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Experimental reproduction of ELEM

A Study to determine the minimum toxic dose in ponies

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Abstract

An experiment to gain insight into the minimum toxic dose of fumonisins was conducted by feeding ponies rations with known fumonisin concentrations. Naturally contaminated corn screenings (CS) were blended with pellets, corn, and molasses to formulate individual daily diets. One group of 4 ponies was fed a ration with fumonisin B_1 (FB_1) varying from <1 ppm to 22 ppm. A second group of 5 ponies was fed a ration at varying rates containing 8 ppm FB_1 for 180 days. A panel of clinical chemistry parameters was evaluated twice weekly for both groups. One pony in the first group died of equine leukoencephalomalacia (ELEM) after 225 days of which the final 55 days' diet contained 22 ppm FB_1. Approximately 9 days prior to death, this animal experienced elevated liver chemistry values. All 5 ponies in the second group experienced mild, transient, clincal signs; were euthanized at 180 days; and had mild, histopathological brain lesions.

Introduction

Prior to the characterization of fumonisins in 1988 [1, 2], suspect feed [3, 4] and autoclaved corn experimentally infected with *Fusarium moniliforme* [5] were used in feeding trials attempting to reproduce ELEM. These studies provided important mycological, clinical, pathological, and toxicological data concerning ELEM despite the inability to identify the mycotoxin involved.

Fundamental research into the toxicity of these mycotoxins is in its infancy. A few reports [6–11] provide a basis for concern. Since the fumonisins appear to be universally present in corn and corn-based feeds [10–16], it is critical that safe levels in foods and feeds be determined. The study described here was designed to gain insight into safe levels of fumonisins in horse feed.

Materials and methods

Experimental diet, feeding regime, and animal husbandry. Several hundred kg of CS were obtained for this study. Lots of approximately 25 kg each were ground in a Romer Mill (Romer Labs, Chesterfield, MO) and mixed thoroughly. Eight subsamples were taken from each lot and analyzed for fumonisins by high-performance liquid chromatography and thin-layer chromatography as previously described [12, 16]. If the FB_1 results showed greater than a 10% variance (relative

Table 1. Feeding regimes for Groups 1 and 2.

Group 1			Group 2		
Phase	Feed (ppm FB_1)	Duration (days)	Phase	Feed (ppm FB_1)	Duration (days)
1	15 ppm	130	1	8 ppm[a]	122
2	<1 ppm	30	2	8 ppm[b]	58
3	22 ppm	13			
4	<1 ppm	7			
5	22 ppm	146			

[a]0.8% BW ration.
[b]1.6% BW ration.

standard deviation), the lot was mixed, sampled, and analyzed a second time or until the variance was less than 10%.

Using the mean FB_1 level for each lot, individual rations representing either 0.8% or 1.6% body weight (BW) were prepared daily by adding CS to pellets (NADC Horse Ration, Code 560, Purina Mills, St. Louis, MO) and molasses (Promolas Easy Mixer Supreme, Pacific Molasses Company, San Francisco, CA) to create FB_1 concentrations as shown in Table 1. In general, the ration contained 60% pellets, 10% molasses, and 30% CS. The control diet consisted of pellets plus molasses.

Eleven clinically normal ponies of varying age and sex were divided into 2 test groups and 1 control group as shown in Table 2. All ponies were conditioned on the control diet for 21 days prior to the trial. During the conditioning period and throughout the trial, all ponies received alfalfa hay free choice. The test groups were fed for varying lengths of time as shown in Table 1. The ponies were individually housed inside at night, in a group paddock during the day, and fed separately. Individual unconsumed rations were measured daily.

Chemical analyses. All portions of the diet, including the CS, were assayed for chlorinated hydrocarbon pesticides (CHC), organophosphate pesticides (OP), carbamate pesticides (CARB), aflatoxins, zearalenol, T-2 toxin, diacetoxyscirpenol, deoxynivalenol, 15-acetyl deoxynivalenol, fusarenone-x, zearalenone, HT-2 toxin, T-2 tetrol, sterigmatocystin, cyclopiazonic acid, ochratoxin A, gliotoxin, sporidesmin, and griseofulvin. Additionally, the hay, molasses, and pellets were tested for FB_1 and fumonisin B_2 (FB_2). All samples were subjected to general screens for toxicants by gas chromatograph/mass spectrometry (GC/MS) and inductively coupled plasma spectrophotometry (ICP).

