, brain, spleen, tibia, and uterus plus ovaries were recorded. Growth parameters measured included initial body weight (IBW), final body weight (FBW), total body weight gain (TBWG), total feed consumption (TFC) and feed conversion efficiency (FCE).

2.4 Hematological and serum biochemical analysis

Hematology and serum biochemical parameters were analyzed by the Clinical Pathology Lab, Texas Veterinary Medical Diagnostics Lab (TVMDL) (College Station, TX). Hematological analysis of whole blood samples was conducted using an Abbott CELL-DYN 3700 Hematology Analyzer (Abbott Laboratories, Abbott Park, IL) and included hemoglobin (Hb) concentration, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), percent corpuscular volume (PCV), platelets, red blood cell (RBC) and white blood cell (WBC) counts. Leukocyte differential analysis was performed manually by microscopic examination of blood smears and included neutrophils, lymphocytes, monocytes, and eosinophils. Serum biochemical parameters were assessed using an automated analyzer Modular P (Roche Diagnostics, Indianapolis, IN) and included albumin/globulin ratio (A/G), albumin (ALB), alkaline phosphatase (ALP), alanine aminotransferase (ALT), amylase (AMYL), aspartate aminotransferase (AST), blood urea nitrogen (BUN), calcium (Ca), cholesterol (CHOL), creatine kinase (CK), chloride (Cl), creatinine (CRT), gamma glutamyl-transferase (GGT), globulins (GLOB), glucose (GLUC), potassium (K), sodium (Na), Na/K ratio, phosphorous (P), total bilirubin (T-BIL), and total serum protein (TSP).

2.5 Iron, zinc, vitamins A and E

Serum micronutrients Fe and Zn were measured with a Hitachi 911 (Roche Laboratories, Indianapolis, IN) and a Perkin Elmer Analyst 100 (PerkinElmer, Shelton, CT), respectively. Vitamin A and E were measured by HPLC according to previously reported methods by Weinman et al. (1999) and Rupérez et al. (2004), respectively.

2.6 Statistical analysis

First, to investigate the weight gained over time among the absolute control and the two doses of UPSN, a linear mixed model was constructed with the fixed effect treatment, sex, and treatment*sex interaction. Time was included as a continuous variable and tested for interactions with treatment and sex. Differences in FBW were investigated with a similar model that included the fixed effect treatment and the covariates IBW and TFC as explanatory variables. Backward selection was done by removing one term at a time, starting with non-significant interactions ($p > 0.05$).

A second analysis was performed to investigate differences in biochemical parameters, relative organ weights and selected vitamins and minerals among the absolute control and the two doses of UPSN for males and females separately. A linear or a generalized linear mixed model was constructed with the fixed effect treatment as the only independent variable. The MIXED and GLIMMIX procedures were used for all statistical analyses using SAS 9.2 with Enterprise Guide 4.1. In all these analyses, the effect of subject (i.e. rat) was considered as random, since all animals came from different litters (non relatives in each

group). All post-test comparisons were adjusted by the method of Tukey-Kramer. Finally, due to the nature of the data, monocyte counts and T-BIL were analyzed with non-parametric tests using SPSS 14.0. Data for T-BIL were classified in categories before analysis. Kruskal-Wallis test followed by Mann-Whitney test for multiple comparisons were used to detect individual differences between groups.

3. Results

3.1 Dioxins/furans and metal analyses

The only dioxins/furans detected in UPSN were octachlorodibenzo-p-dioxin (OCDD) and heptachlorodibenzo-p-dioxin (HpCDD). The average toxic equivalents (TEQs) for these compounds were calculated to be 0.0015 pg g^{-1} . According to previous reports (Afriyie-Gyawu et al., 2008a), HpCDD and OCDD were the only dioxins in NS present above the limits of detection ($\text{LOD} = 1.11 \text{ pg g}^{-1}$ for HpCDD and 1.91 pg g^{-1} for OCDD). The combined toxic equivalent (TEQ) value calculated for both dioxins was 0.0466 pg g^{-1} . In general, the metal analyses showed a similar profile for both clays (Table 1).

3.2 Mineralogical analyses

Differences were observed in moisture content, electrical conductivity, CEC, sulfate precipitation, extractable bases and percentages of the different fractions in both, dry and wet states (Table 2). The major minerals identified in both samples were montmorillonite and quartz (Fig 1). A lower relative intensity of quartz was observed in UPSN suggesting less content of this mineral. Calcite and potassium feldspars (e.g. sanidine) were also observed in both samples. Quartz was the major mineral identified in the sand fractions of both samples; however a broad smectite peak was still evident in both samples, suggesting the presence of smectite aggregates. Similarly, smectite, along with sanidine, albite, mica, and quartz minerals, were identified in the silt fractions of both samples. Montmorillonite presence was confirmed in both samples after XRD analysis of the clay fraction exchanged with Mg and K. After treatment with Mg and glycerol, the d-space of the most intense montmorillonite peak increased from ~ 15 to $\sim 18 \text{ \AA}$. Air dried samples exchanged with K showed a peak of $\sim 12 \text{ \AA}$. After heating at 330°C and 550°C , the K exchanged samples depicted a peak of $\sim 10 \text{ \AA}$ and $\sim 9.8 \text{ \AA}$, respectively. This pattern of collapse of the major $15 \text{ \AA}$ peak was expected for montmorillonites. Montmorillonite was also confirmed with KBr-DRIFT and ATR. Both samples showed a characteristic band of montmorillonite of 3631 cm^{-1} (UPSN) and 3619 cm^{-1} (NS) in the ATR pattern. Similarly, KBr-DRIFT patterns displayed a confirmatory montmorillonite band at 3632 cm^{-1} (UPSN) and 3623 cm^{-1} (NS). Additionally, smectite bands of AlFeOH deformation (884 and 882 cm^{-1}) and AlMgOH (842 and 845 cm^{-1}) were observed for UPSN and NS, respectively.

