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Evaluation of Pathological Effects in Broilers During Fumonisins and Clays Exposure

Jesús Enrique Vizcarra-Olvera · Humberto Astiazarán-García · Armando Burgos-Hernández · Norma Violeta Parra-Vergara · Francisco Javier Cinco-Moroyoqui · Reyna Isabel Sánchez-Mariñez · Eber Addi Quintana-Obregón · Mario Onofre Cortez-Rocha

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Abstract This study was conducted to evaluate the possible protector effect of bentonite and zeolite in Bovans chicks fed a diet containing 59 mg kg^{-1} of fumonisin B1 (FB1) during 3 weeks. A total of 200 one-day-old male chicks were treated varying the amount of bentonite and zeolite. Chick weight was registered weekly. At the end of the experiment, all the chicks were killed, and the livers were analyzed for gross examination and histopathological changes. Plasmatic activity of alanine amino transferase and aspartate amino transferase (AST) were also determined. Sphinganine and the sphinganine-to-sphingosine ratio in serum were evaluated. Both, bentonite and zeolite showed a protector effect against FB1 adsorption in the digestive tract of chicks. Chicks fed with FB1-contaminated feed, amended either with zeolite or bentonite, were heavier, and no macroscopic lesions were observed in the livers. AST activity might be considered as an indicator for FB1 exposition because AST levels were affected when only FB1 was present in the basal diet. These results indicate that both, zeolite and bentonite can be added into feed to diminish the effects of FB1.

Keywords Fumonisin B1 · *Fusarium verticillioides* · Zeolite · Bentonite

J. E. Vizcarra-Olvera · A. Burgos-Hernández · N. V. Parra-Vergara · F. J. Cinco-Moroyoqui · E. A. Quintana-Obregón · M. O. Cortez-Rocha (✉)
Department of Food Research and Graduate Program, University of Sonora, Blvd, Luis Encinas y Rosales s/n. Col. Centro, CP 83000 Hermosillo, Sonora, Mexico
e-mail: mcortez@guayacan.uson.mx

H. Astiazarán-García
CIAD (Research Center in Food and Development), Carretera a la Victoria, Km. 0.6, Hermosillo, Sonora, Mexico

R. I. Sánchez-Mariñez
Department of Chemical-Biological Sciences, University of Sonora, P.O. Box 1658, CP 83000 Hermosillo, Sonora, Mexico

Introduction

Fumonisins are mycotoxins that were first isolated in South Africa [1]. They are produced primarily by strains of *Fusarium verticillioides* (formerly *Fusarium moniliforme*), *F. proliferatum*, and other *Fusarium* species [2, 3]. These *Fusarium* species have been reported as natural contaminants of cereal worldwide and are mainly found in corn and its by-products [4–7]; sorghum and oat [8, 9]; rice [10]; and wheat [11, 12]. Fumonisin concentration varied greatly depending of the commodities, geographic location, and climatic condition.

Twenty-eight fumonisin analogues have been characterized and separated into four main groups [3]. However, the fumonisin B group involves those analogues that are toxicologically important. The fumonisin

B1 (FB1) accounts for 70–80% of the total fumonisin produced, FB2 may comprise 15–25%, and FB3 that makes up from 3 to 8% have been reported to occur naturally in significant levels in corn and corn-based products [13–16].

