

# Sodium bentonite and monensin under chronic aflatoxicosis in broiler chickens

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**ABSTRACT** Clay feed additives have been increasingly incorporated into animal diets to prevent aflatoxicosis. Due to the nonselective nature of the binding interaction, many important components of the diets could also be made unavailable because of these feed additives. The anticoccidial monensin (MON) could also be sequestered by these clays. The use of sodium bentonite (Na-B) from a mine in the province of Mendoza, Argentina, was investigated as a sequestering agent to prevent the effects of 100 µg/kg of dietary aflatoxin B<sub>1</sub> (AFB<sub>1</sub>). In vitro studies demonstrated that the above Na-B was a good candidate to prevent aflatoxicosis. They also showed that MON competes with AFB<sub>1</sub> for the adsorption sites on the clay surface and effectively displaces the toxin when it is in low concentration. Even though the levels of MON in diets, approximately 55 mg/kg,

are high enough to not be significantly changed as a consequence of the adsorption, they can further affect the ability of the clays to bind low levels of AFB<sub>1</sub>. An in vivo experiment carried out with poultry showed that 100 µg/kg of AFB<sub>1</sub> does not significantly change productive or biochemical parameters. However, liver histopathology not only confirmed the ability of this particular Na-B to prevent aflatoxicosis but also the decrease of this capacity in the presence of 55 mg/kg of MON. This is the first report stressing this fact and further research should be performed to check if this behavior is a characteristic of the assayed Na-B or of this type of clay. On the other hand, the presence of MON should also be taken into account when assaying the potential AFB<sub>1</sub> binding ability of a given bentonite.

**Key words:** aflatoxin B<sub>1</sub>, monensin, competitive adsorption, detoxification, broiler chicken

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## INTRODUCTION

Aflatoxins (AF), a group of potent mycotoxins produced by *Aspergillus* section *Flavi*, mainly *Aspergillus flavus* and *Aspergillus parasiticus* strains, are of major concern in poultry production. The natural incidence of potential aflatoxigenic strains and AF in Argentinian and Brazilian poultry feed was previously reported (Dalcerio et al., 1997, 1998; Magnoli et al., 2002; Oliveira et al., 2006; Fraga et al., 2007). The most important

member of the family is AFB<sub>1</sub>, whose toxicity has been widely investigated by the determination of its carcinogenic, mutagenic, and teratogenic and immunosuppressive effects both in humans and animals (Council for Agricultural Science and Technology, 2003). Various symptoms have been associated with aflatoxicosis in poultry. The most common ones are lethargy, anorexia, lower growth rate, and poor feed utilization. Acute aflatoxicosis, performed with dietary AFB<sub>1</sub> doses ranging from 2.5 to 5 mg/kg, adversely affects relevant productive and biochemical-hematological parameters (Keçeci et al., 1998; Oğuz et al., 2000a; Oğuz and Kurtoglu, 2000; Rosa et al., 2001; Mazzio et al., 2005). Aflatoxin B<sub>1</sub> intake causes significant gross and microscopic changes in liver, such as hepatomegaly, paleness, hydropic degeneration, fatty change, bile duct hyperplasia, and periportal fibrosis (Fernandez et al., 1994;

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Kiran et al., 1998; Ledoux et al., 1999; Ortatatl and Oğuz, 2001; Ortatatl et al., 2005).

Different approaches, including physical, chemical, and biological treatments of contaminated feeds and feedstuffs, have been employed to detoxify AF (Jouany, 2007). Worldwide adsorbent-based methodologies have been used to reduce the effects of AF in contaminated feed and to minimize the toxicity of AF in poultry (Ibrahim et al., 2000; Parlat et al., 2001; Daković et al., 2005). The Argentine poultry feed industry has been using different national bentonites, either as binders or as AF-sequestering agents. These layered aluminosilicates have high swelling capacity and, therefore, are poorly adsorbate-selective (Pasha et al., 2005).