SEM findings for the silt fraction of UPSN confirmed the presence of feldspars and mica. Additionally, smectite aggregates were noted in the silts (Fig 2A). The main feature observed in the TEM images was the folding tendency of montmorillonite particles (Fig 2B). The particles are shown as thin films of considerable length ($3\text{--}5 \text{ }\mu\text{m}$) that fold many times and can be described like a “veil fabric”.

3.3 AFB₁ sorption analyses

The parameters of Q_{max} and K_d were similar for both samples at both pH levels. Samples fitted the Langmuir model ($r^2 \geq 0.92$) and depicted the favorable AF sorption pattern characterized as L-shape (Fig 3). Specifically at pH 6.5, an L2-pattern was documented for both samples (plateau has been reached); while at pH 2 the sorption pattern more closely resembled an L1-pattern (in process of reaching the plateau). The theoretical Q_{max} values calculated for UPSN and NS at pH 2 were 0.39 ± 0.01 and $0.40 \pm 0.03 \text{ mol AFB}_1 \text{ kg}^{-1}$,

while at pH 6.5 Q_{max} values were 0.44 ± 0.05 and 0.41 ± 0.02 mol AFB₁ kg⁻¹, respectively. These values (at both pHs) are similar, but the curves are clearly separated. This separation is better represented by the “observed” maximum binding capacities for UPSN and NS, i.e., 0.45 and 0.38 mol AFB₁ kg⁻¹ (pH 6.5); 0.32 and 0.28 mol AFB₁ kg⁻¹ (pH 2), respectively. A slight higher binding capacity was noted for UPSN at both pH levels when compared to NS. The K_d values for UPSN and NS were 6.29×10^5 and 4.90×10^5 (pH 6.5); 1.93×10^5 and 9.88×10^4 (pH 2), respectively.

3.4 Rodent study

No apparent adverse effects due to consumption of either dose of UPSN were noticed throughout the study. All rats remained healthy and active without noticeable changes in behavior.

3.5 Growth parameters

Males gained more weight than the females ($p < 0.0001$), as expected. Gained weight across time was different in at least one of the treatment groups ($p = 0.0099$) and the effect of treatment was different between males and females ($p = 0.0007$). There was no difference in weight gained in the males across all time points (a total of 30 time points recorded) and groups. In contrast, females in the control group gained more weight than the females in the 2% UPSN group by time 15 (day 43) ($p = 0.0351$) and consecutively up to time 30 (day 88) ($p = 0.0026$), but not on time 10 (day 28) or before. Similarly, females in the 0.25% UPSN group gained more weight than the females in the 2% UPSN group by time 30 (day 88) ($p = 0.0362$) but not on time 25 (day 73) or before. Significant differences were observed between the control and the treatment groups for some of the growth parameters in the females (Table 3). TFC and IBW were not significant factors for explaining the differences in FBW of the females.

3.6 Hematological and serum biochemistry analysis

No statistically significant differences were observed between the control and the UPSN groups for most of the biochemical parameters measured (Tables 4 and 5). Nevertheless, some significant differences were registered in at least one of the treatment groups, for either females or males. Importantly, most of the parameters measured in this study fell between the normal ranges reported for control animals (< 6 month old) in several studies and did not show patterns of dose dependency.

3.7 Iron, zinc, vitamin A and E analyses

No statistically significant differences were observed between the control and the treatment groups for Fe and vitamin A, in either males or females. Males of the 2% UPSN group had significantly lower levels of Zn when compared to both the control and the 0.25% UPSN (Table 6). Similarly, the females of 2% UPSN group had significantly reduced Zn levels when compared to the 0.25% group, but the levels were not statistically different from the control. However, Zn values were still within the normal ranges reported by other authors for 28 week old control animals (Afriyie-Gyawu et al. 2005). Interestingly, serum vitamin E was significantly increased in both sexes of the UPSN groups (Table 6), but no dose-dependency was noted.

3.8 Relative organ weights

The relative organ weights (ROWs) were calculated by dividing the wet weight of each organ by the final body weight of the rat just before termination. ROWs were multiplied by a factor of 10^2 . Few differences were observed in relative organ weights for both males and females (Table 7) and none of them suggested dose dependency.

