

In Vitro and In Vivo Assessment of Humic Acid as an Aflatoxin Binder in Broiler Chickens

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ABSTRACT The in vitro affinity and adsorption capacity of a humic acid, oxihumate, for aflatoxin B₁ (AFB₁) was evaluated, utilizing Langmuir and Freundlich adsorption isotherms. Oxihumate showed a high in vitro affinity for AFB₁. The Freundlich isotherm fitted the data better than the Langmuir isotherm, and binding capacities of 10.3, 7.4, and 11.9 mg of AFB₁/g of oxihumate at pH 3, 5, and 7, respectively, were calculated. The in vivo efficacy of oxihumate as an aflatoxin binder in male broiler chickens exposed to aflatoxin-contaminated feed from 7 to 42 d of age was also assessed. The efficacy of oxihumate was compared with a commercially available product with a brewers dried yeast (BDY) and brewers fermentation solubles as main active ingredients. A total of 420 birds were assigned to 28 pens, with 15 birds per pen. The following treatments were applied: 1) 0 mg of AFB₁ + 0 additives, 2) 1 mg of AFB₁/kg of feed + 0 additives, 3) 1 mg of AFB₁/kg of feed + 3.5 g of oxihumate/kg of feed,

4) 1 mg of AFB₁/kg of feed + 3.5 g of BDY/kg of feed, 5) 2 mg of AFB₁/kg of feed + 0 additives, 6) 2 mg of AFB₁/kg of feed + 3.5 g of oxihumate/kg of feed, and 7) 2 mg of AFB₁/kg of feed + 3.5 g of BDY/kg of feed. Each treatment consisted of 4 replicates. Oxihumate was effective in diminishing the adverse effects caused by aflatoxin on BW of broilers ($P < 0.05$). Oxihumate also showed protective effects against liver damage, stomach and heart enlargement, as well as some of the hematological and serum biochemical changes associated with aflatoxin toxicity ($P < 0.05$). Results indicated that oxihumate, but not BDY, could alleviate some of the toxic effects of aflatoxin in growing broilers. Oxihumate might, therefore, prove to be beneficial in the management of aflatoxin-contaminated feedstuffs for poultry when used in combination with other mycotoxin management practices. Additional studies are warranted to assess its efficacy under a wide variety of circumstances.

Key words: broiler, humic acid, oxihumate, aflatoxin binder

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INTRODUCTION

Aspergillus species infect economically important crops and forages in the field and during storage, transportation, and processing. These fungi produce aflatoxins, which contaminate food and animal feeds worldwide, causing serious health problems and livestock production losses. Aflatoxin B₁ (AFB₁), the most toxic of the aflatoxins, is often encountered in food and animal feeds at alarming concentrations around the world (Marquardt, 1996).

Unfortunately, discontinuing the feeding of aflatoxin-contaminated grain is not always practical, especially when alternate feedstuffs are not readily available or affordable (Ramos et al., 1996b). A variety of physical, chemical, and biological techniques for mycotoxin decon-

tamination of agricultural commodities have been used, but they have had limited success (Doyle et al., 1982). One of the most practical approaches is the use of nonnutritive adsorbents, which bind the mycotoxins and inhibit their absorption from the gastrointestinal tract, thus minimizing the toxic effects in livestock and the carryover of these fungal metabolites into animal products (Ramos et al., 1996a). Aluminosilicates; activated charcoal; polymers, such as cholestyramine and polyvinylpyrrolidones; and yeast and yeast products have been extensively studied with promising, but varying, results (Huwig et al., 2001). For an adsorbent to successfully prevent the absorption of aflatoxins from the gastrointestinal tract, it should have a high affinity for aflatoxin, resulting in the formation of a strong complex with little risk of dissociation. The adsorbent should also have a high binding capacity, to prevent saturation (Ramos and Hernández, 1996).

Humic acids are ubiquitous and are found wherever matter is being decomposed or has been transposed, as in the case of sediments. Humic substances have demonstrated a strong affinity to bind various substances, such

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as heavy metals (Madronová et al., 2001), herbicides (Nègre et al., 2001), different mutagens (Sato et al., 1987; Cozzi et al., 1993), monoaromatic (Nanny and Maza, 2001) and polycyclic aromatic compounds (Kollist-Siigur et al., 2001), minerals (Elfarissi and Pefferkorn, 2000), and *Bacillus subtilis* bacteria (Fein et al., 1999). In spite of its known binding characteristics, humic acids have never been evaluated previously as a mycotoxin adsorbent.

Enerkom (Pty.) Ltd. (Pretoria, South Africa) developed an effective large-scale process to regenerate pure, high-quality humic acids from bituminous coal by reversing the process whereby the coal was formed. Humic acids produced in this manner, called oxihumates, differ only slightly chemically from humic acids obtained from other sources (Bergh et al., 1997).