Monensin (**MON**) is the sodium salt of a carboxylic acid ionophore. Its main therapeutic application is as a poultry feed additive to prevent coccidiosis (Schwarz et al., 2001). Because much of the economic losses associated with coccidiosis take place before diagnosis, prevention becomes more important than treatment (Elliott et al., 1998). There are limited data regarding clay feed additives and their effect on the efficacy of anticoccidials. It has been suggested that sodium bentonite (**Na-B**) may interfere with the efficacy of some coccidiostats when these compounds are added at half the minimum level approved for dietary inclusion (Gray et al., 1998). Thus, at the coccidiostat approved dietary inclusion level, the drug bioavailability should be high enough to prevent any practical problem. However, what is not known is how the binding of these coccidiostats to Na-B may affect the ability of the clays to bind AF.

In previous works, different bentonites showed high AFB<sub>1</sub> binding capacities both in vitro (Magnoli et al., 2008a) and in vivo (Rosa et al., 2001; Miazzo et al., 2005; Magnoli et al., 2008b). The adsorption ability of these clays varies from one geological deposit to another. All of those experiments were conducted with AF levels higher than 1 mg/kg. There is little information with respect to the protective effect of Na-B against experimentally induced chronic aflatoxicosis in the presence of MON in broiler chickens. Therefore, the objective of this study was to determine if MON affects the adsorption efficacy of a natural Na-B used to prevent aflatoxicosis in broiler chickens.

## MATERIALS AND METHODS

Aflatoxins were produced via fermentation of rice by *A. parasiticus* NRRL 3000 (USDA, Agricultural Research Service, Peoria, IL) following previously described methodology (Miazzo et al., 2000). The AFB<sub>1</sub> levels in the ground culture material were measured by HPLC according to Trucksess et al. (1994) and AOAC (1995), respectively. The ratio of AFB<sub>1</sub> to AFG<sub>1</sub> in the culture was 2:1. The ground culture material was added to the basal diet to provide 100 µg of AFB<sub>1</sub>/kg of feed. Commercial Na-B was obtained from a mine situated in the province of Mendoza, Argentina. The chemical,

structural, and morphological characterization of the clay was previously reported (Magnoli et al., 2008a). A MON sodium salt (Sigma-Aldrich, St. Louis, MO) was used for in vitro study. Monensin standards were prepared in methanol and stored at 4°C until use.

### ***In Vitro Study***

For in vitro studies, the AFB<sub>1</sub> extract was purified according to AOAC (1995). The adsorption isotherms were carried down following the previously described methodology (Rosa et al., 2001). The AFB<sub>1</sub> concentration in each supernatant was determined by HPLC (Trucksess et al., 1994). Aflatoxin B<sub>1</sub> adsorption was estimated by standard solution-depletion method.

### ***In Vivo Study***

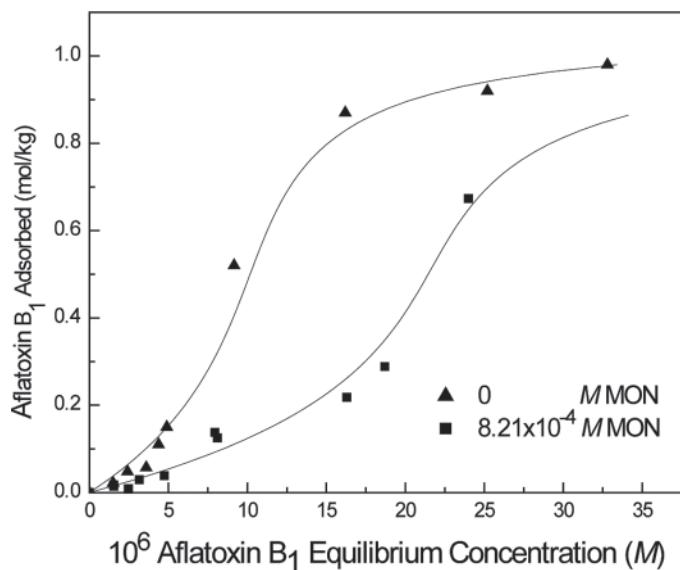
In the in vivo experiment, 1-d-old male chicks (Cobb) vaccinated against Marek's disease were obtained from a commercial hatchery. The birds were kept under continuous incandescent electric lamps with basal diet and water available for ad libitum consumption until they were 5 d old. A standard starter corn-soybean meal diet, which met NRC requirements, formulated without the coccidiostat MON, was fed from d 1 until the end of the experiment (NRC, 1994).