4. Discussion and conclusions

4.1 Mineral characteristics and AFB₁ sorption analyses

The levels of dioxin/furans in UPSN were lower than in parent NS. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has placed a provisional tolerable daily intake (PTDI) value for dioxins of ~ 2.3 pg WHO-TEQ kg-bw⁻¹ day⁻¹ (JECFA, 2001) for humans. The levels found in both UPSN and NS are below these PTDIs, hence negligible risk of dioxin/furan exposure can be expected from these clays. The metal analysis for both clays also showed similarities. The amount of NS previously used in human trials was equal to 3 g per day per participant. In 3 g of either clay, the concentrations of trace metals were below the TDIs or PTDIs calculated for a 70 kg BW subject, according to reported values from different sources (Table 1). More importantly, most of these metals are not expected to be bioavailable upon ingestion. This is particularly important for future applications of UPSN in studies involving human subjects. As expected, the mineralogical analyses for UPSN and parent NS revealed similar structural, morphological and chemical characteristics. Similar pH values were obtained for both clays. Previous studies have found pH values ranging from 6.7 to 9.6 (Marroquín-Cardona et al., 2009) and 7.1 to 9.6 (Kannevischer et al., 2006) for similar bentonite samples. The differences observed in moisture content may be related to batch to batch variations of the clay, while the greater electrical conductivity values obtained for NS are likely related to the presence of gypsum in this sample. Nevertheless, no gypsum was evident in the XRD patterns. The slightly higher CEC obtained for UPSN (89.2 mEq per 100 g clay) could be related to the increased proportion of < 2 μ m particles in this clay, as revealed by the wet state fractionations. The CEC measured for UPSN and NS are within the range reported for similar smectites (Deer et al., 1978; Kannevischer et al., 2006; Sparks, 1995). High CEC values reflect the high capacity of the clays to potentially exchange/interact with diverse cationic components. These components have to be able to reach the interlayer galleries of the clays, implying that there are size, charge and polarity restrictions. The selection procedure used for UPSN explains differences of the dry fractions reported in table 2. Importantly, in the wet state using water, UPSN had more ≤ 2 μ m particles (75%) than in the dry state. This suggests that previous dry selection of particle size does not alter wet state particle size, which is the expected “active” state of these clays upon ingestion by animals and humans. Although not considering safety, Dixon et al. (2008) proposed a criteria for selection of effective aflatoxin adsorbents based on several mineral characteristics, including Langmuir adsorption effectiveness of 0.30 (30 points), smectite predominance by XRD (30 points), CEC of 70 (5 points), pH ranging from 6.5–8.5 (5 points), and FTIR evidence of structural Mg and Fe (10 points). According to this criterion, both UPSN and NS are very effective aflatoxin adsorbents.

The pattern of aflatoxin sorption for both samples was the same, and the Q_{max} values at two pH levels were similar. Similar findings have been previously reported for other NS samples (Marroquín-Cardona et al. 2009; Phillips et al. 2009), well within the range of other smectite materials (Kannevischer et al., 2006). The slight increase in Q_{max} observed for UPSN may be related to the enrichment of smectite particles in this sample, as revealed in the wet state fractionation analyses. At pH 2, an apparent delay in reaching the plateau was observed for both samples and this has been previously noted by other authors (Marroquín-Cardona et al., 2009; Pimpukdee et al., 2004). More importantly, this effect of pH does not seem to impair the effectiveness of these types of clays, as revealed in several animal and human studies (Harvey et al., 1991; Kutz et al., 2009; Phillips et al., 1990; Wang et al., 2008).

4.2 Animal study

None of the differences observed for the growth parameters occurred in both sexes at both levels of UPSN. The statistical analysis of hematological and serum biochemistry parameters revealed some statistically significant differences in both sexes. Nevertheless, all of these differences fall between the ranges reported for control animals in different studies (Almodovar-Cuevas et al., 1985, Wolford et al. 1986). The Na concentration and Na/K ratio in males and females of UPSN groups were significantly greater than the control. According to the extractable bases determinations, these clays are able to leach some cations by cation exchange processes and water solubilization of some components. The K levels in the females of 2% UPSN and males of both, 0.25% and 2% UPSN, were significantly lower than the controls, but no dose dependency was observed for this parameter. A possible explanation for the lower serum K levels in the males could be related to the cation exchange capacity of UPSN. It is possible that UPSN was able to exchange cations (in the clay) for K ions (in the diet). This phenomenon has been demonstrated with Na-cation-exchange resins that are orally administered to treat hyperkalemia (Elder, 2005).

Serum Ca levels were significantly greater in females treated with 0.25% UPSN and in males from both UPSN groups. Despite this, the Ca levels were within the normal range for < 6 month old rats and no evidence of dose dependency was found. In a similar study using SD rats, Afriyie-Guwau et al. (2005) found a significant increase in serum Ca levels in females ingesting 0.5 and 2% of NS in the diet. Although not significant, the males of that study also showed increased levels of serum Ca. Using simulated gastric conditions for edible soils, Hooda et al. (2004) showed that bioavailability of Ca was correlated with amounts of Ca originally present in the soils. The potential supplementation of Ca by clays has also been suggested by Wiley and Katz (1998) and Wilson (2003). In our study, based on the XRD patterns, we suggest that calcite dissolution and exchangeable Ca from montmorillonite may act as potential sources of Ca supplementation.

