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Glucose Reaction with Fumonisin B_1 Partially Reduces Its Toxicity in Swine

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Acute and subacute intraperitoneal doses of fumonisin B_1 (FB_1) were administered to test the efficacy of the FB_1−glucose reaction products in detoxifying FB_1 in swine. In the acute study at 11 μmol of FB_1/kg of body weight, five of six pigs administered FB_1 and four of six pigs administered FB_1−glucose died from acute pulmonary edema. Analysis of weight gain, serum aspartate aminotransferase and γ-glutamyltransferase, total cholesterol, and pathological evaluation did not provide evidence of protection against FB_1 toxicity by the FB_1−glucose reaction products. In the subacute study at 5.5 μmol of FB_1/kg of body weight, one pig administered FB_1 died from liver damage. Analysis of serum aspartate aminotransferase, γ-glutamyltransferase, and total bilirubin showed protection against FB_1 toxicity by the FB_1−glucose reaction products. The levels of sphinganine and sphinganine/sphingosine ratios in serum and liver as well as pathologic findings provided definitive evidence of protection against the FB_1 toxic effects by this detoxification procedure ($p < 0.05$).

KEYWORDS: Fumonisin B_1; fumonisin B_1−glucose; detoxification; swine

INTRODUCTION

The fumonisins are a group of mycotoxins mainly produced by the fungi *Fusarium verticillioides* (= *F. moniliforme*) and *Fusarium proliferatum* in corn (*1*). Among other structurally related homologues, fumonisin B_1 (FB_1) is the most abundant natural contaminant of corn-based products intended for human or animal consumption throughout the world (*2*). This mycotoxin has been associated with a high incidence of human esophageal cancer in some regions of China and the Transkei in South Africa, where corn-based foods are the staple diet (*3*). Hence, FB_1 has been declared a class 2B carcinogen, a probable human carcinogen (*4*). Fumonisin B_1 has species-specific toxic effects. In rodents, FB_1 is hepatocarcinogenic, hepatotoxic, and nephrotoxic (*5*). Horses are the most sensitive species, known to develop leukoencephalomalacia (ELEM) after consuming corn contaminated with FB_1 at levels >0.001 μmol/g (*6*, *7*). In pigs, subacute dietary levels, in the range of 0.1 μmol of FB_1/g, can cause liver disease in ∼15 days, whereas acute dietary levels, >0.14 μmol of FB_1/g, may produce fatal porcine pulmonary edema (PPE) in <1 week (*8*, *9*). In the United States, widespread large-scale outbreaks of ELEM and PPE occurred during the fall of 1989 and the winter of 1990. Significant numbers of horses and pigs died from consuming commercial mixed feeds containing FB_1-contaminated corn (*10*).

Fumonisin B_1 disrupts sphingolipid metabolism by potently inhibiting the enzyme ceramide synthase (sphinganine *N*-acetyltransferase) in the *de novo* biosynthesis pathway of major sphingolipids, thus causing a rapid increase in free sphinganine (Sa), one of the base precursors (*11*, *12*). The levels of free Sa and sphingosine (So) in tissues and biological fluids may be compared by constructing a ratio of Sa to So (Sa/So ratio) that is used as an early biomarker of exposure to the mycotoxin (*13*). The inhibition of ceramide synthase is also likely to disrupt overall sphingolipid metabolism and, theoretically, sphingolipid-mediated regulation of important cell events such as apoptosis and mitosis, which are probably in part responsible for the cytotoxic and carcinogenic properties of the mycotoxin (*11*, *14*).

In swine, the animal model used in the present study, FB_1 alters sphingolipid biosynthesis with the greatest alterations in Sa and So concentrations occurring in kidneys, liver, lungs, and heart (*13*). In a recent swine study, diets that were amended with a fungal culture of *F. verticillioides* to achieve FB_1 concentrations of 0.006 and 0.01 μmol/g caused a significant increase in the Sa/So ratio at days 15 and 8 of the study, respectively (*15*). Serum markers of liver injury, such as aspartate aminotransferase (AST), γ-glutamyltransferase (GGT), total bilirubin (BIL), and total cholesterol (CHOL), increase after exposure to either acute or subacute levels of FB_1. Hepatic lesions consist of apoptosis, necrosis, and hepatocyte proliferation (*8*, *9*). The toxicological effects of feeding 0.04 μmol of

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FB_1/g of feed to weaned piglets for 28 days were recently evaluated. Animals presented typical signs of pulmonary edema with reduced feed consumption and weight gain as well as typical gross and microscopic lesions. Increases in erythrocyte count, hematocrit, BIL, total protein, CHOL, and activities of serum alkaline phosphatase (SAP), AST, and alanine aminotransferase (ALT) were detected (*16*).

The primary amine group of FB_1 has been suggested as the site of toxicity for the molecule (*17*, *18*). Detoxification of FB_1 by blocking the amine group with a reducing sugar such as fructose or glucose via the nonenzymatic browning (NEB) or Maillard reaction has been suggested (*19*). This detoxification strategy was used to demonstrate a significant reduction in cancer promotion of diethylnitrosamine (DEN)-initiated Fischer344/N rats treated with FB_1−fructose when compared to rats treated with FB_1 alone (*20*). The effect of extrusion cooking on the chemical recovery of FB_1 was tested by HPLC analysis of the FB_1 amine. Results showed more apparent loss of the FB_1 amine in samples processed with mixing screws and heat generated than with nonmixing screws. The brown color of samples after extrusion suggested that loss of FB_1 might be due to the NEB reaction (*21*, *22*). The products of the FB_1−glucose chain reaction were recently characterized as *N*-carboxymethylfumonisin B_1 (*23*, *24*), *N*-(1-deoxy-D-fructos-1-yl)fumonisin B_1, *N*-methylfumonisin B_1, *N*-(3-hydroxyacetonyl)fumonisin B_1, and *N*-(2-hydroxy-2-carboxyethyl)fumonisin B_1 (*24*).