Table 2. Anamnestic data on ponies for test and control groups.

Pony No.	Weight (kg)			
	Starting	Ending	Age (yrs)	Sex
Group 1				
336	143	207	5	Stallion
338	150	195	6	Mare
341[a]	152	175	4	Gelding
343	173	193	6	Gelding
Group 2				
337	159	182	9	Gelding
340	146	182	5	Gelding
342	114	152	5	Stallion
344[b]	118	137	3	Gelding
345	123	186	8	Stallion
Control Group				
299	205	215	12	Mare
300	100	200	1	Stallion

[a]Died of ELEM on day 59 of Phase 5 (Table 1).
[b]Euthanized on day 92 of Phase 1 (Table 1).

Clinical chemistry, evaluation, and tissue collection. The animals were observed intermittently throughout each day, 7 days per week. Blood was drawn approximately twice a week, and the following clinical chemistry parameters were evaluated: total bilirubin (BT), direct bilirubin (BD), urea nitrogen (BUN), alkaline phosphatase (ALP), SGOT/AST, creatinine kinase (CK), gamma glutamyl transaminase (GGT) (a-gent™, Abbott Laboratories, South Pasadena, CA), and bile acids (BA) (Enzabile™, Nycomed, Diagnostica, Oslo, Norway). All clinical chemistry tests were done at 37 °C except BD which was done at 30 °C. A routine necropsy was performed on the animals that died or that were euthanized. A wide range of tissues was collected at necropsy, but only brain, liver, and kidney were prepared for

microscopic study. The entire brain was fixed in 10% buffered formalin, then sectioned and embedded in paraffin, cut to 5 μm thickness, and stained with hematoxylin and eosin.

Animal care. The animal care and use protocol was reviewed by the National Veterinary Services Laboratories Animal Care and Use Committee. Experiments were conducted in accordance with provisions of the 'Guide for Care and Use of Laboratory Animals', NIH publication No. 88–23, 1985.

Results

Fumonisins assay and other analytical results. The level of FB_1 detected in the subsamples of 17 lots of CS ranged from 61 to 268 μg/g. Fumonisin B_2 was detected in all subsamples at concentrations ranging from 19 to 86 μg/g. Fumonisin B_2 was consistently present at 32% of FB_1. The hay, CS, molasses, and pellets had concentrations of FB_1 and FB_2 below the 1 μg/g detection limits. The results of CHC, OP, CARB, GC/MS, and ICP screens were negative on all samples. Results of testing for mycotoxins other than fumonisins were negative for all samples.

Group 1. Pony 341 died acutely of ELEM after 58 days of Phase 5 while consuming a diet containing 22 ppm FB_1. During most phases of the feeding trial, it consumed all of its daily feed; however, in the several days prior to death, 50% or less was consumed. Between days 20 and 40 of Phase 1, the animal periodically became mildly aggressive toward the other ponies and was head shy. During Phase 3, the pony was mildly depressed and reluctant to move or walk into the pen. The final 30 days of Phase 5 were characterized by mild ataxia, aggressiveness, and confusion with eventual acute death.

Pony 341 consumed 4519 mg FB_1 throughout the 3 critical phases. During Phase 5, the dose rate was 0.18 mg FB_1/kg BW/day. Clinical pathology parameters remained within normal ranges until the final 9 days of Phase 5 when all values, with the exception of CK and BUN, increased significantly (Table 3). Necropsy revealed an oval, very friable lesion in the left cerebral hemisphere about 3 × 3 cm in diameter. The lesion, located in the upper left quadrant of the brain at the level of the optic chiasma, was predominantly in the subcortical white matter and was approximately 1.5 to 2.0 cm in length (Fig. 1). Microscopically, the lesion consisted of rarefaction of the white matter, multiple foci of perivascular hemorrhage (mainly in both the grey and white matter), and a very mild, focal leptomeningeal mononuclear infiltrate.

Table 3. Selected clinical pathology values for pony 341.