The objective of this study was to evaluate the efficacy of oxihumate as an aflatoxin binder, both in vitro and in vivo. The binding capability of oxihumate for AFB₁ was tested, and the Langmuir and Freundlich adsorption isotherms of oxihumate were determined for AFB₁ to describe surface adsorption (Ramos and Hernández, 1996). The second objective of this study was to evaluate the effects of oxihumate on growth performance, liver morphology, and serum biochemical and hematological variables in broiler chickens exposed to aflatoxins.

MATERIALS AND METHODS

In Vitro Study

Experiment 1. The use of isotherms is one of the most efficient mathematical approaches to describe surface adsorption, in which the amount of compound adsorbed per unit of weight of adsorbent is plotted against the concentration of the compound in the external phase, under equilibrium conditions (Ramos and Hernández, 1996). Multiple isotherm equations have been proposed for the modeling of the adsorption of compounds in aqueous solutions to the surfaces of solids (Kinniburgh, 1986), of which the Langmuir and Freundlich isotherms are the most extensively used (Ramos and Hernández, 1996). The Langmuir equation is most applicable to a single ligand adsorbed to a single type of site on a particular adsorbent (Langmuir, 1916), whereas the Freundlich equation relates to a heterogeneous adsorbent surface, or the coexistence of different adsorption mechanisms (Ramos and Hernández, 1996).

The binding of oxihumate [Enerkom (Pty.) Ltd.] to AFB₁ (Sigma-Aldrich Inc., St. Louis, MO) was determined under different pH conditions, and a study of the Langmuir and Freundlich adsorption isotherms was carried out. Primary stock solutions of 1,000 mg of AFB₁/L of methanol were prepared. Oxihumate was weighed into clean, 15-mL screw-cap test tubes, and 10 mL of 0.1 M phosphate buffer (pH 3, 5, or 7), containing 2 mg/L of AFB₁, was added to the tubes. To correct for possible exogenous peaks, controls were prepared by adding 10 mL of 0.1 M phosphate buffer plus 100 mg of adsorbent to test tubes. The tubes were vortexed, shaken for 30 min at room

temperature, and centrifuged at 675 × g for 5 min. The aqueous supernatants were analyzed for AFB₁ levels by HPLC, which was performed by a Perkin-Elmer 250 pump (Perkin-Elmer Life and Analytical Sciences Inc., Wellesley, MA) with a Perkin-Elmer ISS-200 autosampler (Perkin-Elmer Life and Analytical Sciences Inc.), fluorescence detection (excitation wavelength of 365 nm and emission wavelength of 430 nm) with a Hitachi F1200 fluorescence spectrophotometer (Hitachi High Technologies America Inc., San Jose, CA), and ultraviolet detection with a Perkin-Elmer LC-90 detector (Perkin-Elmer Life and Analytical Sciences Inc.). Separations were achieved on a 100 × 4.6 mm Hypersil BDS 3-μm C₁₈ column (Phenomenex, Torrance, CA) or a Perkin-Elmer 3-cm C₁₈ column (3-μm particle size, Perkin-Elmer Life and Analytical Sciences Inc.). A water:MeOH:isopropanol (40:17:2) mobile phase was pumped at 1 mL/min. Percentage of AFB₁ bound was calculated from the difference between the initial and final concentration in the aqueous supernatant. All samples were run in triplicate. An aliquot of the original buffered AFB₁ test solution was used as standard.

Experiment 2. The binding of AFB₁ by oxihumate was determined when mixed with a commercial poultry feed to simulate practical conditions. If a high proportion of oxihumate was bound by the feed, it could inhibit the adsorption of AFB₁ by oxihumate. Oxihumate and feed were weighed into clean, 15-mL screw-cap test tubes at a concentration of 3.5 g of oxihumate/kg of feed. Ten milliliters of 0.1 M phosphate buffer (pH 3 or 7) was added to the tubes. The tubes were vortexed and shaken for 30 min at room temperature to allow binding of oxihumate to the feed. Aflatoxin B₁ was then added to the tubes at a final concentration of 2 mg/L. The tubes were vortexed, shaken, and centrifuged at 675 × g for 5 min. The aqueous supernatant was analyzed for aflatoxin levels by HPLC, as previously described. All tests were run in triplicate.