Fumonisins have been epidemiologically and experimentally associated with equine leucoencephalomalacia (ELEM) and porcine pulmonary edema syndrome [17, 18], as well as hepatocarcinomas and cholangiocarcinomas in rats [19]. Fumonisin have been associated with increased risk of human esophageal cancer in the Transkei region in the Republic of South Africa [20, 21] and China [22, 23], where FB1-contaminated corn was consumed as a dietary staple. The mechanism of FB1 toxicity is not completely understood. FB1 resembles the sphingoid base backbone of sphingolipids, a class of membrane lipids that play an important role in cell signal transduction pathways, cell growth, differentiation, and cell death [24, 25]. FB1 is a potent, competitive inhibitor of ceramide synthase, the enzyme that catalyzes the acylation of sphinganine in the de novo biosynthesis of sphingolipid and sphingolipid turnover and also causes sphinganine accumulation in serum of exposed animals. Sphinganine concentration (Sa) and sphinganine-to-sphingosine ratio (Sa/So) are sensitive biomarkers of fumonisin B1 exposure in animals and have been proposed to reveal FB1 exposure in humans. These parameters correlate well with liver and kidney toxicity and often precede signs of toxicity [26, 27]. Several attempts have been done to develop an effective preventive decontamination technology to minimize the toxic effects of fumonisins. Since the beginning of 1990, the adsorbent property of clay has been used to remove aflatoxins from contaminated poultry feed [28]. Aluminosilicates such as natural zeolites [29], natural bentonites [30], montmorillonite, and clinoptilolite are preferred because of their reducing effect on aflatoxin B1 absorption from the intestinal tract. The efficacy of binding mycotoxins depends on physical properties and on the crystal structure of the adsorbent, along with the chemical and physical characteristics of the mycotoxin. Aluminosilicates are very effective in preventing aflatoxicosis, but their efficacy against other mycotoxins is limited. Therefore, the aim of the present study was to evaluate the gross and microscopic changes caused by FB1 exposure and determine the preventive role of natural zeolite and natural bentonite in FB1-exposed broiler chicks.

Materials and Methods

Two hundred kg of commercial broiler chick feed obtained locally in Hermosillo, Sonora, Mexico, was used in the study. Bentonite and zeolites were purchased from JAV Minerales y Nutrientes, S.A. de C.V. in Hermosillo, Sonora. Feed was amended with an autoclaved cultured material of *Fusarium verticillioides* to obtain a final concentration of 59 mg kg^{-1} of FB1. Feed was divided into 10 lots of 20 kg each. Nine dietary treatments were prepared: (1) Two controls, basal diet with no FB1 added; (2) Basal diet/bentonite 0.5%; (3) Basal diet/bentonite 1.0%; (4) Basal diet/zeolite (0.5%); (5) Basal diet/zeolite (1.0%); (6) Basal diet contaminated with FB1 + zeolite (0.5%); (7) Basal diet contaminated with FB1 + zeolite (1.0%); (8) Basal diet contaminated with FB1 + bentonite (0.5%); and (9) Basal diet contaminated with FB1 diet + Zeolite (1.0%). Every lot was homogenized by mixing in a 200 kg capacity horizontal mixer for 15 min.

Two hundred male Bovans chicks (*Gallus gallus*) 2 days old ranging from 38 to 42 g were obtained from a commercial hatchery. Chicks were wing banded for identification and randomly distributed into 20 groups of 10 animals each. Then, they were housed in compartments with continuous lighting of 23 h, at 32°C and 45% relative moisture content for the first week. For the second week, the relative moisture content was adjusted to 45%, at 30°C and with 22 h continuous lighting. For the third week, moisture content was adjusted to 40%, at 28°C and 20 h of continuous lighting. Birds were started with the amended rations since the first day and continued on their respective ration until day 21. Chicks were allowed to access feed and water ad libitum. They were observed twice daily and weighted once a week (after 7, 14, and 21 days). Two separated feeding trials with 2 replicates were done.

AST and ALT Determination

When the feeding trial was over, all the chicks were humanly euthanized. Four chicks from each group were randomly taken for blood collection from the jugular vein using Vacutainer® tubes. Serum was

separated by centrifugation at 400×*g* for 10 min. The activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) was evaluated using a spectrometric method.