	Phase 1[a] 15 ppm FB_1	Phase 3[b] 22 ppm FB_1	Phase 5[c] 22 ppm FB_1
Bilirubin			
total (mg/dL)	0.6	0.7	2.1
direct (mg/dL)	0.4	0.4	0.9
CK (IU/L)	261	388	488
BUN (mg/dL)	24	29	18
ALP (IU/L)	187	204	696
SGOT/AST (IU/L)	273	316	2082
Bile acid (μmol/L)	2	7	118
GGTP (IU/L)	19	17	172

[a]Day 14 of Phase 1.
[b]Day 13 of Phase 3.
[c]Day 48 of Phase 5.

The 3 remaining ponies in Group 1 periodically showed signs of depression, dullness, incoordination, decreased proprioception in their gait, confusion, apathy, hypersensitivity, and aggressiveness. Clinical chemistry parameters were within normal ranges throughout all 5 phases. On day 106 of Phase 5, pony 343 experienced tremors, head bobbing, impaired vision, ataxia, and incoordination and was off feed. By day 107, it had recovered. The 3 remaining ponies in Group 1 were euthanized and necropsied on day 146 of Phase 5. On necropsy, no significant gross lesions were noted in any of the 3 ponies. All 3 had similar histopathological lesions including: (1) diffuse, mild vacuolation of the hepatocytes, (2) mild, focal accumulation of plasma and lymphoid cells in the periportal areas, (3) perivascular

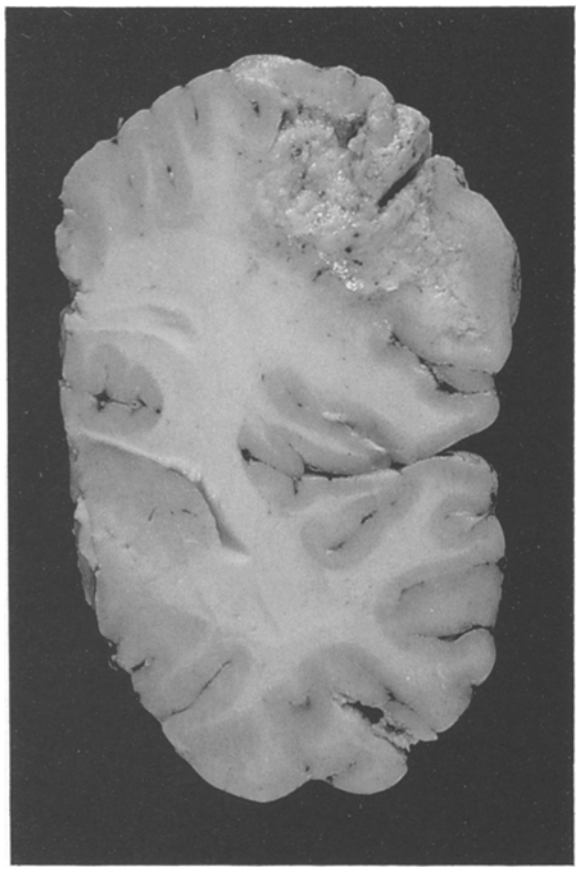

Fig. 1. Soft, necrotic, focal lesion in the left cerebral hemisphere of pony 341. Figure is oriented so that the left cerebral hemisphere is at the top.

edema, most prominent in the cerebral cortical vessels, and (4) punctate hemorrhagic foci in the grey and white matter, most common and most prominent in the cerebral cortex.

Group 2. With a few exceptions, all 5 ponies in Group 2 consumed all of their daily rations throughout the entire trial with a few exceptions. Clinical chemistry parameters for the entire group were within normal ranges for the entire period. All 5 gained weight during Phase 1 (8 ppm at 0.8% BW) but did not gain weight during Phase 2 (8 ppm at 1.6% BW).

Pony 344 was euthanized (embutramide) on day 92 of Phase 1. It had exhibited mild symptoms of confusion, circling, incoordination, agitation in the stall, and facial twitching perodically during Phase 1. The 4 remaining ponies in Group 2 were euthanized (embutramide) over a period of 2 weeks after Phase 2 was complete. During Phases 1 and 2, these ponies also exhibited periodic episodes of confusion, apathy, dullness, abnormal reactions to minor changes in handling patterns, hyperexcitability, incoordination, stupidity, head shyness, and depression. On necropsy, no significant gross lesions were noted in any of the ponies.