Experiment 3. The stability of the aflatoxin–oxihumate adsorption complex in the presence of a series of solvents was determined. Ten milligrams of oxihumate was weighed into clean, 15-mL screw-cap test tubes, and 10 mL of 0.1 M phosphate buffer (pH 3), containing 2 mg/L of AFB₁, was added to each tube. The tubes were vortexed, shaken, and centrifuged at 675 × g for 5 min. The aqueous supernatants were removed, and 10 mL of chloroform, acetonitrile, or acetone were added. After centrifugation, 5 mL of solvent was removed and evaporated. One milliliter of methanol was added to each tube and vortexed, after which 4 mL of buffer (pH 3) was added to restore the original volume. Samples were analyzed for AFB₁ levels by HPLC, as previously described. All tests were run in triplicate.

In Vivo Study

Aflatoxin Production. *Aspergillus parasiticus* strain NRRL 2999 (kindly donated by W.M. Hagler, College of Agriculture and Life Sciences, North Carolina State

University, Raleigh) was grown on rice, as described by Shotwell et al. (1966). This strain is very stable and consistently yields high levels of aflatoxin, especially AFB₁, even after many transfers. Fermented rice was autoclaved to stop fungal growth and dried in stainless steel pans in a forced-air oven at 40°C for 24 h. After several batches of aflatoxin-contaminated rice were produced, it was ground, thoroughly mixed, and tested for aflatoxin levels by HPLC, as previously described. The ground rice was added to the ration to provide the required AFB₁ level. The concentration of ground rice culture material never exceeded 1% of the total diet.

Pilot Studies. After a pilot trial to determine the oxihumate inclusion level, 3.5 g of oxihumate/kg of feed was chosen as dietary concentration for the main trial, as it showed the same efficacy against the effects of 2 mg of AFB₁/kg of feed as the higher levels (data not shown). A toxicity study was conducted, in which 120 Ross broilers were divided into 2 treatment groups, with 4 replicates per treatment and 15 birds per replicate. One group received a commercial broiler ration with 3.5 g of oxihumate/kg of feed added, and the other group received the same ration without oxihumate. Parameters measured in this study included BW gain, hematocrit, serum profile (albumin, globulin, total protein, and γ -glutamyltransferase), and mortality rate.

Experimental Design, Birds, and Diets. A total of 500 d-old male Ross 788 chicks were adapted for a 7-d period before commencement of the trial. During this period, the birds were submitted to conventional broiler chicken management and housed in floor pens in an environmentally controlled broiler house with litter floors. They received a commercial broiler starter diet formulated to meet or exceed the nutritional requirements of broilers, as recommended by the NRC (1994). This diet, as well as all basal diets used subsequently, was analyzed and tested negative for aflatoxins. Day-old chicks were spray-vaccinated against infectious bronchitis and Newcastle disease, but no further vaccination was applied during the trial. At 7-d of age, 420 chicks of similar weight were randomly assigned to 28 clean pens in the same broiler house used for the adaptation period. Birds were maintained on a 23L:1D schedule and allowed to consume feed and water ad libitum. Air temperature was controlled according to Ross recommendations. The basal diet used throughout the study was a 3-phase commercial corn and soybean meal-based ration, formulated to meet or exceed the nutritional requirements of broilers, as recommended by the NRC (1994) and contained a coccidiostat. The birds were divided into 7 treatment groups, with 4 replicates per treatment and 15 birds per replicate. Three levels of AFB₁ were used (0, 1, and 2 mg of AFB₁/kg of feed). The AFB₁-contaminated diets contained either no additives, 3.5 g of oxihumate/kg of feed, or 3.5 g of a well-known commercially available adsorbent with a brewers dried yeast (BDY) and brewers fermentation solubles as main active ingredients per kilogram of feed. This product is also known as yeast glucomannan (Karaman et al., 2005), esterified glucomannan (Raju and Devegowda, 2000; Ara-

vind et al., 2003; Diaz et al., 2005) or modified glucomannan (Dvorska et al., 2003) in the literature.

The oxihumate, BDY, and aflatoxin-contaminated rice powder were mixed into the different treatment diets to required concentrations. Samples were collected from each treatment and analyzed, as previously described, for confirmation of AFB₁ levels.

The experiment was terminated when the broilers were 42 d of age. The birds were weighed weekly, and mortalities were recorded as they occurred.

Hematological and Serological Analysis. On d 38, all chicks were bled by puncture of the brachial vein. Whole blood was collected in EDTA blood tubes for hematocrit determination, using a Jouan microhematocrit centrifuge (Scientific Group, SA Scientific Products Pty. Ltd. Trading, Johannesburg, South Africa). Another blood sample was collected from all birds in tubes without anticoagulant. Serum was obtained from these samples and analyzed for serum albumin, total serum protein, γ -glutamyltransferase (E.C. 2.3.2.2), and aspartate aminotransferases (E.C. 2.6.1.1) with a Technicon RA-1000 system (Miles Inc., Diagnostics Division, Tarrytown, NY) according to standard procedures, as described by Technicon RA Systems (1994).