A recent study compared three groups of DEN-initiated Fischer rats fed diets containing no FB_1, highly purified FB_1 at 0.03 μmol of FB_1/g of feed, and FB_1 reacted with glucose containing a residual 0.01 μmol of FB_1/g of feed, respectively. The Sa/So ratio in the FB_1−glucose group was the same as in the controls, whereas the FB_1 group presented a ratio that indicated an alteration in the synthesis of complex sphingolipids (*25*).

This study describes the effects of FB_1−glucose reaction products in swine using acute and subacute intraperitoneal (IP) doses of FB_1 in completely randomized designs. Toxicological endpoints were defined as PPE and liver damage, respectively. Our hypothesis was that the chemical reaction of FB_1 with glucose would decrease FB_1 toxicity in swine. The goal was to evaluate the efficacy of the FB_1−glucose reaction products in the detoxification of acute and subacute FB_1 poisoning of swine.

MATERIALS AND METHODS

Preparation of Purified Fumonisin B_1. Liquid cultures of *F. proliferatum* strain M5991 were prepared following the method of Dantzer et al. (*26*) by inoculating capped baffled Erlenmeyer flasks containing 500 mL of modified Myro medium with a 4 day shake flask culture of the fungus for 100 days. FB_1 was isolated and purified to >95% according to the procedure of Dantzer et al. (*27*).

Preparation of Fumonisin B_1−Glucose Adducts. Two batches of FB_1−glucose adduct were prepared according to the method of Lu et al. (*24*). Briefly, 1.39 mM FB_1 (total = 6.144 mmol or 4.435 g in the acute toxicity study and 2.061 mmol or 1.488 g in the subacute toxicity study) was mixed with 0.1 M D-glucose and dissolved in 50 mM potassium phosphate buffer, pH 7.0. After incubation at 80 °C for 48 h for the acute toxicity study and for 200 h for the subacute toxicity study, the pH of the reaction mixture was adjusted to pH 2.7 with 2 N HCl to stop the reaction. The reaction was conducted for a longer time in preparation for the subacute toxicity study to achieve 89% free amine FB_1 reduction by glucose. High-capacity C18 SPE cartridges (Alltech, Deerfield, IL) were preconditioned with 50 mL of 100% methanol at pH 2.7 followed by 100 mL of deionized water at pH 2.7. An aliquot of 50 mL of the 1.39 mM FB_1−0.1 M D-glucose reaction mixture was loaded onto the cartridge. The cartridge was washed with 100 mL of deionized water and 100 mL of 30% methanol at pH 2.7. The excess D-glucose was washed out in this step. The FB_1−glucose was eluted with 50 mL of 40% methanol and 100 mL of 100% methanol at pH 2.7. The eluent was evaporated to dryness with a Brinkmann rotavapor R110 (Westbury, NY) at 37 °C. The percentages of free and reacted FB_1 were determined according to the method of Dantzer et al. (*27*). For the acute toxicity study, the residue was redissolved in 0.9% sterile saline to obtain an FB_1 concentration of 11 μmol/mL. For the subacute toxicity study, the residue was redissolved in sterile 0.05 M potassium phosphate buffer, pH 7.35, to achieve an FB_1 concentration of 5.5 μmol/mL. We used potassium phosphate buffer in the subacute toxicity study after we determined it would improve the solubility of the FB_1−glucose residue compared to saline solution.

Experimental Animals. The use of animals and experimental procedures were approved by the Iowa State University Laboratory Animal Care Committee in 2000. In the acute toxicity study, 18 3-week-old healthy pigs with initial body weights between 6.2 and 7.7 kg were randomly assigned to three treatment groups (6 pigs/treatment), housed in groups of 2 per pen, and allowed to acclimate to the animal room for 3 days. Room temperature was maintained at 27 °C with water and feed supplied ad libitum. In the subacute toxicity study, 12 3-week-old pigs with initial body weights between 4.9 and 8.6 kg were divided into similar treatment groups (4 pigs/treatment), individually housed in pens, and exposed to the same conditions as in the acute toxicity study.

Experimental Treatments. All doses were administered intraperitoneally (ip) once a day. Volume injected never exceeded 1 mL/kg of body weight (BW) to minimize abdominal discomfort. Treatments consisted of the vehicle, FB_1, and the FB_1−glucose reaction products (FB_1-G). In the acute toxicity study with PPE as endpoint, doses were administered for a maximum of 9 days and were, in the control group, 1 mL of vehicle/kg of BW; in the FB_1 group, 11 μmol of FB_1/kg of BW; and in the FB_1-G group, 11 μmol of FB_1-G/kg of BW. The FB_1-G products contained ~30% unreacted FB_1 determined by HPLC and ELISA = 3.3 μmol of FB_1/kg of BW. In the subacute toxicity study with liver damage as endpoint, doses were administered for a maximum of 7 days and were in the control group, 1 mL of vehicle/kg of BW; in the FB_1 group, 5.5 μmol of FB_1/kg of BW; and in the FB_1-G group, 5.5 μmol of FB_1-G/kg of BW. The FB_1-G products contained ~11% unreacted FB_1 determined by HPLC = 0.6 μmol of FB_1/kg of BW.