All 5 ponies had similar histopathological lesions including (1) mild, focal to diffuse, random, hepatocyte vacuolation, (2) mild, focal, interstitial infiltrates of mononuclear cells in the renal cortex and medulla, (3) focal, hemorrhagic foci (often perivascular) in transverse sections of the brain stem at the level of the vestibular, olivary, and hypoglossal nuclei (these foci were deep in the neuropile as well as adjacent to the floor of the fourth ventricle), and (4) reactive astrocytes, glial cells, and edema.

Discussion

Experimental reproduction of ELEM from a naturally contaminated source with known fumonisin concentrations has not been reported previously. During Phases 3 and 5, pony 341 was given daily doses of 0.18 mg FB_1/kg BW when all the ration was consumed; an oral dose lower than indicated by other reports. Researchers in South Africa have reproduced the disease by IV injection (0.125 mg/kg/day) [8] and oral dosing (1.00–4.0 mg/kg/day) of pure FB_1 [7]. We have previously shown that 0.36 mg FB_1/kg/day caused toxic hepatosis and encephalopathy in 2 of 4 ponies (9 and 43 days) consuming a naturally contaminated ration with 44 ppm FB_1 (FB_2 present at 13 ppm) [P.F. Ross, personal communication]. We have also reported on an outbreak of ELEM in which 14 of 66 Arabian horses consumed FB_1 estimated from 0.6–2.1 mg/kg/day (FB_2 was also present) for approximately 26 days [16]. Ponies

in Group 2 consumed 0.065 mg/kg/day and 0.130 mg/kg/day during Phases 1 and 2, respectively. Although none from Group 2 died of ELEM, they developed some clinical neurological symptoms, and all 5 had microscopic brain lesions suggestive of antemortem toxicity.

In both groups, ponies experienced transient, clinical signs followed by a return to normal behavior. ELEM may not progress beyond mild clinical signs, but long-term behavior may be significantly altered. This observation has also been reported in horses that were orally dosed with FB_1 [7]. The question of subsequent and/or interrupted exposure is very important; further study is essential.

The evidence of CNS signs (Group 2) without changes in the clinical chemistry parameters is consistent with reports that long-term, low-level exposure will produce brain damage but no hepatic involvement [7]. Confounding this is that all 5 ponies in Group 2 showed very mild, nonspecific, microscopic liver lesions. Pony 341 (Group 1) consumed apparently low levels yet developed classical neurological lesions and clinical pathology values indicative of significant liver disease suggesting that 22 ppm is not a low level or that there is always liver involvement with low level.

The CS in this study contained FB_2 concentrations that were 32% of FB_1. Scant information is available on FB_2 toxicity, and its significance is presently unclear. Its presence at a known constant amount compared to FB_1 facilitates easy recalculation of doses if future work determines that FB_2 is involved with ELEM. Workers elsewhere have suggested that FB_2 should be considered of equal toxigenic potential [7] until further data is available. As presented in this volume by W.C.A. Gelderblom, FB_2 and fumonisin B_3 are at least as effective as FB_1 in the rat initiation-promotion bioassay.

The variable dosing regime used in the current study was selected to represent the variability that could occur in the field. Rarely would diets come from the same lot or source over extended time periods. The FB_1 concentrations here were selected based on our previous work with ELEM and ponies, as well as reports on FB_1 in feeds associated with confirmed ELEM field cases [13, 15]. We have reported on the study of 36 confirmed cases from 1989–90 and 9 cases from 1984–85 [13] which suggested that for levels above the 10–20 ppm range, there should be concern for toxicity. Although the significance of the brain lesions in the 8 ppm ponies is not known, there should be concern for feeding that level or lower. Additionally, with the exception of the 8 ppm ponies, daily rations here were always 0.8% body weight, which is considered maintenance only. Many producers use corn-based rations at high rates to effect weight gain and enhance appearance; concentration and feeding rate are to be considered in determining toxicity.

Many questions concerning the fumonisins are yet to be answered. The work described here provides basic information that will assist in eventually determining safe concentrations in horse feeds.

Acknowledements

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