Histopathology. At the termination of the study at 42 d, all chicks were killed by cervical dislocation, and the liver, heart, proventriculus, and gizzard were removed and weighed. The contents of the proventriculus and gizzard were removed before weighing to determine the stomach weight. The organ weights were expressed as a percentage of BW.

Samples of the right liver lobe of all birds were fixed in 10% neutral buffered formalin and examined for liver lesions by the Pathology Laboratory, Faculty of Veterinary Sciences, Onderstepoort, South Africa, using standard histological and staining techniques. The evaluation was done as a double-blinded study. Two opposite sections of all formalin fixed samples were examined and macroscopically judged for color after formalin fixation. The microscopic appearance of the samples was evaluated for fatty degeneration, hepatocyte necrosis, bile-duct proliferation, fibrosis, architectural disturbances, anisonucleosis, chromatin margination, prominent nucleoli, mitosis, nodular hyperplasia, and heterophil and lymphocyte aggregation. The degree of severity of each of the different lesion types was expressed as 0 (no lesions), 1 (mild), 2 (moderate), or 3 (severe). The sum of the numeric values of all the lesions together for each sample was used for statistical analysis to compare treatment groups.

Ethical Approval. Ethical approval was obtained from the Ethics Committee of the Faculty of Natural and Agricultural Sciences, University of Pretoria (EC010607-006).

Statistical Analysis. For BW data, a repeated measure ANOVA with the GLM model (SAS Institute, 1994) was used to determine the significance (5%) between treatment and pens within treatment effects for the repeated weekly BW. Least square means and \pm SE were calculated.

For all other measurements, an ANOVA with the GLM model (SAS Institute, 1994) was used to determine the

Table 1. In vitro binding (%) of aflatoxin B₁ (AFB₁) by oxihumate at pH 3, 5, and 7¹

Oxihumate ² (mg)	Binding of AFB ₁ (%)		
	pH 3	pH 5	pH 7
50	99.8	99.8	89.7
10	95.4	91.9	85.5
5	83.7	85.5	82.1
4	75.8	82.6	81.3
3	71.2	81.4	76.4
2	58.2	59.9	74.0
1	46.7	36.4	58.1
0.6	39.9	24.5	42.9
0.4	27.5	18.1	33.5
0.08	8.3	5.2	9.8
0.04	4.3	2.6	4.9

¹Each value is the average of 2 replicates compared with a control without added oxihumate.

²In 10 mL of buffer containing 2 mg of AFB₁/L.

significance between treatment and pens within treatment effects for the unbalanced data. Least square means and \pm SE were calculated for treatments. Significance of difference (5%) among least square means was determined by the Bonferroni test (Samuels, 1989).

RESULTS AND DISCUSSION

Aflatoxins are a cause of concern in the poultry industry due to health problems in flocks and potential economic losses (Bailey et al., 1998). Consequently, poultry producers are in need of aids and methods to assist them in the protection of their flocks against these toxins. It is important to adequately test a potential mycotoxin adsorbent, not only for its in vitro binding capabilities, but also for its in vivo ability, because results in the past have indicated that there is great variability in the efficacy of adsorbents in vivo, even though the compounds may show potential for toxin binding in vitro (Bailey et al., 1998). This emphasizes the importance for industry scrutiny of new products purported to ameliorate the effects of mycotoxins in poultry, to ensure that sound scientific principles have been applied in the evaluation of these products (Dale, 1998).

In Vitro Study

Aflatoxin is a relatively low molecular-weight, lipophilic molecule that appears to be absorbed rapidly (Kumagai, 1989) and completely (Wogan et al., 1967) from the gastrointestinal tract. Adsorbents used to hinder the gastrointestinal absorption of mycotoxins should have a high affinity for the specific mycotoxins, resulting in the formation of a strong complex to minimize the risk of any rupture of the complex. The chosen compound should also have a high capacity to prevent saturation (Ramos and Hernández, 1996).

The adsorption and equilibrium concentrations of AFB₁ in solution at room temperature and at different pH levels for different levels of oxihumate are presented in Table 1. Each value is the average of 2 replicates compared with

Table 2. The Langmuir and Freundlich oxihumate adsorption isotherm parameters obtained for aflatoxin B₁ (AFB₁) at pH 3, 5, and 7 at room temperature

Isotherm parameters ¹	AFB ₁		
	pH 3	pH 5	pH 7
Freundlich isotherm			
$C_a = k_1 \cdot C_s^{k_2}$			
k_1	10.268	7.408	11.892
k_2	0.614	0.557	1.517
r^2	0.913	0.9627	0.899
Langmuir isotherm			
$C_a/C_a = (C_s/k_1) + (1/k_1k_2)$			
k_1	81.967	16.529	-10.965
k_2	0.151	0.970	-0.432
r^2	0.064	0.701	0.205