Diet. All pigs in the acute and subacute toxicity studies received the same balanced diet consisting of weaning feed (Nevada Feed and Seed Co., Nevada, IA) with the following proximate composition: dry matter (88.8%), protein (18.76%), lysine (1.35%), fat (4.74%), fiber (3.22%), calcium (0.91%), phosphorus (0.75%), salt (0.55%), zinc (139.4 ppm), iodine (0.02 ppm), iron (19.8 ppm), copper (0.9 ppm), manganese (3.42 ppm), selenium (0.28 ppm), and vitamin E (3.96 IU/kg).

The general health of the swine was evaluated using a checkup list form specifically designed to record simple clinical observation parameters including behavior, mucous membrane coloration, respiratory rate (resp/min), heart rate (beats/min), and rectal temperature (°C) on a daily basis.

Weight gain was calculated on day 3 of the acute study and on day 7 of the subacute study by subtracting initial body weight from final body weight.

Clinical Chemistry. In the acute toxicity study, blood samples were taken on days 0 and 3 by jugular venipuncture. In the subacute toxicity study, pigs were bled from the orbital venous sinus on days 0, 4, and 7. Blood was allowed to clot for at least 45 min and then centrifuged at 3100 rpm for 15 min to separate serum from the clot. All samples were evaluated for serum chemistry analysis. The liver enzymes aspartate aminotransferase (AST) and γ-glutamyltransferase (GGT), total bilirubin (BIL), and total cholesterol (CHOL) were measured using a programmable, automated clinical analyzer (Hitachi 912, Roche Diagnostics, Basel, Switzerland). In the subacute toxicity study, duplicate serum samples were stored at −20 °C for extraction and HPLC analysis of sphingolipids.

Sphingolipid Analysis. The concentrations of Sa and So and their ratio in serum and liver were measured only in the subacute toxicity

study because tissues from acute toxicity study were lost in a freezer failure. Extraction, internal standard curve, and HPLC analysis were as follows.

The method in appendix 1 of Riley et al. (*28*) was followed with some modifications. All samples were analyzed in triplicates. Three hundred microliters of serum was added to 1.2 mL of cold 0.05 M potassium phosphate buffer, pH 7.0, for homogenization. One hundred microliters of the solution was transferred to a 13 × 1000 mm acid-washed glass tube with a PTFE-faced, rubber-lined cap. Thirty microliters of 5 μM *d,l*-erythro-C20-dihydrosphingosine (C-20, 150 pmol) internal standard was added. Samples were stirred every 15 min during incubation. Centrifugation speed was 4000 rpm. The $CHCl_3$ residue was dried at 37 °C under N_2 using a nitrogen evaporator (Organomation Associates, Inc., Berlin, MA). Samples were stored at 4 °C overnight. The next day, samples were base-hydrolyzed and incubated at 37 °C for 1 h. Chloroform and alkaline water were added, and then the samples were stirred and centrifuged at 4000 rpm for 15 min. The final $CHCl_3$ residue was dried at 37 °C under N_2 using a nitrogen evaporator. Samples were stored at −80 °C until HPLC analysis. A C-20 internal standard curve was constructed for So and Sa analysis using C-20 as the internal standard.

Samples were resuspended 2 h prior to HPLC analysis with 300 μL of 85% methanol and 30 μL of ethanol, stirred for 30 s, and sonicated for 5 min. Twenty microliters of OPA was added, followed by stirring for 30 s and sonication for 1 min. The samples were kept for 2 h at room temperature in a dark environment. Samples were transferred to a 0.45 μm Eppendorf nylon filter tube and centrifuged at 10000 rpm for 30 s in a microcentrifuge (Beckman Instruments, Inc., Fullerton, CA). Injection volume was 250 μL. The mobile phase consisted of HPLC-grade methanol (pump A, 85%) and 1% acetic acid (pump B, 15%). Flow rate was 1 mL/min with a run time of 15 min. The column used was a C-18 reversed phase Dynamax 50 × 4.6 mm (Varian, Walnut Creek, CA) heated at 42 °C. A fluorescence detector was set at 335 nm emission and 440 nm excitation.

Pathological Evaluation. In the acute toxicity study pigs were necropsied on day 9, unless euthanized due to poor health or found dead. In the subacute toxicity study necropsies were performed on day 7. In both studies, pigs were euthanized with a lethal intravenous dose of 0.5 mL of 7.8% sodium pentobarbital solution. In the subacute toxicity study, weights of potential target organs liver, lungs, heart, and kidneys were obtained and compared to final body weight to construct organ weight/body weight ratios. Tissue samples from organs were fixed in 10% neutral buffered formalin, processed routinely, sectioned at 5 μm, and stained with hematoxylin and eosin for histological evaluation. Additional tissue samples were stored at −20 °C for further sphingolipid extraction.

Statistical Analysis. Within-day comparisons of the least-squares means of the response variables among the three treatment groups were made by a repeated measurements analysis using SAS software v. 8.02 (SAS Institute Inc., Cary, NC, 2001). A p value of 0.05 was considered to be significant except where noted.

RESULTS

In the acute toxicity study, pigs in the FB_1 and FB_1-G groups received 11 μmol of FB_1/kg of BW. The dose of free unreacted FB_1 in the FB_1-G group was 3.3 μmol of FB_1/kg of BW, representing a 70% free amine FB_1 reduction. No clinical signs were observed in any of the pigs on day 1 of the study. Five pigs in the FB_1 group and four in the FB_1-G group died from day 2 through day 6. Eight of the nine pigs died acutely with clinical signs typical of PPE with duration of <4 h. The ninth pig, from the FB_1 group, was mildly icteric and weak late on day 6 and was found dead early on day 7. From days 7 to 9, the remaining pig in the FB_1 group and the two remaining in the FB_1-G group were free of clinical signs of PPE except that one of the FB_1-G pigs was moderately depressed with evident mild icterus of skin, sclera, and mucous membranes. The control group had the highest weight gain, 1.2 ± 0.1 kg, whereas the FB_1 and FB_1-G groups gained an average of 0.5 ± 0.14 and 0.7 ± 0.14 kg, respectively. Weight gains of the FB_1 and FB_1-G groups were not statistically different ($p < 0.05$) (**Figure 1**).