In the mineral and vitamin analysis, no significant differences were observed for Fe and vitamin A in the UPSN groups for either sex. However, significantly lower levels of serum Zn were detected in the males from the 2% UPSN treatment group when compared to the control. Normal ranges of serum Zn for 3 month old rats are not reported in the literature, but the Zn levels in our study fell in the ranges previously reported for control SD rats that were 28 weeks old, according to Afriyie-Gyawu et al., (2005). Nonetheless, these findings are important since previous studies using a similar bentonite (hydrated sodium calcium aluminosilicate; HSCAS) reported potential interactions with Zn. Chung et al., (1990) observed that chickens fed 1% HSCAS in the diet had significantly ($p < 0.05$) lower Zn levels measured in the tibia. Zn tibia concentration is a more reliable indicator of Zn levels in the body (Wedekind and Baker, 1990) since plasma Zn levels represent short-term changes in dietary zinc and can be influenced by factors independent of diet intake (Wallwork, 1987). Based on results from this study and Chung et al., (1990), it can be suggested that Zn interactions may become important at higher inclusion levels of bentonites but not at lower levels. Regarding vitamin E, a significant increase in serum levels was noted for both doses of UPSN in both sexes. Afriyie-Gyawu et al. (2008b) observed a similar phenomenon in men during a human 3 month trial with NS clay. In our study, a treatment effect was clear and this could be related to a potential protective function the clay may exert on the vitamin. Specifically, it is possible that UPSN may prevent complete oxidation of the vitamin in the stomach, hence making it more bioavailable in the intestines. More work is warranted to determine the mechanism of this effect.

In summary, UPSN showed similar aflatoxin sorption and mineral characteristics as parent NS; they are mainly composed of montmorillonite but also contained mica, feldspars and quartz minerals. We did not find evidence of overt toxicity due to the addition of UPSN at

the levels used in the present study. Nevertheless, our findings with Na, Zn, K and vitamin E are important and further research on this clay is warranted.

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Research Highlights

- We investigated mineral and aflatoxin sorption characteristics of two bentonites
- We tested the safety of a refined bentonite in a 3-month rodent study
- Refined bentonite and its parent material had similar *in vitro* characteristics
- Consumption of refined bentonite resulted in no overt toxicity *in vivo*

Figure 1.

Powder mounts XRD patterns of UPSN and parent NS. Minerals identified in both samples included montmorillonite (15.23, 5.1, 4.48, 2.5, 1.49 Å), mica (9.9 Å), quartz (3.3 Å), calcite (3.0 Å), orthoclase feldspars (3.7 Å) and sanidine (3.24, 3.20 Å). Quartz relative intensities in UPSN were lower than in parent NS.

Figure 2.

A) SEM image of smectite particle in UPSN sample. The particle is depicting a globular morphology with dimensions of $\sim 25 \mu\text{m} \times 15 \mu\text{m}$, according to the scale bar. B) TEM image of smectite particle displaying complex morphology of sheets and rolls. This morphology is commonly observed for effective aflatoxin adsorbents, according to Mulder et al. (2008).

Figure 3.

Aflatoxin B₁ adsorption isotherms onto UPSN and NS samples at pH 6.5 and 2. Both samples depicted an L-shape pattern characteristic of planar arrangement of molecules adsorbed on the surface. The bend on the pattern indicates that the sorbent is reaching saturation as the concentration of the solute is increased. The Q_{max} values calculated for both bentonites were similar at both pH levels, ranging from 0.39 to 0.44 mol AFB₁ kg⁻¹.

Table 1

Levels of important metals in UPSN and NS

Metal	Conc. in UPSN (mg kg ⁻¹)	Conc. in NS (mg kg ⁻¹)	Amt. in 3g UPSN (mg)	Amt. in 3g NS (mg)	TDI or PTDI (mg kg ⁻¹ BW)	Based on 70 kg BW (mg)	Reference TDI or PTDI
As*	2.5	1.7	0.007	0.005	0.002	0.14	WHO, 1989
Ba	72.1	77.5	0.216	0.233	0.051	3.57	NSCFS, 2004
Cd	0.1	0.1	0.000	0.000	0.001	0.07	WHO, 1989
Co	1.8	1.4	0.005	0.004	0.001	0.07	RIVM, 2001
Cr	1.2	1.7	0.004	0.005	0.005	0.35	RIVM, 2001
Hg	0.003	0.009	0.000	0.000	0.0002	0.01	JECFA, 2007
Mn	223.0	172.0	0.669	0.516	0.030	2.10	OEHA, 2004
Mo	0.2	0.1	0.001	0.000	0.010	0.70	RIVM, 2001
Ni	3.5	3.0	0.011	0.009	0.005	0.35	WHO, 1993
Pb	12.6	10.5	0.038	0.031	0.003	0.21	JECFA, 2000
Sr	1150.0	1040.0	3.450	3.120	0.130	9.1	WHO, 2010
Zn	53.8	38.0	0.160	0.114	1.000	70.0	JECFA, 1982

* Metalloid. TDI: Tolerable daily intake; PTDI: Provisional tolerable daily intake; RIVM: National Institute of Public Health and the Environment; NSCFS: Norwegian Scientific Committee for Food Safety; JECFA: Joint FAO/WHO Expert Committee on Food Additives; OEHA: Office of Environmental Health Hazard Assessment; WHO: World Health Organization.