Sphinganine-to-Sphingosine Ratio Determination

Free sphinganine and sphingosine were measured in serum by HPLC [31]. Briefly, 0.5 mL of serum was added to 1.5 mL of KCl (0.8%), 50 μL KOH (1 M), and 4 mL of ethyl acetate and mechanically agitated by 20 min. Then, they were centrifuged at 354.8×*g* for 15 min, and the organic phase removed and evaporated under nitrogen. Evaporated samples were redissolved in 1.0 mL of mobile phase [K_2HPO_4-Methanol, (1:9)], and 50 μL of OPA was added. Samples were let to stand for 30 min and then injected into the HPLC. A Kromasil C18 column (5 μm i.d., 25 cm × 4.6 mm)with a fluorescence detector was used (excitation wavelength, 340; emission wavelength 455 nm). Two mobile phases were used, which were as follows: Solvent A (K_2HPO_4-Methanol, (1:9), and solvent B, methanol HPLC grade according to Castagnaro et al. [31].

Histopathological Analysis or Histopathological Study

Livers were removed and kept in 10% neutral-buffered formalin, embedded in paraffin, and processed for microscopical analyses. Thin section (2 mm) was stained with routine hematoxylin-eosin stain (H&E), mounted and inspected by optical microscopy. In order to detect any presence of fibrous tissue, selected tissue sections were stained according to the method of trichromic-Periodic acid Schiff.

From each slide, 1.0 cm^2 was marked and analyzed for cellular changes. For inflammation, cellular infiltrates were counted and registered either as lobular or portals according to the site. Three categories were established for portal infiltrates: (a) when more than 20 mononuclear cells were observed; (b) when less than 20 mononuclear cells were observed; and (c) presence of nodules. By other hand, in lobular infiltrates, there were more than 20 mononuclear cells and nodular infiltrates, so the number of portal spaces was considered and thus the percentage of cellular infiltrates determined.

Fibrosis and vacuolar degradation were null when they were not observed: Slight when 1–25% was observed; moderate from 26 to 50%, and severe when more than 51% was observed [32].

Statistical Analysis

Data were analyzed for all variables using JMP 4.0 (SAS Institute Inc. Cary, NC. 2002). Data were subjected to ANOVA using the general linear model procedure to establish differences between means. Significance was based on probability $P \leq 0.01$.

Results and Discussion

Feed was tested for aflatoxin B1 (AFB1) and fumonisins before the experiment. AFB1 was detected in 2.5 mg kg^{-1} level, which is low. The maximum tolerated levels for AFB1 in food is 20 mg kg^{-1} [33]. Proximal analysis of feed indicated 21.5% of protein (N × 6.25), 3.5% of fiber, 5.1% ether extract, and 3,100 kcal, which agrees to the recommendations of the National Research Council [29, 34].

Body Weight

Neither mortality nor signs of micotoxicosis were observed throughout the 21-day period of the trial. Since the first week of experimentation, chicks fed with diets containing bentonite and zeolite presented differences in the body weight. In the third week, chicks fed with FB1-contaminated feed showed the lowest body weight and were statistically different to the other treatments ($P \leq 0.01$) (Table 1). Our results indicated that zeolite and bentonite in diets increased the body weight of the chicks compared with control diet. This result agrees to previous finding [35], who studied the effect of fumonisin B1, B2, and moniliformin, which markedly reduced body weight gain.

Sphinganine-to-Sphingosine Ratio Determination

This biomarker is particularly important because it is indicative for the exposure to fumonisins and the toxic effects from biochemical disruption of sphingolipid metabolism. Results for SA/SO in our study are presented in Table 2. Control diet, basal diet + zeolite (0.5%), and basal diet + FB1 + bentonite (1.0%)

Table 1 Chicks weight (grams) receiving a diet amended with fumonisin B1 without or with zeolite or bentonite during 21 days