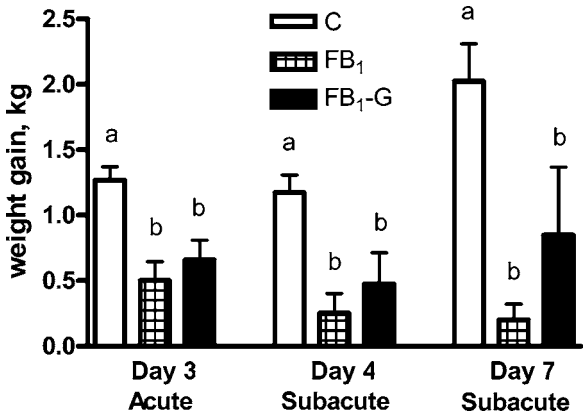

Figure 1. Comparison of weight gain among the three treatment groups in the acute ($n = 6$) and subacute ($n = 4$) studies. The error bar represents the standard error of the mean (SEM). Different letters above error bars indicate statistically significant differences between treatment groups at a given time point ($p < 0.05$). In both studies, average initial body weights (day 0) did not differ significantly among the groups.

Table 1. Aspartate Aminotransferase (AST), γ-Glutamyltransferase (GGT), Total Bilirubin (BIL), and Total Cholesterol (CHOL) Levels in Serum on Days 0 and 3 of the Acute Toxicity Study[a]

	AST (IU/L)	GGT (IU/L)	BIL (mg/dL)	CHOL (mg/dL)
		Day 0		
control	37.8 ± 4.8 **a**	56.3 ± 5.3 **a**	0.18 ± 0.01 **a**	157.5 ± 33.4 **a**
FB_1	51 ± 6.7 **a**	62.5 ± 7.9 **a**	0.29 ± 0.04 **a**	213 ± 13.8 **b**
FB_1-G	42.7 ± 5.9 **a**	70 ± 13.8 **a**	0.25 ± 0.04 **a**	242.3 ± 35.2 **b**
		Day 3		
control	54 ± 9.3 **a**	55.3 ± 2.1 **a**	0.19 ± 0.01 **a**	84.7 ± 3.6 **a**
FB_1	191 ± 72.5 **b**	69.8 ± 7.5 **a**	1.11 ± 0.33 **b**	136.8 ± 9.9 **b**
FB_1-G	174.2 ± 62.7 **b**	76.6 ± 9.7 **a**	0.48 ± 0.13 **a**	127.8 ± 17.2 **b**

[a] Data are expressed as mean ± SEM. $n = 6$. Different letters indicate statistically significant differences between the group means at a given time point ($p < 0.05$). FB_1 dose: 11 μmol/kg of BW. Free FB_1 in FB_1-glucose dose: 3.3 μmol/kg of BW.

On day 0 of the acute toxicity study, the serum levels of AST, GGT, and BIL were similar among the treatment groups (**Table 1**). CHOL was moderately elevated in FB_1 and FB_1-G pigs with respect to controls. On day 3, AST was moderately elevated in FB_1 and FB_1-G pigs as compared to controls, GGT levels were similar among the treatment groups, BIL was moderately elevated only in FB_1 pigs, and CHOL levels were slightly elevated in FB_1 and FB_1-G groups with respect to controls ($p < 0.05$). The analysis of sphingolipids could not be carried out in this study because the serum and liver tissue samples stored for this purpose were lost in a freezer failure.

In the acute toxicity study, five FB_1 and four FB_1-G pigs showed characteristic signs of acute PPE, which included massive hydrothorax with abundant clear straw-colored fluid high in serum proteins within the thoracic cavity and marked interstitial pulmonary edema with broad bands of gelatinous straw-colored fluid separating the major interlobular spaces. One additional FB_1-G pig was determined to have died due to exanguination into the peritoneal cavity as a probable result of trauma from ip injection. Necropsy of the remaining pigs in the FB_1 and FB_1-G groups revealed no lesions of PPE, although one pig in the FB_1-G group showed mild icterus of skin, sclera, and mucous membranes.

Table 2. Aspartate Aminotransferase (AST), γ-Glutamyltransferase (GGT), Total Bilirubin (BIL), and Total Cholesterol (CHOL) Levels in Serum on Days 0, 4, and 7 of the Subacute Toxicity Study[a]