¹ C_a = amount of aflatoxin adsorbed per unit of weight of adsorbent (mg/g); C_s = concentration of unadsorbed aflatoxin at equilibrium (mg/g); k_1 = capacity constant (mg/g); and k_2 = affinity constant.

a control without added oxihumate. These data were used to obtain the Langmuir and Freundlich oxihumate adsorption isotherm parameters for AFB₁ at pH 3, 5, and 7 at room temperature. The Freundlich oxihumate adsorption isotherm fits the data better than the Langmuir oxihumate isotherm, as demonstrated by the higher coefficients of determination values obtained (Table 2). According to Ramos and Hernández (1996), this might indicate the presence of adsorption centers within the oxihumate with different affinities for aflatoxin, resulting in a heterogeneous adsorbent surface or the coexistence of different adsorption mechanisms. Adsorptions of 10.3, 7.4, and 11.9 mg of AFB₁/g of oxihumate at pH 3, 5, and 7, respectively, were calculated. According to Decker and Corby (1980), activated charcoal adsorbed at a rate of 10 mg of AFB₁/g, whereas a gram of montmorillonite silicate was able to adsorb about 1 mg of AFB₁ at pH 7 (Ramos and Hernández, 1996). In contrast, the maximum AFB₁ adsorption capacity of a sodium bentonite from Southern Argentina was estimated to be 45 mg/g at pH 2 (Rosa et al., 2001).

The second in vitro experiment showed that oxihumate mixed with poultry feed adsorbed AFB₁ with the same efficacy as in buffer alone, at pH 3 and pH 7, suggesting that oxihumate does not bind feed molecules. In this experiment, oxihumate bound 92.0% of the AFB₁ at pH 3 and 82.2% at pH 7, whereas 92.9% and 87.5% of the AFB₁ were bound at pH 3 and pH 7, respectively, in the presence of both oxihumate and poultry feed. All oxihumate particles mixed into mycotoxin-contaminated feed should, therefore, be available for the formation of an oxihumate-mycotoxin complex. The complex may, however, not be very stable, as it was ruptured to a high degree in the presence of acetonitrile (61%) and acetone (75%), but not chloroform (3%). The failure of chloroform to rupture the complex may be attributed to the high capacity of oxihumate to hold water in and around its structure with a consequential gel formation, thus preventing the hydrophobic chloroform from penetrating the water layer. The aflatoxin, in association with oxihumate, is water-insoluble, and the chloroform can, therefore, not reach the complexes.

Table 3. Body weight at 35 d of age, hematocrit, and serum profile of broilers fed diets with or without oxihumate¹

Treatment	BW (g)	Hematocrit ² (%)	Serum globulin ³ (g/L)	Serum albumin ³ (g/L)	Serum γ -glutamyltransferase (IU/L) ³	Total serum protein ³ (g/L)
0 g of oxihumate/kg of feed	1,743.5 \pm 20.5	31.1 \pm 0.82	15.5 \pm 0.41	16.5 \pm 0.35	9.82 \pm 1.25	32.15 \pm 0.46
3.5 g of oxihumate/kg of feed	1,781.2 \pm 19.5	31.7 \pm 0.79	16.3 \pm 0.50	17.9 \pm 0.37	8.40 \pm 1.19	33.85 \pm 0.49

¹Each value represents the mean \pm SE of 4 replicates with 15 birds per replicate.

²Hematocrit determined using a Jouan microhematocrit centrifuge (Scientific Group, SA Scientific Products Pty. Ltd. Trading, Johannesburg, South Africa).

³Serum analyzed with a Technicon RA-1000 system (Miles Inc., Diagnostic Division, Tarrytown, NY) according to standard procedures.

In Vivo Study

Aflatoxins were produced by fermentation of rice by the NRLL 2999 strain of *Aspergillus parasiticus*, under constant stirring and controlled temperature. A total of 1,116 mg of aflatoxin/kg of rice material was obtained, containing 82.6% AFB₁, 3.2% aflatoxin B₂, 13.6% aflatoxin G₁, and 0.7% aflatoxin G₂.

For the toxicity study, no significant differences were noted between birds receiving oxihumate and those who did not receive oxihumate, for all parameters measured (Table 3). The effect of BDY alone was not studied in this trial, but results from previous trials on similar products revealed conflicting results. Raju and Devegowda (2000) found that the additive included at 1 g/kg of feed did not affect BW, feed intake, feed conversion ratio, organ weight, total serum protein, total serum cholesterol, blood hemoglobin, or activity of the serum enzymes, γ -glutamyltransferase, alanine aminotransferase, and aspartate aminotransferase of broilers at 35 d of age. Aravind et al. (2003), however, reported that 0.5 g of a similar product per kilogram of feed resulted in an increased BW, improved feed conversion ratio, increased kidney weight, and increased total serum protein and cholesterol levels in broilers at 35 d of age. Serum enzyme activity, hemoglobin, and hematocrit were not affected by the feed additive.