On d 6, a total of 240 birds were selected by weight and randomly distributed to 30 pens in five 3-tier batteries with raised floors. Each pen represents 1 replicate and each tier represents 1 block. Eight chickens were housed per pen (90 × 100 × 39 cm). All pens had a 1-cm<sup>2</sup> wire mesh bottom with a removal tray placed underneath to allow cleaning. The pens were also equipped with a feeding trough placed outside and a water cup placed inside them. Six replicas per treatment were placed in 2 vertical arrangements of the 3-tier batteries to minimize cross-contamination. These arrangements were randomly distributed.

From d 6 until 33, chickens received the corresponding experimental diets. The level of AFB<sub>1</sub> in the basal diet (4.2 µg/kg) was determined as described previously (Magnoli et al., 2002). The experimental diets for each treatment were as follows: treatment 1 = basal diet; treatment 2 = basal diet plus AFB<sub>1</sub> (100 µg/kg); treatment 3 = basal diet plus AFB<sub>1</sub> plus Na-B (0.3%); treatment 4 = basal diet plus AFB<sub>1</sub> plus Na-B plus MON (55 mg/kg); and treatment 5 = basal diet plus MON.

Chickens were monitored daily for signs of morbidity and mortality. The inhibitory effect of MON on the ability of Na-B to prevent aflatoxicosis was evaluated by measuring BW gain (BW), feed consumption, and feed:gain ratio during 3 periods evenly distributed over the entire experiment.

At the end of the trial, 3 chickens from each treatment were randomly selected, weighed, and killed by cervical dislocation. Blood samples, taken by cardiac puncture, with anticoagulant addition were collected before eutha-



**Figure 1.** Effect of monensin (MON) on the adsorption isotherm of aflatoxin B<sub>1</sub> on sodium bentonite at pH 2 and 39.5 ± 0.5°C.

nasia, centrifuged, and the plasma was stored at -20°C until use. Total protein, albumin (**ALB**), and globulin (**GLOB**) concentrations and ALB:GLOB ratio for each group were determined with a clinical chemistry analyzer (commercial kit, Wiener Laboratory, Rosario, Argentina) according to the manufacturer's recommended procedure (Rojkin and Alguin, 1974).

After necropsy, 5 livers per treatment (1 liver for each replicate) were excised, weighed, and fixed in 10% neutral-buffered formalin. Fixed tissues were trimmed, embedded in paraffin, and stained with hematoxylin and eosin for histopathological examination. As suggested by Oğuz et al. (2003), the hepatocellular degeneration in livers was graded in a blinded test according to the following score: slight (degree 1) = mild hepatocellular swelling due to hydropic degeneration and fatty changes only in centrilobular areas; moderate (degree 2) = clear hepatocellular swelling in both centrilobular and midzonal areas; and severe (degree 3) = diffuse and severe hepatocellular swelling, cytoplasmic palleness, and rupture.

The experiment was conducted as a randomized complete block design with the single pen representing the experimental unit (replicate). Data were subjected to ANOVA for randomized complete block design statistical analysis to determine whether significant differences exist in the group means of the observed variables (SAS Institute, 1997). All statements of significance were based on the 0.05 level of probability (Quinn and Keough, 2002).

## RESULTS

In this study, the interference of MON in the adsorption efficacy of a natural bentonite to prevent the effects of low levels of AFB<sub>1</sub> in broiler chicken diets was undertaken. To in vitro test the AFB<sub>1</sub> adsorption

ability of Na-B, isotherms were determined at pH 2 and 39.5°C (Figure 1). The AFB<sub>1</sub> coverage for a saturated surface was 1.07 mol of AFB<sub>1</sub>/kg of Na-B.