	AST (IU/L)	GGT (IU/L)	BIL (mg/dL)	CHOL (mg/dL)
		Day 0		
control	116 ± 33.3 **a**	40.5 ± 7.3 **a**	0.3 ± 0.05 **a**	220.3 ± 22.9 **a**
FB_1	34 ± 2.6 **a**	42 ± 3.7 **a**	0.2 ± 0.01 **a**	218.8 ± 12.4 **a**
FB_1-G	47.2 ± 15 **a**	45.5 ± 5.3 **a**	0.9 ± 0.26 **a**	245.8 ± 18.6 **a**
		Day 4		
control	63 ± 19.6 **b**	43.5 ± 2.7 **a**	0.2 ± 0.02 **a**	104 ± 6 **a**
FB_1	201 ± 55 **a**	74.3 ± 7.3 **a**	2.3 ± 0.7 **b**	316.3 ± 71.8 **b**
FB_1-G	48.5 ± 11 **b**	56.3 ± 8.6 **a**	0.7 ± 0.23 **a**	252.5 ± 75.5 **b**
		Day 7		
control	47.5 ± 8.4 **a**	37.3 ± 2.8 **a**	0.2 ± 0.01 **a**	85 ± 6.1 **a**
FB_1	1804 ± 661 **b**	128 ± 26.2 **b**	7.6 ± 2.13 **b**	378.8 ± 57.5 **b**
FB_1-G	48.5 ± 6.9 **a**	48.3 ± 11.7 **a**	1 ± 0.45 **a**	274.5 ± 111.4 **c**

[a] Data are expressed as mean ± SEM. $n = 4$. Different letters indicate statistically significant differences between the group means at a given time point ($p < 0.05$). FB_1 dose: 5.5 μmol/kg of BW. Free FB_1 in FB_1-glucose dose: 0.6 μmol/kg of BW.

In the subacute toxicity study, pigs in the FB and FB_1-G groups received 5.5 μmol of FB_1/kg of BW. The dose of free unreacted FB_1 in the FB_1-G group was 0.6 μmol of FB_1/kg of BW representing an 89% free amine FB_1 reduction. The control pigs had no apparent signs of disease throughout the duration of the experiment. Pigs receiving FB_1, on the other hand, showed weakness, rough hair, and lateral recumbency by day 4. Their condition continued to worsen on a daily basis as they refused to eat and icterus became evident in the ventral abdominal and inguinal region of the body and in the ocular sclera. Early on day 7, one FB_1 pig was found dead. Three of four FB_1-G pigs showed rough hair and mild weakness from days 4 to 7, but in general all pigs in this group were considered to be in good health until the end of the experiment. Pigs in the control group had the highest weight gain, 2.02 ± 0.28 kg, whereas pigs in the FB_1 and FB_1-G groups gained only 0.2 ± 0.12 and 0.85 ± 0.51 kg, respectively. Weight gains of FB_1 and FB_1-G groups were not statistically different ($p < 0.05$, **Figure 1**).

On day 0 of the subacute toxicity study, the serum levels of AST, GGT, BIL, and CHOL were similar among the treatment groups (**Table 2**). On day 4, the FB_1 pigs showed moderately elevated AST and BIL with respect to controls. GGT levels were similar among the treatment groups, and CHOL was lower in controls as compared to FB_1 and FB_1-G, which shared moderately elevated levels. AST and BIL were highly elevated in FB_1-treated pigs as compared to controls and FB_1-G-treated pigs on day 7. GGT was higher in the FB_1 group but to a lesser extent. CHOL was higher in FB_1 pigs followed by FB_1-G and controls, with all groups different from each other ($p < 0.05$).

In the subacute toxicity study, serum So levels remained stable throughout and were not statistically different among the treatment groups at any time point (**Table 3**). On day 0, Sa concentration and the Sa/So ratio were similar between the treatment groups. On day 4, Sa concentration in FB_1 pigs was greater than in control but similar to that in FB_1-G pigs. Control and FB_1-G pig results were not different from each other. The Sa/So ratio in FB_1 pigs was higher than in control but similar to FB_1-G pigs, whereas control and FB_1-G pigs had similar ratios. On day 7, the Sa concentration and Sa/So ratio were higher in FB_1 pigs as compared to control and FB_1-G pigs, which results were not different from each other ($p < 0.05$). In liver, So levels in FB_1-G pigs were higher than in FB_1 and control pigs (**Table 4**). FB_1 pigs had lower levels of So than control and FB_1-G pigs. The So concentration in control pigs was intermediate between those of FB_1 and FB_1-G pigs. Levels of Sa were higher in FB_1 as compared to controls and FB_1-G, which were not different from each other. The Sa/So ratio followed a trend similar to the Sa concentration ($p < 0.05$).

Table 3. Sphinganine (Sa) and Sphingosine (So) and Sa/So Ratio in Serum on Days 0, 4, and 7 of the Subacute Toxicity Study[a]

	Sa (nmol/mL)	So (nmol/mL)	Sa/So
		Day 0	
control	0.8 ± 0.2 **a** ($n = 12$)	12.4 ± 1.9 **a** ($n = 12$)	0.07 ± 0.01 **a** ($n = 12$)
FB_1	0.8 ± 0.2 **a** ($n = 12$)	9.3 ± 1.2 **a** ($n = 11$)	0.08 ± 0.01 **a** ($n = 12$)
FB_1-G	0.7 ± 0.08 **a** ($n = 13$)	10.5 ± 2.6 **a** ($n = 13$)	0.11 ± 0.02 **a** ($n = 13$)
		Day 4	
control	0.92 ± 0.2 **a** ($n = 12$)	8.8 ± 1.2 **a** ($n = 11$)	0.11 ± 0.03 **a** ($n = 12$)
FB_1	2.77 ± 0.9 **b** ($n = 12$)	8.25 ± 1.7 **a** ($n = 11$)	0.33 ± 0.07 **b** ($n = 12$)
FB_1-G	1.9 ± 0.9 **ab** ($n = 11$)	9.88 ± 4.9 **a** ($n = 9$)	0.25 ± 0.05 **ab** ($n = 12$)
		Day 7	
control	1 ± 0.1 **a** ($n = 14$)	15.22 ± 2 **a** ($n = 8$)	0.05 ± 0.006 **a** ($n = 16$)
FB_1	7.67 ± 2.5 **b** ($n = 10$)	26.57 ± 5.7 **a** ($n = 9$)	0.25 ± 0.07 **b** ($n = 10$)
FB_1-G	2.02 ± 0.8 **a** ($n = 9$)	18.04 ± 4.1 **a** ($n = 7$)	0.1 ± 0.03 **a** ($n = 9$)

[a] Sphingolipids are expressed as nanomoles per milliliter of serum and expressed as mean ± SEM. Different letters indicate statistically significant differences between the group means at a given time point ($p < 0.05$). FB_1 dose: 5.5 μmol/kg of BW. Free FB_1 in FB_1-glucose dose: 0.6 μmol/kg of BW.