Table 2

Mineral characterization of UPSN and NS

Parameter	UPSN	NS
Moisture %	6.7	12.1
pH	7.5	7.1
EC ($\mu\text{S cm}^{-1}$)	304.1	535.0
HCl reaction	Little	Little
H ₂ O ₂ reaction	ND	Little
Magnetic minerals	ND	Little
Sulfate precipitation	ND	Evident
Dry state fractions (%)		
> 100 μm	2	20
100-45 μm	67	30
< 45 μm	31	50
Wet state fractions (%) (water + Na ₂ CO ₃)		
> 53 μm	1.0	15.0
2-53 μm	23.0	20.0
< 2 μm	76.0	65.0
Wet state fractions (%) (water)		
> 50 μm	0	25
2-50 μm	25	67
< 2 μm	75	8
CEC (mEq/100 g clay)	89.2	82.9
Extractable bases (mEq/100 g clay)(NH ₄ OAc)		
Ca	98.4	85.8
Mg	8.7	9.1
Na	0.5	0.5
K	1.7	1.5
Extractable bases (mEq/100 g clay/L) (DI-water)*		
Ca	8.1	20.4
Mg	0.7	1.6
Na	0.9	1.4
K	0.6	0.8

* Extractable bases were measured in DI-water vacuum extracts from a paste made with 100 g of clay according to Rhoades and Clark, 1978; ND: non-detected.

Table 3

Growth parameters of SD rats receiving dietary UPSN at 0.25 and 2% for 13 weeks

Group	Control	UPSN 0.25%	UPSN 2%
<i>Females</i>			
FBW (g)	243.85 ± 13.49	242.41 ± 10.29 ^a	231.42 ± 6.67 ^{*b}
FCE	13.24 ± 2.74	13.60 ± 2.33 ^a	16.45 ± 2.72 ^{*b}
IBW (g)	99.52 ± 4.75	95.88 ± 4.93	94.72 ± 3.25 [*]
TBWG (g)	144.33 ± 10.12	146.53 ± 9.43 ^a	136.70 ± 7.23 ^b
TFC (g)	1899.17 ± 341.57	1982.55 ± 292.75	2244.73 ± 368.36
<i>Males</i>			
FBW (g)	406.45 ± 19.99	423.65 ± 13.8	424.88 ± 13.89
FCE	7.42 ± 0.70	7.42 ± 0.32	7.19 ± 0.25
IBW (g)	120.87 ± 3.65	122.37 ± 4.20	124.42 ± 4.64
TBWG (g)	285.58 ± 20.18	301.28 ± 12.95	300.46 ± 12.14
TFC (g)	2110.08 ± 104.45	2233.62 ± 102.72	2160.43 ± 42.07

* Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between treatment groups at the 0.05 level. Data are reported as mean values ± standard deviation. FBW: Final body weight; FCE: Feed conversion efficiency; IBW; Initial body weight; TBWG: Total body weight gain; TFC: Total feed consumption.

Table 4

Hematological parameters of SD rats receiving dietary UPSN at 0.25 and 2% for 13 weeks