As can be observed from the isotherm data,  $8.21 \times 10^{-4}$  M of MON remarkably reduces the adsorption of AFB<sub>1</sub> by this particular Na-B. In fact, for a toxin equilibrium concentration of approximately  $20 \times 10^{-6}$  M, which is 20 times lower than that of MON, a reduction of approximately 25% in the adsorption was observed. This competition between adsorbates for the adsorbent is likely to be more important when the relationship between MON and AFB<sub>1</sub> increases. This fact is particularly important because, in poultry diets, this relationship could be much greater than the one used in the isotherm.

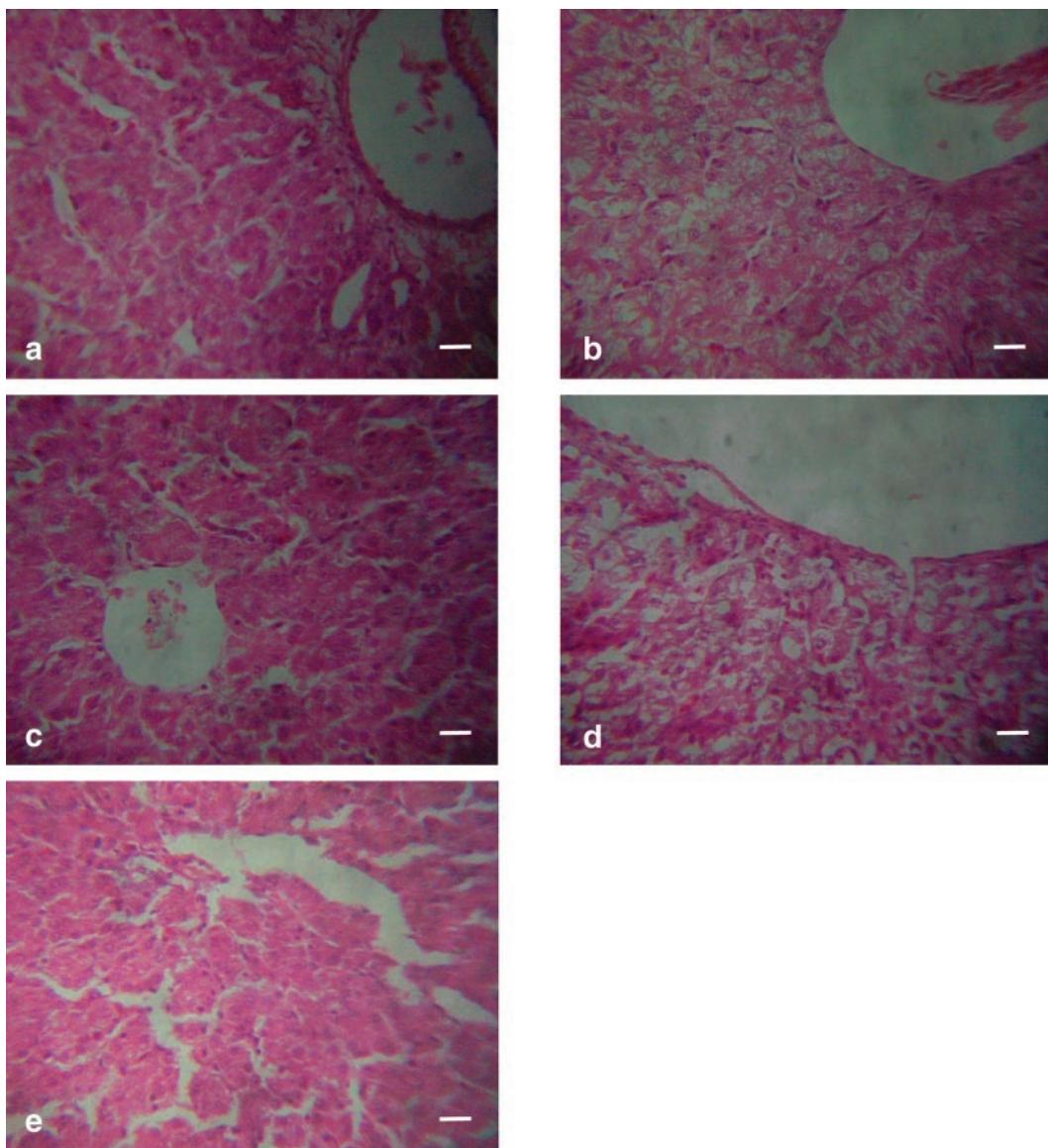
*In vivo* assay did not show significant differences on productive parameters (BW gain, feed intake, and feed:gain ratio) compared with controls either among blocks or treatments ( $P < 0.05$ ; data not shown). Mortality did not occur, nor were significant changes observed in biochemical parameters (total protein, ALB, and GLOB concentrations and ALB:GLOB ratio in blood plasma) nor in the weight of livers.