Table 4. Liver Levels of Sphinganine (Sa) and Sphingosine (So) and Sa/So Ratio on Day 7 of the Subacute Toxicity Study[a]

	Sa (nmol/g)	So (nmol/g)	Sa/So
control	0.1 ± 0.05 **a** ($n = 8$)	45.1 ± 4.1 **ab** ($n = 13$)	0.01 ± 0.008 **a** ($n = 6$)
FB_1	13.7 ± 2.1 **b** ($n = 12$)	37 ± 12.1 **a** ($n = 10$)	1.2 ± 0.4 **b** ($n = 9$)
FB_1-G	2.7 ± 0.4 **a** ($n = 13$)	71 ± 10.04 **b** ($n = 12$)	0.05 ± 0.01 **a** ($n = 12$)

[a] Sphingolipids are expressed as nanomoles per gram of liver tissue and expressed as mean ± SEM. Different letters indicate statistically significant differences between the group means ($p < 0.05$). Sa/So ratios between control and FB_1 groups were different at a 10% significance level. FB_1 dose: 5.5 μmol/kg of BW. Free FB_1 in FB_1-glucose dose: 0.6 μmol/kg of BW.

Gross inspection of the FB_1 pigs in the subacute toxicity study revealed severe yellow discoloration of subcutaneous, abdominal, and pericardial fat. Lungs had a normal diffuse pink color, were not edematous, and had clear airways and trachea. Pale-yellowish irregular areas could be observed in the liver, which did not appear to be enlarged. The surface of the kidneys appeared pale tan to gray and in some cases had a very congested cortex and very yellow-tinged renal papillae on cut surface. Serosal surfaces of the gastrointestinal tract appeared to be mildly congested and edematous but not hemorrhagic. FB_1-G and control pigs showed no obvious icterus and were in general free of lesions. Microscopic liver changes in the FB_1 group consisted primarily of mild to moderate, multifocal random necrosis of individual hepatocytes or small groups of hepatocytes with resultant disorganization of hepatic sinusoids. Hepatocytes individualized, became rounded or hypereosinphilic, and had pychnotic, karryorrhexic, or karryolytic nuclei. Mild to moderate karyomegaly was often observed in adjacent

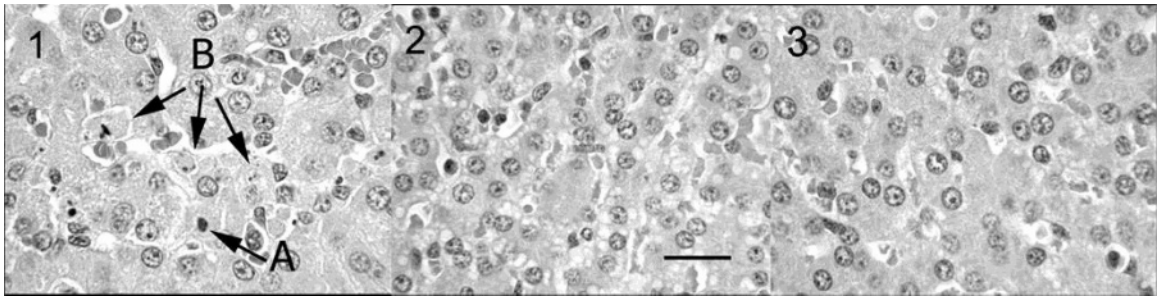

Figure 2. Subacute study: (**1**) liver from a fumonisin B_1-treated pig with multifocal, individual hepatocyte degeneration and necrosis [several degenerate hepatocytes with pyknotic nuclei (**A**) and nuclear karyorrhexis (**B**) are illustrated]; (**2**) liver from control pig (no significant microscopic lesions were observed); (**3**) liver from a fumonisin B_1–glucose-treated pig (no significant microscopic lesions were observed). Hematoxylin and eosin stain were used. Bar = 30 μm.

hepatocytes. A comparison of the three treatment groups is presented in **Figure 2**. In the FB_1-G group, two of four pigs showed large, clear, well-demarcated vacuoles in the distal nephron but were otherwise free of lesions. No microscopic lesions were observed in control pigs. Ratios of organ weight to total body weight for heart, lungs, liver, or kidney showed no significant differences between the treatment groups (data not shown).