Parameter	Group			Reference
	Control	UPSN 0.25%	UPSN 2.0 %	
Females				
Eosinophils μL^{-1}	25.30 $\pm$ 29.91	25.30 $\pm$ 37.83	25.10 $\pm$ 35.23	~ 196.50 $\pm$ 216.10 B
Hb (g dL^{-1})	14.19 $\pm$ 0.33	13.96 $\pm$ 0.59	13.77 $\pm$ 0.34 *	14.30 $\pm$ 0.83 C
Lymphocytes μL^{-1}	3755.10 $\pm$ 907.92	5654.00 $\pm$ 1992.89 *	4497.20 $\pm$ 855.67	6880.00 $\pm$ 2630.00 B
MCH (pg)	18.44 $\pm$ 1.70	18.33 $\pm$ 2.30	19.14 $\pm$ 1.91	19.8 $\pm$ 0.94 C
MCHC (g dL^{-1})	34.43 $\pm$ 0.72	33.30 $\pm$ 1.00 *	33.36 $\pm$ 0.55 *	34.40 $\pm$ 0.50 B
MCV (fl)	55.85 $\pm$ 1.76	57.72 $\pm$ 2.40	58.36 $\pm$ 1.55 *	57.90 $\pm$ 2.10 B
Monocytes μL^{-1}	0 $\pm$ 0.00	81.60 $\pm$ 70.85 ^a	0 $\pm$ 0.00 ^b	~19.60 $\pm$ 58.90 B
Neutrophils μL^{-1}	163.70 $\pm$ 111.49	288.30 $\pm$ 373.30 ^a	44.70 $\pm$ 62.04 ^b	870.00 $\pm$ 660.00 B
PCV (%)	41.24 $\pm$ 1.53	41.96 $\pm$ 2.07	41.30 $\pm$ 1.42	38.10 $\pm$ 2.43 C
Platelets ($\times 10^3$) μL^{-1}	468.20 $\pm$ 77.83	619.40 $\pm$ 224.76 ^a	344.80 $\pm$ 85.57 ^b	471.68 $\pm$ 38.77 A
RBCs ($\times 10^6$) μL^{-1}	7.39 $\pm$ 0.29	7.27 $\pm$ 0.29	7.08 $\pm$ 0.31	7.02 $\pm$ 0.40 B
WBC μL^{-1}	3944.00 $\pm$ 962.96	6049.00 $\pm$ 2267.71 *	4567.00 $\pm$ 872.60	7840.00 $\pm$ 2950.00 B
Males				
Eosinophils μL^{-1}	134.00 $\pm$ 109.88	49.30 $\pm$ 86.83	39.60 $\pm$ 60.57	~89.30 $\pm$ 114.80 B
Hb (g dL^{-1})	14.51 $\pm$ 0.46	14.40 $\pm$ 0.36	15.05 $\pm$ 0.78	14.70 $\pm$ 1.22 C
Lymphocytes μL^{-1}	6265.60 $\pm$ 1994.52	6951.40 $\pm$ 1839.27	6047.30 $\pm$ 1083.73	6880.00 $\pm$ 2630.00 B
MCH (pg)	18.78 $\pm$ 0.45	18.05 $\pm$ 0.41 *	18.46 $\pm$ 0.50	19.00 $\pm$ 1.09 C
MCHC (g dL^{-1})	33.77 $\pm$ 0.71	32.59 $\pm$ 0.64 ^a	33.82 $\pm$ 0.58 ^b	34.10 $\pm$ 0.40 B
MCV (fl)	55.78 $\pm$ 2.25	55.49 $\pm$ 1.63	54.61 $\pm$ 1.70	59.50 $\pm$ 2.00 B
Monocytes μL^{-1}	18.80 $\pm$ 54.70	95.30 $\pm$ 96.04	19.10 $\pm$ 31.57	~38.20 $\pm$ 63.80 B
Neutrophils μL^{-1}	819.90 $\pm$ 669.78	517.00 $\pm$ 623.21 ^a	79.10 $\pm$ 50.89 ^b	870.00 $\pm$ 660.00 B
PCV (%)	43.10 $\pm$ 1.73	44.22 $\pm$ 1.48	44.50 $\pm$ 1.96	39.90 $\pm$ 3.49 C
Platelets ($\times 10^3$) μL^{-1}	418.60 $\pm$ 133.39	692.50 $\pm$ 145.47 ^a	374.00 $\pm$ 81.09 ^b	471.68 $\pm$ 38.77 A
RBCs ($\times 10^6$) μL^{-1}	7.73 $\pm$ 0.32	7.97 $\pm$ 0.31	8.15 $\pm$ 0.28 *	7.82 $\pm$ 0.83 C

Parameter	Group			Reference
	Control	UPSN 0.25%	UPSN 2.0 %	
WBC μL^{-1}	7238.00 $\pm$ 2462.55	7613.00 $\pm$ 2293.36	6185.00 $\pm$ 1106.74	7840.00 $\pm$ 2950.00 B

* Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between UPSN treatment groups at the 0.05 level. Data are reported as mean values $\pm$ standard deviation. Hb: hemoglobin; MCH: mean cell hemoglobin; MCHC: mean corpuscular hemoglobin concentration; MCV: mean corpuscular volume; PCV: packed cell volume; RBC: red blood cell; WBC: white blood cell. References: A) Almodovar et al., 1985; B) Lillie et al., 1996; C) Wolford et al., 1986.