The gross and microscopic changes in the livers during chronic aflatoxicosis were also evaluated. The macroscopic appearances of livers from chickens fed different diets (not shown) were similar, except for treatment 3 (AFB<sub>1</sub> plus Na-B), which was slightly darker in color.

Figure 2 shows the photomicrographs of hematoxylin-and-eosin-stained liver sections of chickens fed dietary treatments. The livers of chickens fed the basal diet showed normal hepatocytes (Figure 2a). Livers of chickens fed the diet containing 100 µg/kg of AFB<sub>1</sub> showed slight lesions, such as multifocal and varied cytoplasmatic vacuolation with perilobular location (Figure 2b), whereas the livers of broilers consuming the AFB<sub>1</sub> plus Na-B diet showed only a moderate hydropic degeneration (Figure 2c). Birds fed with AFB<sub>1</sub> plus Na-B and MON showed moderate lesions showing hepatocytes with small fatty vacuoles and diffuse and severe necrosis were observed (Figure 2d). Normal hepatocytes were observed in the basal plus MON treatment (Figure 2e). None of the treatments showed severe lesions.

## DISCUSSION

Results of the *in vitro* study showed that the Na-B was a good candidate to adsorb low levels of AFB<sub>1</sub> *in vitro* (Magnoli et al., 2008a). However, the adsorption isotherm of AFB<sub>1</sub> was significantly changed by the presence of MON in the system. As can be observed in Figure 1, the competition of MON for adsorption sites on the surface diminishes both the adsorption ability and the maximum capacity of the Na-B for the AF. The decrease is important at low toxin concentrations. It should be noted that the *in vitro* assay was conducted using a MON concentration around the recommended levels of the coccidiostat in prophylactic management



**Figure 2.** Photomicrographs of hematoxylin-and-eosin-stained chicken liver sections: (a) basal diet, (b) basal diet plus aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), (c) basal diet plus AFB<sub>1</sub> plus sodium bentonite (Na-B), (d) basal diet plus AFB<sub>1</sub> plus Na-B plus monensin (MON), (e) basal diet plus MON, normal hepatocytes. Bar equals 10  $\mu\text{m}$ . Color version available in the online PDF.

of broiler chickens (Dusi and Gamba, 1999). Gray et al. (1998) reported the interactive effect of Na-B and MON in coccidiosis-infected chickens. In fact, they found that dietary Na-B may reduce the efficacy of MON when it is added below recommended levels. The present results agree with our in vitro findings suggesting a decrease in the bioavailability of MON due to Na-B adsorption.