Data presented in Table 4 show the effect of dietary treatments on BW. A concentration of 1 mg of AFB₁/kg of feed did not affect the BW and, thus, growth of the broilers up to 42 d of age. However, the 2 mg of AFB₁/kg of feed level depressed growth, with significantly lower BW evident from an age of 21 d on, 2 wk after intake of the contaminated feed commenced. The reduction in

weight gain continued throughout the study. These results support various researchers, who found that aflatoxin ingestion inhibits growth in chickens (Ledoux et al., 1999; Miazzi et al., 2000). The decrease in BW gain caused by the addition of 2 mg of AFB₁/kg of feed was diminished at 42 d of age by the addition of 3.5 g of oxihumate/kg of feed to the diet. In the present study, no indication of any improvement in weight gain was observed when the chicks consumed diets containing aflatoxin plus BDY, contrary to the report of Stanley et al. (1993).

The stunted growth associated with aflatoxin intoxication may be a secondary effect, as the liver is considered to be the main target organ for aflatoxicosis (Coulombe, 1994). The toxic metabolites of aflatoxin bind to nucleic acids and nucleoproteins, essential to cellular viability, and result in an excessive buildup of hepatic lipids, with enlargement of the liver, proliferation of bile duct epithelium (Adav and Godinwar, 1997), necrosis (Kichou and Walser, 1994), and hepatocellular carcinoma (Van Rensburg et al., 1985; Peers et al., 1987). In poultry, the relative weight of the liver is increased by aflatoxin ingestion more than that of any other organ (Huff et al., 1986; Kubena et al., 1990a). The present data (Table 5) indicate that the relative weights for the livers were significantly increased by almost 75% for the chicks consuming diets contaminated with 2 mg of AFB₁/kg of feed. The livers of these chicks also appeared to be friable and pale yellow as a result of fat accumulation in the cytoplasm of the hepatocytes. Oxihumate showed significant protective effects with respect to liver damage, as indicated by an inhibition of liver enlargement. Microscopically, aflatoxin ingestion had a pronounced dose-response effect on the quantity and severity of hepatic lesions, with a lesion

Table 4. Body weight (g) of broilers fed aflatoxin B₁- (AFB₁) contaminated diets supplemented with oxihumate, or brewers dried yeast (BDY)¹

Treatment			Day					
AFB ₁ (mg/kg)	Oxihumate (g/kg)	BDY (g/kg)	7	14	21	28	35	42
0	0	0	153 \pm 1.8 ^a	380 \pm 4.8 ^a	724 \pm 10 ^a	1,137 \pm 18 ^a	1,630 \pm 26 ^a	2,194 \pm 39 ^a
1	0	0	155 \pm 1.8 ^a	382 \pm 4.9 ^a	717 \pm 10 ^{ac}	1,143 \pm 18 ^a	1,647 \pm 27 ^a	2,227 \pm 40 ^a
1	3.5	0	157 \pm 1.8 ^a	387 \pm 5.1 ^a	723 \pm 11 ^a	1,147 \pm 19 ^a	1,642 \pm 28 ^a	2,226 \pm 41 ^a
1	0	3.5	158 \pm 1.8 ^a	385 \pm 4.9 ^a	725 \pm 11 ^a	1,127 \pm 18 ^a	1,602 \pm 27 ^a	2,123 \pm 39 ^a
2	0	0	158 \pm 1.9 ^a	384 \pm 5.1 ^a	689 \pm 11 ^{bc}	995 \pm 19 ^b	1,317 \pm 28 ^b	1,692 \pm 41 ^c
2	3.5	0	158 \pm 1.8 ^a	380 \pm 5.0 ^a	669 \pm 11 ^b	983 \pm 19 ^b	1,361 \pm 27 ^b	1,821 \pm 40 ^b
2	0	3.5	157 \pm 1.8 ^a	382 \pm 5.0 ^a	681 \pm 11 ^b	993 \pm 19 ^b	1,339 \pm 27 ^b	1,727 \pm 40 ^{bc}

^{a-c}Values within a column with no common superscript differ significantly ($P < 0.05$).

¹Each value represents the mean \pm SE of 4 replicates with 15 birds per replicate.