DISCUSSION

The acute toxicity study did not clearly demonstrate reduction of FB_1 toxicity by its reaction with glucose in IP dosing of swine. This was shown by the death from acute PPE of five of six pigs in the FB_1 group, dosed at 11 μmol of FB_1/kg of BW, and four of six pigs in the FB_1-G group, dosed at 3.3 μmol of FB_1/kg of BW. On day 3 weight gains were similar among the FB_1 and FB_1-G groups, and the clinical parameters AST and CHOL were above normal levels (*29*) and not significantly different in pigs from these two groups. The toxic effects observed in this study were consistent with acute FB_1 toxicosis in swine and have been reported by several authors (*30*−*34*). The partial failure of the FB_1−glucose adduct in protecting the pigs in the acute toxicity study from the effects of FB_1 could be explained by the 30% free FB_1 in the FB_1-G mixture. If this was the case, FB_1-G pigs received an ip dose of ∼3.3 μmol of FB_1/kg of BW. This dose of FB_1 is significantly higher than the intravenous (iv) dose level of 0.55 μmol of FB_1/kg of BW used by Harrison et al. (*35*) to characterize acute PPE. It is worth mentioning that on day 3 of the acute toxicity study, BIL results in FB_1-G pigs were within normal range (*29*), similar to results in the control group and lower than in the FB_1 group, providing some evidence of protection of the liver in the FB_1-G group (**Table 1**).

In contrast to the acute FB_1 dose, the subacute toxicity study revealed reduction of FB_1 toxicity in pigs. Only one pig died from apparent liver damage in the 5.5 μmol of FB_1/kg of BW group, whereas no pigs administered the FB_1−glucose products at a dose of 0.6 μmol of FB_1/kg of BW group died. We believe that two main factors, dose level and amount of free unreacted FB_1 in the FB_1−glucose mixture, contributed to the success of the detoxification treatment in protecting the pigs from FB_1-induced liver damage. The ip dose of 5.5 μmol of FB_1/kg of BW was half the dose used in the acute toxicity study, and the amount of free unreacted FB_1 in the FB_1−glucose adduct was only 11%, meaning that FB_1-G pigs received an FB_1 dose of ∼0.6 μmol/kg of BW. The high mortality observed in the FB_1-G pigs of the acute toxicity study, which received 3.3 μmol of FB_1/kg of BW, as compared to the FB_1 pigs of the subacute toxicity study, which received a dose of 5.5 μmol of FB_1/kg of BW, could be explained by the toxicity of the FB_1−glucose products. In preparation for the acute toxicity study, the FB_1−glucose mixture was heated for 48 h, resulting in 70% free amine FB_1 reduction by glucose. Additionally, other reaction products that were not chemically characterized and of which toxicity remains unknown may have also been formed. It is likely that these additional uncharacterized products in the acute toxicity study may have inhibited the enzyme ceramide synthase in the pigs treated with FB_1−glucose, disrupting the sphingolipid metabolism and leading to left-sided heart failure and ultimately PPE. Although in the subacute toxicity study the FB_1−glucose products were prepared following the same method, the mixture was instead heated for 200 h, resulting in 89% free amine FB_1 reduction. The additional FB_1−glucose reaction products formed in the subacute toxicity study were not chemically characterized. However, we observed that these did not cause any obvious signs of toxicity in the pigs of the FB_1−glucose group. Howard et al. (*36*) fed female B6C3F mice 14, 70, and 140 μmol/kg of diet of several fumonisin derivatives, including FB_1 and *N*-carboxymethylfumonisin B_1, one of the fumonisin B_1−glucose reaction products previously characterized (*23*, *24*). The study found that only FB_1 was hepatotoxic, whereas all other fumonisin derivatives did not alter serum analytes, organ weights, or hepatic structure. The high mortality of pigs administered the FB_1−glucose products in the acute toxicity study as compared to the pigs administered FB_1 in the subacute toxicity study could also be explained by a higher susceptibility to FB_1 exposure. The pigs used in the acute toxicity study were obtained from a different source than the pigs used in the subacute toxicity study, possibly having a different genetic background and a higher susceptibility to FB_1 at a 3.3 μmol/kg of BW dose. In pilot studies not previously published we have exposed crossbred pigs to levels as high as 145 μmol of FB_1/kg of diet and found no evidence of alteration in AST, GGT, or BIL. These findings contrast with the results of several studies (*8*, *9*) that have found liver damage after pigs were fed diets containing <145 μmol FB_1/kg, which leads us to believe that susceptibility to FB_1 could be different in pigs from different genetic lines. Harrison et al. (*35*) reported the observation of pulmonary edema in pigs administered iv pure FB_1 at a dose of 0.55 μmol/kg of BW for 4 days, a dose much lower than our ip dose of 3.3 μmol/kg of BW. Toxicokinetics of pure FB_1 after iv or ip administration appear to be comparable. Shephard et al. (*37*) dosed rats ip with 10.4 μmol/kg of BW of ^{14}C-labeled FB_1 and after 24 h recovered 66% of radioactivity in feces and 32% in urine. Prelusky et al. (*38*) administered iv ^{14}C-labeled FB_1 to pigs at a dose of 0.5 μmol/kg of BW. After 72 h, 58% of radioactivity was recovered in feces and 21% in urine.

Although in different species, the toxicokinetics results from these two studies appear to indicate similar excretions of FB_1 after either iv or ip administration.