Table 5

Serum parameters of SD rats receiving dietary UPSN at 0.25 and 2% for 13 weeks

Parameter	Group			Reference
	Control	UPSN 0.25%	UPSN 2.0 %	
Females				
A/G Ratio	2.41 ± 0.11	2.18 ± 0.21*	2.41 ±0.22	2.13 ± 0.18 A
ALB (g dL ⁻¹)	3.74 ± 0.15	3.79 ± 0.10	3.77 ± 0.13	3.41 ± 0.23 C
ALP (U L ⁻¹)	58.40 ± 14.23	67.60 ± 17.10	69.20 ± 7.87	117 ± 41.7 B
ALT (U L ⁻¹)	54.70 ± 9.47	51.00 ± 8.75	51.60 ± 9.77	44.00 ± 23.9 D
AMYL (U L ⁻¹)	1258.40 ± 112.41	1413.50 ± 268.12	1341.70 ± 138.95	1703 ± 164.32 A
AST (U L ⁻¹)	138.20 ± 42.38	98.40 ± 24.23*	103.40 ± 24.96	93.00 ± 30.3 D
BUN (mg dL ⁻¹)	18.44 ± 1.70	18.33 ± 2.30	19.14 ± 1.91	21.00 ± 3.90 D
Ca (mg dL ⁻¹)	9.34 ± 0.32	9.80 ± 0.25 ^a	9.53 ± 0.22 ^b	10.36 ± 0.32 C
CHOL (mg dL ⁻¹)	86.73 ± 9.33	92.43 ± 11.80	90.35 ± 10.52	89.00 ± 23.00 D
CK (U L ⁻¹)	815.11 ± 360.20	262.70 ± 102.96*	558.20 ± 421.90	210.00 ± 109 C
Cl (meq L ⁻¹)	94.50 ± 19.54	101.90 ± 1.37	101.50 ± 0.84	104.00 ± 2.40 B
CRT (mg dL ⁻¹)	0.29 ± 0.03	0.30 ± 0.03	0.32 ± 0.02	0.55 ± 0.10 C
GGT (U L ⁻¹)	<3.0	<3.0	<3.0	<3.0 A
GLOB (g dL ⁻¹)	1.55 ± 0.08	1.75 ± 0.16*	1.57 ± 0.14	1.91 ± 0.09 A
GLUC (mg dL ⁻¹)	180.10 ± 15.62	192.00 ± 20.48	190.00 ± 20.79	199.80 ± 21.60 C
K (meq L ⁻¹)	5.71 ± 1.33	4.89 ± 0.32	4.68 ± 0.19*	4.76 ± 0.44 C
Na (meq L ⁻¹)	129.80 ± 25.68	140.30 ± 1.06*	141.60 ± 0.70*	142.00 ± 2.00 C
Na/K Ratio	23.04 ± 2.99	28.80 ± 1.78*	30.32 ± 1.28*	~29 C
P (mg dL ⁻¹)	5.56 ± 0.87	4.94 ± 0.61	5.30 ± 0.37	5.80 ± 1.10 B
T-BIL (mg dL ⁻¹)	0.09 ± 0.05	0.07 ± 0.06	0.13 ± 0.02	0.30 ± 0.24 D
TP (g dL ⁻¹)	5.29 ± 0.21	5.54 ± 0.17*	5.34 ± 0.19	6.17 ± 0.33 C
Males				
A/G Ratio	1.93 ± 0.15	1.75 ± 0.17	1.82 ± 0.10	1.37 ± 0.18 A
ALB (g dL ⁻¹)	3.42 ± 0.15	3.67 ± 0.20*	3.70 ± 0.16*	3.40 ± 0.20 B

Parameter	Group				Reference
	Control	UPSN 0.25%	UPSN 2.0 %	Normal values	
ALP (U L ⁻¹)	79.20 ± 9.47	60.40 ± 14.35*	58.40 ± 8.45*	130 ± 43.7	B
ALT (U L ⁻¹)	61.30 ± 19.38	49.20 ± 8.79 ^a	40.20 ± 3.82 ^{ab}	49.00 ± 24.1	B
AMYL (U L ⁻¹)	2316.00 ± 211.13	2329.80 ± 274.48	2305.20 ± 192.53	2671.00 ± 89.00	A
AST (U L ⁻¹)	116.80 ± 49.97	104.40 ± 31.13 ^a	70.30 ± 13.42 ^{ab}	97.00 ± 28.40	D
BUN (mg dL ⁻¹)	19.41 ± 2.63	20.98 ± 2.50	20.88 ± 3.08	20.00 ± 3.20	D
Ca (mg dL ⁻¹)	9.12 ± 0.39	9.90 ± 0.18*	9.82 ± 0.30*	10.48 ± 0.28	C
CHOL (mg dL ⁻¹)	85.37 ± 10.16	95.70 ± 9.79	97.69 ± 12.03*	75.00 ± 19.80	D
CK (U L ⁻¹)	432.22 ± 227.32	338.80 ± 151.83	271.60 ± 92.34	222.00 ± 109.00	C
Cl (meq L ⁻¹)	98.30 ± 2.26	100.20 ± 0.92	101.00 ± 2.30*	103.00 ± 1.90	B
CRT (mg dL ⁻¹)	0.26 ± 0.03	0.29 ± 0.03	0.28 ± 0.02	0.54 ± 0.08	C
GGT (U L ⁻¹)	<3.0	<3.0	<3.0	<3.0	A
GLOB (g dL ⁻¹)	1.78 ± 0.15	2.11 ± 0.14*	2.04 ± 0.11*	2.51 ± 0.25	A
GLUC (mg dL ⁻¹)	192.70 ± 37.05	214.20 ± 39.70	192.10 ± 19.83	212.40 ± 28.8	C
K (meq L ⁻¹)	7.23 ± 0.66	5.11 ± 0.38*	5.38 ± 0.27*	5.30 ± 0.39	C
Na (meq L ⁻¹)	135.20 ± 2.74	140.80 ± 0.63*	142.30 ± 3.30*	142.00 ± 2.00	C
Na/K Ratio	18.86 ± 1.74	27.69 ± 2.11*	26.51 ± 1.36*	~26	C
P (mg dL ⁻¹)	5.92 ± 0.39	6.08 ± 0.59	6.15 ± 0.22	7.30 ± 1.50	B
T-BIL (mg dL ⁻¹)	0.01 ± 0.03	0.07 ± 0.06	0.09 ± 0.05*	0.30 ± 0.16	D
TP (g dL ⁻¹)	5.20 ± 0.25	5.78 ± 0.17*	5.74 ± 0.22*	5.85 ± 0.23	C

* Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between UPSN treatment groups at the 0.05 level. Data are reported as mean values ± standard deviation. ALB: albumin; ALP: alkaline phosphatase; ALT: alanine aminotransferase; AMYL: amylase; AST: aspartate aminotransferase; BUN: blood urea nitrogen; CHOL: cholesterol; CK: creatine kinase; CTR: creatinine; GGT: gamma-glutamyl transferase; GLOB: globulin; GLUC: glucose; T-BIL: total bilirubin; TP: total protein. References: A) Afriyie-Gyawu et al., 2005; B) Kohn and Clifford, 2002; C) Lillie et al., 1996; D) Wolford et al., 1986.