Diet	Day 1	Day 7	Day 14	Day 21	% of weight gain	Difference to the control
Control (basal diet)	39.9 ± 1.2a	74.1 ± 3.3a,b	139.1 ± 8.7b,c,d	230.1 ± 15.7a,b	100.00	0
Basal diet + FB1	40.1 ± 1.2a	66.7 ± 4.8c	127.6 ± 9.8d	216.8 ± 11.3b	94.23	-5.77
Basal diet + zeolite (0.5%)	40.1 ± 1.4a	77.8 ± 8.6a,b	152.7 ± 12.0a	246.2 ± 28.7a	107.00	6.99
Basal diet + zeolite (1.0%)	40.0 ± 1.4 a	75.9 ± 4.0a,b	149.6 ± 10.8a,b,c	245.1 ± 20.9a	106.21	6.21
Basal diet + FB1 + zeolite (0.5%)	40.2 ± 1.2a	74.4 ± 6.3a,b	139.9 ± 11.1a,b,c,d	241.5 ± 21.5a	104.95	4.95
Basal diet + FB1 + zeolite (1.0%)	40.0 ± 1.4a	75.9 ± 4.0a,b	149.6 ± 10.8a,b,c	245.1 ± 20.9a	106.52	6.52
Basal diet + bentonite (0.5%)	40.0 ± 1.1a	72.7 ± 5.2a,b	139.5 ± 10.5b,c,d	238.4 ± 14.9a	103.60	3.60
Basal diet + bentonite (1.0%)	39.9 ± 1.2a	75.6 ± 6.0a,b	143.2 ± 10.4a,b,c	240.0 ± 15.1a	104.32	4.32
Basal diet + FB1 + bentonite (0.5%)	39.8 ± 1.0a	79.2 ± 5.3 a	137.3 ± 10.7c,d	228.8 ± 19.5a,b	99.43	-0.47
Basal diet + FB1 + bentonite (1.0%)	40.0 ± 1.1a	79.2 ± 3.3a	151.4 ± 8.3a,d	241.6 ± 14.7a	105.02	5.02

Means followed by the same letter within a row and a column are nor significantly different at $P < 0.01$

Table 2 Sphinganine, sphingosine, and sphinganine-to-sphingosine ratio in chicks receiving a diet amended with fumonisin B1, without or with zeolite or bentonite during 21 days

Diet	SA(ng)[a]	SO(ng)[a]	SA/SO[a]
Control (basal diet)	2.9453	43.9323	0.07
Basal diet + FB1	6.0433	37.7898	0.16
Basal diet + zeolite (0.5%)	0.4166	6.4543	0.07
Basal diet + zeolite (1.0%)	3.4089	18.0278	0.19
Basal diet + FB1 + zeolite (0.5%)	0.8686	1.6356	0.54
Basal diet + FB1 + zeolite (1.0%)	6.1461	51.2412	0.12
Basal diet + bentonite (0.5%)	11.542	69.1199	0.17
Basal diet + bentonite (1.0%)	5.4059	23.3887	0.24
Basal diet + FB1 + bentonite (0.5%)	6.5079	35.6666	0.19
Basal diet + FB1 + bentonite (1.0%)	1.1454	11.8111	0.10

[a] Values are means of triplicates

showed similar SA/SO ratio ($P > 0.01$). All the other treatments presented higher SA/SO ratios that indicate sphingolipids disruption. This result indicates only a protective effect by the bentonite (1.0%). Wang et al. [26] reported that the first biological effect of FB1 is the inhibition of sphingolipids, ceramides, and complex sphingolipids. Riley et al. [36, 37] indicate that this inhibition causes an increase in the SA/SO ratio in exposed animals. Henry et al. [38] added pure FB1 into the diets of broiler chicks and found that liver sphinganine concentration, the sphinganine/sphingosine ratio, and free sphinganine in the serum were significantly increased in chicks fed on FB1, which agrees with our results. This also agrees with studies in humans [31] and ducks [39]. Solfrizzo et al. [40] and Riley et al. [37] reported that the increase in the SA/SO ratio in rats and pigs have a consistent relationship with FB1 in the diets.