Table 5. Histopathological and hematological parameters of broilers fed aflatoxin B₁- (AFB₁) contaminated diets supplemented with oxihumate or brewers dried yeast (BDY)¹

	Treatment			Relative liver weight (% of BW)	Relative heart weight (% of BW)	Relative stomach weight (% of BW)	Liver lesions ²	Hematocrit ³ (%)
	AFB ₁ (mg/kg)	Oxihumate (g/kg)	BDY (g/kg)					
0	0	0	2.44 ± 0.20 ^c	0.622 ± 0.02 ^b	3.00 ± 0.11 ^d	1.75 ± 0.67 ^d	31.58 ± 0.52 ^a	
1	0	0	2.31 ± 0.20 ^c	0.591 ± 0.02 ^b	3.43 ± 0.11 ^{bc}	4.13 ± 0.71 ^c	32.03 ± 0.52 ^a	
1	3.5	0	2.27 ± 0.20 ^c	0.595 ± 0.02 ^b	3.15 ± 0.11 ^{cd}	3.79 ± 0.69 ^c	30.83 ± 0.52 ^a	
1	0	3.5	2.41 ± 0.20 ^c	0.598 ± 0.02 ^b	3.31 ± 0.11 ^{bcd}	3.85 ± 0.67 ^c	31.98 ± 0.52 ^a	
2	0	0	4.25 ± 0.20 ^a	0.689 ± 0.02 ^a	3.77 ± 0.11 ^a	9.60 ± 0.67 ^a	28.97 ± 0.46 ^b	
2	3.5	0	3.08 ± 0.20 ^b	0.631 ± 0.02 ^b	3.53 ± 0.11 ^{ab}	6.30 ± 0.67 ^b	30.88 ± 0.52 ^a	
2	0	3.5	4.08 ± 0.20 ^a	0.639 ± 0.02 ^{ab}	3.46 ± 0.11 ^{ab}	9.85 ± 0.67 ^a	28.81 ± 0.52 ^b	

^{a-d}Values within a column with no common superscript differ significantly ($P < 0.05$).

¹Each value represents the mean ± SE of 4 replicates with 15 birds per replicate.

²Mean numeric value expressing both presence and degree of severity of liver lesions.

³Hematocrit determined using a Jouan microhematocrit centrifuge (Scientific Group, SA Scientific Products Pty. Ltd. Trading, Johannesburg, South Africa).

value of 1.75 for the controls, 4.13 for the broilers that received 1 mg of AFB₁/kg of feed, and 9.60 for the treatment group that received 2 mg of AFB₁/kg of feed (Table 5). Supplementation of oxihumate improved the liver lesion value of the latter significantly, to 6.30. Brewers dried yeast treatment of the contaminated diets did not show any positive effect on liver enlargement or liver lesion values.

The feed contaminated with 1 mg of AFB₁/kg of feed had no effect on the relative weight of the liver and heart, but it did cause a significant increase in stomach weight, indicating, possibly, a higher sensitivity of the stomach to aflatoxin contamination than the other organs (Table 5). Mycotoxins have been known to irritate the proventriculus and gizzard of the gastrointestinal tract, thus causing an increase in the relative weight of these organs (Huff and Doerr, 1981). The additions of oxihumate and BDY (to a lesser extent) inhibited this effect at the contamination level of 1 mg of AFB₁/kg of feed and prevented the enlargement of the heart at 2 mg of AFB₁/kg of feed.

Verma and Raval (1991) showed a concentration-dependent increase in the rate of hemolysis, indicating AFB₁-induced cytotoxicity, which could be due to lipid peroxidation of plasma membranes, permeability alterations, and cell lyses. According to the data presented in Table 5, 1 mg of AFB₁/kg of feed did not affect hematocrit levels of the broilers. Hematocrit was, however, significantly reduced by the treatment that provided 2 mg of AFB₁/kg of feed. Oxihumate supplementation to this diet significantly improved hematocrit values, probably as a result of effective adsorption in the gut to reduce the amount of aflatoxin absorption by the body. Brewers dried yeast did not improve the hematocrit of the contaminated birds.

The observed reduction in serum concentration of total protein and albumin in all groups fed aflatoxin (Table 6) indicates impaired protein synthesis in the liver (Tung et al., 1975) caused by the blockage of RNA synthesis (Clifford and Rees, 1967), resulting from the hepatotoxicity seen in aflatoxicosis (Bailey et al., 1998). Albumin and total protein levels in the serum proved to be sensitive