Table 6

Levels of Fe, Zn, vitamin A and E in serum of SD rats receiving dietary UPSN at 0.25 and 2% for 13 weeks

Parameter	Group			
	Control	UPSN 0.25%	UPSN 2%	Normal values
<i>Females</i>				
Iron ($\mu\text{g dL}^{-1}$)	254.67 $\pm$ 38.20	245.74 $\pm$ 33.98	246.47 $\pm$ 60.02	220 $\pm$ 130
Zinc ($\mu\text{g mL}^{-1}$)	1.24 $\pm$ 0.18	1.34 $\pm$ 0.09 ^a	1.12 $\pm$ 0.10 ^b	1.05 $\pm$ 0.09
Vitamin A ($\mu\text{g L}^{-1}$)	267.31 $\pm$ 27.78	275.50 $\pm$ 33.92	271.01 $\pm$ 19.11	254.10 $\pm$ 6.00
Vitamin E ($\mu\text{g L}^{-1}$)	3560.00 $\pm$ 40.00	9150.00 $\pm$ 1350 [*]	8500.00 $\pm$ 1040.00 [*]	NA
<i>Males</i>				
Iron ($\mu\text{g dL}^{-1}$)	115.89 $\pm$ 10.92	126.87 $\pm$ 19.99	123.45 $\pm$ 16.62	152 $\pm$ 70
Zinc ($\mu\text{g mL}^{-1}$)	1.50 $\pm$ 0.09	1.51 $\pm$ 0.13 ^a	1.24 $\pm$ 0.09 ^{*b}	1.29 $\pm$ 0.04
Vitamin A ($\mu\text{g L}^{-1}$)	530.80 $\pm$ 39.44	489.50 $\pm$ 80.10	509.40 $\pm$ 36.22	567.40 $\pm$ 24.80
Vitamin E ($\mu\text{g L}^{-1}$)	3440.00 $\pm$ 50.00	7860.00 $\pm$ 2690.00 [*]	7430.00 $\pm$ 870.00 [*]	2106.17 $\pm$ 1261.98

* Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between UPSN treatment groups at the 0.05 level. Data are reported as mean values $\pm$ standard deviation. NA: Not available. References: A) Afriye-Gyawu et al. 2005; B) Kohn and Clifford 2002; E) Seft et al. 2009.

Table 7

Relative organ weights of SD rats receiving dietary UPSN at 0.25 and 2% for 13 weeks

Group	Control	UPSN 0.25%	UPSN 2%
<i>Females</i>			
Brain	1.63 ± 0.11	1.60 ± 0.07 ^a	1.66 ± 0.04 ^{*b}
Heart	0.82 ± 0.06	0.81 ± 0.06	0.81 ± 0.05
L. kidney	0.80 ± 0.07	0.79 ± 0.08	0.71 ± 0.04 [*]
Liver	6.82 ± 0.40	6.92 ± 0.71	6.35 ± 0.27
Lung	1.20 ± 0.10	1.17 ± 0.06	1.11 ± 0.07
R. kidney	0.79 ± 0.06	0.77 ± 0.07	0.72 ± 0.05
Spleen	0.52 ± 0.06	0.53 ± 0.06	0.51 ± 0.06
Tibia	0.75 ± 0.13	0.70 ± 0.04	0.62 ± 0.07
Uterus	0.63 ± 0.13	0.59 ± 0.12	0.55 ± 0.12
<i>Males</i>			
Brain	1.65 ± 0.09	1.75 ± 0.15	1.69 ± 0.24
Heart	1.36 ± 0.09	1.32 ± 0.10 ^a	1.42 ± 0.11 ^b
L. kidney	1.31 ± 0.11	1.33 ± 0.12	1.32 ± 0.08
Liver	12.34 ± 0.82	12.10 ± 0.89	11.35 ± 0.63 [*]
Lung	1.64 ± 0.21	1.65 ± 0.22	1.61 ± 0.14
R. kidney	1.35 ± 0.14	1.39 ± 0.12	1.33 ± 0.09
Spleen	0.77 ± 0.07	0.78 ± 0.08	0.79 ± 0.09
Tibia	1.04 ± 0.04	1.06 ± 0.03	1.14 ± 0.10

* Means with asterisk are significantly different from the control at the 0.05 level. Means with different letters are significantly different between UPSN treatment groups at the 0.05 level. The relative organ weights (ROWs) were calculated by dividing the wet weight of each organ by the final body weight of the rat just before termination. ROWs have been multiplied by a factor of 10². Data are reported as mean values ± standard deviation.