AST and ALT Determination

The results of enzymes ALT and AST activity in the sera of experimental chicks are presented in Table 3. Control chicks levels were 249 U/L for AST and 15.5 U/L for ALT, which is in agreement with those reported in sound chicks [41]. FB1 affected AST concentration, an increase in the level was detected, and it was statistically significative ($P < 0.01$). This agrees with the chicks' weight reduction and the effects on liver because AST is considering a marker for liver damage. Chicks fed with FB1-contaminated feed presented the highest AST concentration and

Table 3 Enzymatic activity in ALT and AST (U/L) in chicks receiving a diet amended with fumonisin B1, without or with zeolite or bentonite during 21 days

[a] Values are means of triplicates ± standard deviation. Means followed by the same letter within a row and a column are not significantly different at $P < 0.01$

Diet	ALT[a]	AST[a]
Control (basal diet)	249.00 ± 10.29a	15.50 ± 11.35a
Basal diet + FB1	290.75 ± 29.27a	34.75 ± 6.07b
Basal diet + zeolite (0.5%)	279.75 ± 24.39a	18.00 ± 2.16a
Basal diet + zeolite (1.0%)	284.00 ± 43.74a	10.75 ± 0.95a
Basal diet + FB1 + zeolite (0.5%)	226.00 ± 6.68a	12.50 ± 2.06a
Basal diet + FB1 + zeolite (1.0%)	240.00 ± 47.44a	12.00 ± 6.45a
Basal diet + bentonite (0.5%)	246.5 ± 23.87a	8.50 ± 7.72a
Basal diet + bentonite (1.0%)	279.00 ± 16.54a	12.50 ± 4.04a
Basal diet + FB1 + bentonite (0.5%)	258.00 ± 25.59a	6.75 ± 1.25a
Basal diet + FB1 + bentonite (1.0%)	271.00 ± 13.63a	11.75 ± 3.10a

lowest weight gain. This is an indication that cellular necrosis could have occurred. ALT levels were not a valuable factor in our study, it did not give us anything to compare or to analyze. There are some studies which reported that AST values in chicks fed with AFB1 increases compared with those from the control chicks [42, 43]. Also, Lana [44] reports that the chicks at this age had variable AST levels, and this could be possible in our study. Similar results were found when evaluated the effects of aflatoxin-contaminated feeds in broiler chickens [45].

Histopathological Analysis or Histopathological Study

No macroscopic lesion was observed in the chicks at the end of the trial whatever the diet used. Microscopic liver observations from chicks fed with control diet presented light vacuolar degradation. In one of the chicks, light fibrosis was detected (Table 4). Also light inflammation was observed caused by mononuclear cells that seemed as an infiltrate. This observation indicates that even when chicks are fed with low levels of mycotoxins, the amount was large enough to produce liver damage. The observation in the liver of chicks fed with control diet amended with zeolite or bentonite (0.5, 1.0%) did not show any kind of damage. However, the liver from chicks fed with FB1-contaminated diet exhibited more damage than the livers from chicks fed with diet containing zeolite or bentonite. Livers from chicks fed with FB1-contaminated diet presented vacuolar degradation from moderate to severe (Fig. 1, Table 5). In addition, high amount of infiltrates and nodules were observed mainly by mononuclear cells in the portal areas and outside of them. This observation agrees with findings of Javed et al. [35], who reported that chicks fed FB1-contaminated feed had dose-responsive hepatic lesions. They reported that hepatocytes were pleomorphic and extremely vacuolated with marked hydropic degeneration and hepatocellular swelling in the higher dosage groups (274 mg kg^{-1} of FB1). These types of damages have also been reported in a study with aflatoxin-contaminated diet [32]. Howard et al. [46] found fibrosis, vacuolation, and infiltrates in livers of mice fed with FB1-contaminated diet. The livers of chicks fed with diet amended with zeolite or bentonite without FB1 did not show fibrosis, and vacuolar degradation was scarce; therefore, suggesting that they were in

Table 4 Fibrosis observed in livers from treated chicks receiving a diet amended with fumonisin B1, without or with zeolite or bentonite during 21 days