indicators of aflatoxicosis in broilers, as significant decreases were observed at 1 mg of AFB₁/kg of feed, a level which did not affect BW gain. The decrease in serum albumin levels in broilers that consumed the diet of 2 mg of AFB₁/kg of feed with added oxihumate was less profound when compared with the group that received aflatoxin alone. The inclusion of BDY with 2 mg of AFB₁/kg of feed in the diet resulted in a significant lower total serum protein level compared with birds that consumed the diet of 2 mg of AFB₁/kg of feed without BDY. This may be an effect of BDY on the total serum protein level, although Raju and Devegowda (2000) and Aravind et al. (2003) found that similar products caused an increase in total serum protein. With continued exposure, intrahepatic biliary epithelial hyperplasia occurs in an attempt to regenerate the hepatic parenchyma when the parenchymal cells themselves have lost their capacity. Such hepatobiliary hyperplasia results in a significant increase of alanine aminotransferase, γ -glutamyltransferase, and total bilirubin (Zaky et al., 1998). Serum γ -glutamyltransferase enzyme activity is a sensitive indicator of liver disease, whether the disorder involves liver inflammation, lesions, or obstruction to the biliary tract (Kubena et al., 1990a,b). In the present study, serum γ -glutamyltransferase activity was significantly decreased in birds consuming 2 mg of AFB₁/kg of diet (Table 6). This observation is in contrast to studies in which an increase in γ -glutamyltransferase activity in the serum was reported (Kubena et al., 1990a,b). Neither oxihumate nor BDY supplementation counteracted the observed decrease in γ -glutamyltransferase. Aflatoxin-contaminated diets in this study did not significantly affect serum aspartate aminotransferase activity (Table 6), which is in contrast to the findings of Huff et al. (1992), in which aflatoxin caused a decrease in the activity of aspartate aminotransferase. Abo-Norag et al. (1995) did not find aflatoxin to have any effect on serum activity of either γ -glutamyltransferase or aspartate aminotransferase. Raju and Devegowda (2000) and Aravind et al. (2003) reported that no significant effects were noted for BDY on γ -glutamyltransferase or aspartate aminotransferase activity in the serum of broilers.

Table 6. Serological parameters of broilers fed aflatoxin B₁- (AFB₁) contaminated diets supplemented with oxihumate or brewers dried yeast (BDY)¹

	Treatment			Albumin ² (g/L)	Total serum protein ² (g/L)	γ-Glutamyltransferase ² (IU/L)	Aspartate aminotransferase ² (IU/L)
	AFB ₁ (mg/kg)	Oxihumate (g/kg)	BDY (g/kg)				
0	0	0	15.19 ± 0.44 ^a	31.30 ± 1.01 ^a	9.66 ± 0.84 ^a	97.4 ± 2.99 ^{bd}	
1	0	0	11.60 ± 0.44 ^b	27.13 ± 1.01 ^{bc}	7.78 ± 0.84 ^{ab}	102.5 ± 2.99 ^{bcd}	
1	3.5	0	11.39 ± 0.44 ^b	26.22 ± 1.01 ^{bc}	7.79 ± 0.84 ^{ab}	106.5 ± 3.01 ^{ac}	
1	0	3.5	11.76 ± 0.44 ^b	27.36 ± 1.01 ^b	7.72 ± 0.84 ^{abc}	104.0 ± 2.99 ^{bc}	
2	0	0	8.01 ± 0.44 ^d	24.44 ± 1.01 ^{cd}	5.94 ± 0.84 ^{bc}	94.3 ± 2.99 ^{de}	
2	3.5	0	9.67 ± 0.44 ^c	23.19 ± 0.99 ^d	5.55 ± 0.83 ^{bc}	97.8 ± 2.95 ^{bd}	
2	0	3.5	7.32 ± 0.45 ^d	19.31 ± 1.03 ^e	5.36 ± 0.86 ^c	88.8 ± 3.06 ^e	

^{a-e}Values within a column with no common superscript differ significantly ($P < 0.05$).

¹Each value represents the mean ± SE of 4 replicates with 15 birds per replicate.

²Serum analyzed with a Technicon RA-1000 system (Miles Inc., Diagnostic Division, Tarrytown, NY) according to standard procedure.

The mortality rate did not differ significantly among treatments. The negative control group had the lowest mortality rate, with a mean of 0.25 birds per pen (1.67%). The highest mortality rate observed was for the group fed 2 mg of AFB₁/kg of feed, with a mean of 1.75 birds per pen (13.3%).

Results demonstrated that oxihumate was able to effectively bind AFB₁ in vitro and was also effective in diminishing the growth inhibitory effects of aflatoxin in vivo. There was apparent protection noted for some of the organ, hematological, and serum biochemical changes associated with aflatoxin toxicity. In this study, oxihumate proved to be much more effective in the amelioration of aflatoxicosis in broilers than the commercially available mycotoxin binder containing BDY. The data suggest that oxihumate may alleviate some of the toxic effects of aflatoxin in growing broilers, and when it is used in combination with other mycotoxin management practices, it might prove to be beneficial in the management of aflatoxin-contaminated feedstuffs for poultry.

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