The results of our subacute toxicity study agree with the FB_1 data of Haschek et al. (*31*), in which pure FB_1 administered iv to pigs at 0.0012 and 0.0016 μmol/kg of BW for 9 and 4 days, respectively, caused mild intermittent respiratory signs but not pulmonary edema or severe liver damage. Osweiler et al. (*8*) also observed subacute hepatotoxicosis in pigs administered iv 0.0011 μmol of FB_1/kg of BW for 14 days. Our weight gain results were equivocal. Pigs in the FB_1-G group had a weight gain similar to that in pigs in the FB_1 group. This observation is because two of four pigs dramatically decreased their feed consumption on days 4−5 of the study, causing the average weight gain to drop, whereas the other two pigs consumed as much feed as the controls (feed consumption data not shown). Although we could not establish a clear explanation for this variability, experiment-related stress events may have contributed to it. We do not believe these two pigs had developed FB_1−glucose adduct-induced feed refusal because two of four pigs ate well. In FB_1 pigs, the liver enzymes AST and GGT started to show a time-dependent elevation on day 4. By day 7, dramatic differences in the liver enzyme levels were evident between the FB_1 and control or FB_1-G groups. This indicated that liver cells in the FB_1 pigs of the subacute study were severely damaged, allowing the intracellular enzymes to leak into the bloodstream, events that presumably did not occur in the control or FB_1-G pigs. These findings are similar to the results of Liu et al. (*25*), in which rats exposed to diets containing 0.011 or 0.034 μmol of FB_1/g for 12 or 20 weeks had significantly higher plasma alanine aminotransferase (ALT) levels than rats fed a basal or FB_1−glucose diet for the same period of time. Our results also agree with a study by Lu et al. (*20*) in which rats fed 0.07 μmol of FB_1/g of diet for 4 weeks had a significantly higher ALT level than rats fed 0.07 μmol of FB_1/g of diet reacted with fructose for the same time. ALT is a specific enzyme for hepatocellular injury in small species, including the rat (*39*). Total cholesterol levels in control pigs showed a time-dependent decrease, whereas levels in FB_1-G pigs remained practically unchanged during the study. This pattern could explain the significant differences observed between control and FB_1-G pigs at days 4 and 7 of the subacute study.

Because FB_1 disrupts sphingolipid metabolism, results of individual levels of serum Sa and So and their ratio showed protection of the FB_1-G pigs in the subacute toxicity study. The levels of Sa, in particular, showed a time-dependent elevation in the FB_1 group throughout the study. On day 7, Sa levels and the Sa/So ratio were higher in the FB_1 pigs as compared to controls and FB_1-G pigs. The higher Sa levels in the FB_1 group were what we expected. Several studies had previously found a significant elevation of Sa after swine had been fed pure FB_1 at 0.002 μmol/g of diet for 5 days (*40*), FB_1 from culture material at 0.027 μmol/g for 1 day (*41*), or pure FB_1 at 0.013 μmol/g for 8 days (*30*). In contrast to Riley et al.'s findings (*13*), the serum Sa levels in the FB_1-treated pigs of our study did not plateau before day 4, and the serum So levels did not continue to increase for the duration of the study. The levels of So were not different among the three groups throughout the subacute toxicity study, which agrees with the findings of Riley et al. (*13*) and Gumprecht et al. (*30*). Blocking of the primary amine group of FB_1 by its reaction with glucose likely prevented the inhibition of the enzyme ceramide synthase in the sphingolipids synthesis pathway. Therefore, accumulation of sphingolipid precursors Sa and So was not observed in the FB_1-G group. A similar scenario was observed in liver, where Sa levels were higher in the FB_1 group but not different between controls and FB_1-G, resulting in Sa/So ratios that followed the same pattern. The results obtained from our FB_1-treated pigs were similar to those of previous studies that have found increased liver Sa, and to a lesser extent So, and elevated Sa/So ratios in pigs fed diets containing 0.031 μmol of FB_1/g for 14 day (*13*) or 0.013 μmol of FB_1/g for 8 weeks (*40*). In addition, pharmacokinetic studies have shown a good correlation between the magnitude of sphingolipid alterations and the distribution of FB_1, which is highest in liver and kidneys (*38*).

Gross lesions in the subacute toxicity study agreed with a study by Colvin et al. (*33*) in which pigs were daily gavaged with 5.5 and 22.2 μmol of FB_1/kg of BW for 45 and 7 days, respectively. In that study, pigs were also severely icteric, as was evident from the bright yellow color of the sclera, oral mucosa, and subcutaneous adipose tissue. Livers had a tan coloration. However, the findings of Colvin et al. (*33*) in kidneys were different from ours in that they found mild to moderate renal tubular necrosis and we did not. These researchers reported that lungs were normal and, although more severe than in our study, their pigs showed gastrointestinal lesions such as ulceration and erosion of the nonglandular mucosa. Microscopic changes consistent with both hepatocyte necrosis and apoptosis were observed in the FB_1 pigs of our subacute toxicity study. These changes matched those found in several dietary and iv studies (*8, 31, 34*). Interestingly, two of our FB_1-G pigs had well-demarcated vacuoles in the distal nephrons. We believe this represented a random finding because the remaining two FB_1-G pigs did not present such lesions and the other kidney structures were free of lesions.

In conclusion, in contrast to the acute ip dose of 11 μmol of FB_1/kg of BW, the subacute ip dose of 5.5 μmol of FB_1/kg of BW yielded clear evidence that derivatization of the FB_1 amine with glucose can protect the livers of pigs against the hepatotoxic effects of FB_1. Further studies are necessary to assess the use of this detoxification strategy in swine feed operations where quality might be adversely affected by FB_1 contamination. Its implementation in the field will depend on the use of appropriate processing equipment that would require additional research.

ABBREVIATIONS USED

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BIL, total bilirubin; C-20, *d*,*l*-erythro-C20-dihydrosphingosine; CHOL, total cholesterol; DEN, diethylnitrosamine; ELEM, equine leukoencephalomalacia; FB_1, fumonisin B_1; FB_1-G, fumonisin B_1−glucose; GGT, γ-glutamyltransferase; NEB, nonenzymatic browning reaction; OPA, *o*-phthalaldehyde; PPE, porcine pulmonary edema; Sa, sphinganine; SAP, alkaline phosphatase; So, sphingosine.

SAFETY

Fumonisin B_1 is a class 2B carcinogen and was handled accordingly.

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