Diet	Absent[a]	Light[a]	Moderate[a]
Control (basal diet)	3	1	0
Basal diet + FB1	1	0	3
Basal diet + zeolite (0.5%)	4	0	0
Basal diet + zeolite (1.0%)	4	0	0
Basal diet + FB1 + zeolite (0.5%)	3	1	0
Basal diet + FB1 + zeolite (1.0%)	4	0	0
Basal diet + bentonite (0.5%)	4	0	0
Basal diet + bentonite (1.0%)	4	0	0
Basal diet + FB1 + bentonite (0.5%)	2	1	1
Basal diet + FB1 + bentonite (1.0%)	4	0	0

[a] Values represent the number of chicks showing effects, 4 were examined in each group

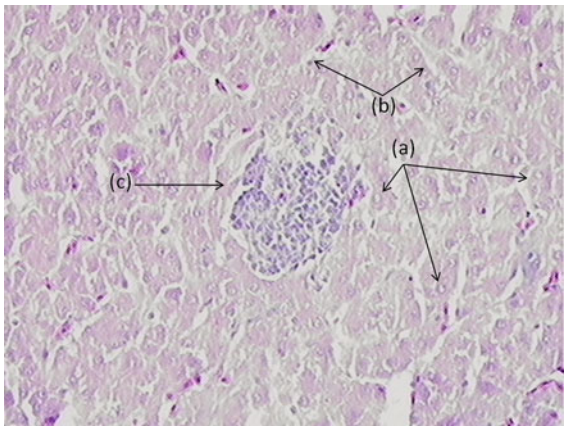

Fig. 1 Damaged liver from 59.9 μg/g FB1-basal diet-treated group after 21 days of feeding. **a** Vacuoles, **b** Fibrosis, **c** cellular infiltrates

apparently better conditions than control-fed chicks. This observation could be the result of a protective effect of the bentonite and zeolite to AFB1 and FB1 avoiding their adsorption and thus the hepatic damage. However, liver from chicks fed with bentonite-amended contaminated FB1 diet had lower damages. There was

Table 5 Vacuolization observed in livers from treated chicks after feeding for 21 days

Diet	Absent[a]	Light[a]	Moderate[a]	Severe[a]
Control (basal diet)	4	0	0	0
Basal diet + FB1	0	0	2	2
Basal diet + zeolite (0.5%)	1	3	0	0
Basal diet + zeolite (1.0%)	4	0	0	0
Basal diet + FB1 + zeolite (0.5%)	0	4	0	0
Basal diet + FB1 + zeolite (1.0%)	2	2	0	0
Basal diet + bentonite (0.5%)	3	1	0	0
Basal diet + bentonite (1.0%)	4	0	0	0
Basal diet + FB1 + bentonite (0.5%)	4	0	0	0
Basal diet + FB1 + bentonite (1.0%)	4	0	0	0

[a] Values represent the number of chicks showing effects, 4 were examined in each group

significant difference ($P < 0.01$) between zeolite and bentonite. Bentonite and zeolite was inert and non-toxic for the chicks because the addition of both of them to the basal diet did not produce any adverse effect on the parameters evaluated.

Conclusions

The results obtained in this study showed that both, zeolite and bentonite positively influenced chicks' weight gain. There was a liver protection effect of zeolite and bentonite since they had good macroscopic and microscopic appearance. An increase in the aspartate aminotransferase level activity was detected only in the serum from chicks fed with FB1-contaminated diets; therefore, AST might be useful as an indicator of exposure to FB1 in this model. However, further studies are necessary to obtain a full correlation between these variables. The sphinganine-to-sphingosine ratio behaves as an indicator for FB1 ingestion. The addition of bentonite and zeolite reduced the mycotoxin effects in chicks, for this reason, our findings shows that they have a FB1 protective effect reducing damages in liver. Bentonite (1.0%) causes a better protective effect than zeolite.

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