Most previously reported in vivo studies have been performed under acute aflatoxicosis (i.e., 1 to 5 mg/kg of dietary AFB<sub>1</sub>; Keçeci et al., 1998; Oğuz et al., 2000a; Oğuz and Kurtoglu, 2000; Rosa et al., 2001; Karaman et al., 2005; Mazzia et al., 2005). Less information is available dealing with lower AFB<sub>1</sub> dose exposure (i.e., 30 to 185  $\mu\text{g}/\text{kg}$ ; Oğuz et al., 2000b, 2003; Ortatatlı et al., 2005; Magnoli et al., 2008b). It would be very important to determine the chronic AFB<sub>1</sub> levels that affect broilers in terms of performance, hematology, en-

zyme biochemistry, immunology, and histology. It has been suggested that clinical, hematological-biochemical, and histopathological changes in broilers began at AFB<sub>1</sub> concentrations ranging from 90 to 100  $\mu\text{g}/\text{kg}$  (Giambrone et al., 1985; Marquez and Hernandez, 1995; Oğuz et al., 2000b; Shi et al., 2009). Our results differ from others reported in the literature, in which productive parameters were affected by 100  $\mu\text{g}/\text{kg}$  of AFB<sub>1</sub> dietary intake (Marquez and Hernandez, 1995; Oğuz et al., 2000b; Shi et al., 2009). The biochemical parameters, serum total protein, ALB, and GLOB were also not significantly different among treatments. Johri et al. (1990) and Abdelhamid et al. (1994) also reported no significant changes in serum biochemistry for induced aflatoxicosis ranging from 50 to 200  $\mu\text{g}/\text{kg}$ .

From macroscopic examination of livers, a beneficial effect of Na-B might be occurring because a slightly

darker brownish color can be appreciated in livers of birds fed the basal diet plus Na-B plus AFB<sub>1</sub>. However, because no macroscopic differences in liver appearance of birds fed the basal diet and basal diet plus AFB<sub>1</sub> were observed, the color may have had nothing to do with AFB<sub>1</sub> exposure. The liver histopathology of chicks receiving 100 µg/kg of AFB<sub>1</sub> alone showed the typical pattern of subclinical aflatoxicosis. The present results agree with those reports by Giambrone et al. (1985), who reported only microscopic lesions in birds given 100 µg/kg of dietary AFB<sub>1</sub>. On the other hand, a rather mild aflatoxicosis was observed in liver of chicks fed the basal diet plus Na-B. These results suggest the ability of Na-B to counteract low levels of AFB<sub>1</sub> at least when no MON is added to the diet. Moreover, the major histopathologic changes were observed in livers of chicks fed diets with Na-B (0.3%) plus AFB<sub>1</sub> (100 µg/kg) plus MON (55 mg/kg). The present results indicate that Na-B in the presence of MON does not ameliorate the effects of the 100 µg/kg of AFB<sub>1</sub>. Therefore, the histopathologic findings agree with those resulting from in vitro studies, that is, MON competes and displaces AFB<sub>1</sub> from adsorption sites at the adsorbent surface at least when AFB<sub>1</sub> levels are low. These results are the first evidence of competition at least for this particular Na-B from a mine in the province of Mendoza in Argentina. This type of inhibition in the binding ability of Na-B due to MON could be also present in other animals, like pigs and cows. These species, which are also sensitive to AFB<sub>1</sub> usually, receive MON as a growth promotor or as control for rumen acidosis. Moreover, the longer life span of these species compared with poultry could make the aflatoxicosis signs worse. Because the adsorbent supplementation levels used worldwide range from 0.05 to as high as 1%, the present results could be of a great concern for the doses lower than 0.3% (Dixon et al., 2008). Results of this study would be less of a concern at higher adsorbent concentrations. It should be stressed that there is no reference in the literature about the existence of such competition, although care should be taken because it could be specific to the particular assayed Na-B. Therefore, the presence of MON should be considered when testing related adsorbents for prevention of aflatoxicosis. These results might reopen discussions about the efficacy of nonselective adsorbents, like this specific Na-B, to prevent subclinical aflatoxicosis and the need to find selective AFB<sub>1</sub> binders. However, taking into account that the clinical signs for aflatoxicosis depend on bird age and both nutritional or health status at the time of exposure to contaminated feed, we suggest that further *in vivo* experiments should be conducted to confirm the present results.

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