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Reproductive and Sphingolipid Metabolic Effects of Fumonisin B_1 and its Alkaline Hydrolysis Product in LM/Bc Mice: Hydrolyzed Fumonisin B_1 Did Not Cause Neural Tube Defects

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Fumonisins are mycotoxins produced by *Fusarium verticillioides*. They are toxic to animals and exert their effects through mechanisms involving disruption of sphingolipid metabolism. Fumonisins are converted to their hydrolyzed analogs by alkaline cooking (nixtamalization). Both fumonisins and hydrolyzed fumonisins are found in nixtamalized foods such as tortillas, and consumption of tortillas has been implicated as a risk factor for neural tube defects (NTD). Fumonisin B_1 (FB_1) induced NTD when given (ip) to pregnant LM/Bc mice; however, neither the NTD induction potential of hydrolyzed fumonisin B_1 (HFB_1) nor its affect on sphingolipid metabolism in pregnant mice have been reported. The teratogenic potential of FB_1 and HFB_1 was therefore compared using the LM/Bc mouse model. Dams were dosed (ip) with 2.5, 5.0, 10, or 20 mg/kg (≤ 49 μmol/kg) body weight (bw) HFB_1 on embryonic day (E)7–E8. Negative and positive control groups were given vehicle or 10 mg/kg (14 μmol/kg) bw FB_1, respectively. The high dose of HFB_1 disrupted sphingolipid metabolism, albeit slightly, but did not cause maternal liver lesions or NTD (n = 8–10 litters per group). In contrast, 10 mg/kg bw FB_1 markedly disrupted maternal sphingolipid metabolism, caused hepatic apoptosis in the dams, increased fetal death rates, and decreased fetal weights. Furthermore, NTD were found in all FB_1-exposed litters (n = 10), and 66 ± 24% of the fetuses were affected. The findings indicate that HFB_1 does not cause NTD in the sensitive LM/Bc mouse model and only weakly disrupts sphingolipid metabolism at doses up to sevenfold higher (micromole per kilogram body weight basis) than the previously reported lowest observed adverse effect level for FB_1.

Key Words: **fumonisins; hydrolyzed fumonisin B_1; nixtamalization; reproductive toxicity; sphingolipids.**

Fumonisins are mycotoxins produced by *Fusarium verticillioides* (formerly *Fusarium moniliforme* Sheldon) and *Fusarium proliferatum* (Gelderblom *et al.*, 1991). They are found worldwide in maize and maize-based foods (Bolger *et al.*, 2001 and references therein). Fumonisin B_1 (FB_1), the most common congener, causes the animal diseases associated with *F. verticillioides* exposure, including leukoencephalomalacia in *Equidae*, pulmonary edema in swine, kidney and liver toxicity in diverse species, and kidney and liver cancer in rodents (Bolger *et al.*, 2001; Gelderblom *et al.*, 1991; Howard *et al.*, 2001; Voss *et al.*, 2007b). The key molecular event underlying toxicity is the inhibition of ceramide synthase with subsequent disruption of sphingolipid metabolism and sphingolipid-dependent physiological processes (Gelineau-van Waes *et al.*, 2009; Riley *et al.*, 2001; Zitomer *et al.*, 2009).

The human health effects of fumonisins are poorly understood. Esophageal cancer rates have been correlated with fumonisin concentrations in local maize (Bolger *et al.*, 2001), and Hendricks (1999) proposed that fumonisins were involved in a cluster of neural tube defects (NTD) that affected babies born to Mexican-American women living in the Texas counties bordering Mexico in 1990–1991. Unusually high incidences of equine leukoencephalomalacia and porcine pulmonary edema were noted in the area during 1989, and residents consumed locally grown maize harvested at that time. A correlation between tortilla consumption (up to 400) during the first trimester of pregnancy and increased risk of NTD was found during a retrospective epidemiological study of the Texas border region (Missmer *et al.*, 2006). Risk was further increased in women consuming homemade tortillas, presumably made from locally grown maize, compared to those eating exclusively commercial products. FB_1 exposure during the critical gestational window for neural tube closure induces NTD in cultured mouse embryos (Sadler *et al.*, 2002) and in the more sensitive LM/Bc (Gelineau-van Waes *et al.*, 2005, 2009) and the less sensitive CD1 (Voss *et al.*, 2007a) mouse strains *in vivo*.

Nixtamalization is the alkaline cooking method for making masa and tortillas from maize. Varying amounts of FB_1 and

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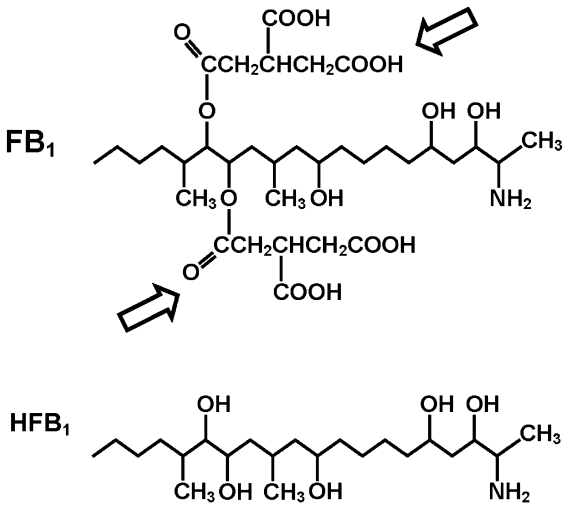

FIG. 1. The chemical structures of FB_1 and its full hydrolysis product. Under alkaline conditions, including those found during cooking by nixtamalization, FB_1 is converted to HFB_1 by hydrolytic cleavage of the carballylic acid (propane 1-2-3 tricarboxylic acid) groups (arrows) attached to the FB_1 hydrocarbon ''backbone'' at the C_{14} and C_{15} positions.

other fumonisins are converted under alkaline conditions to their hydrolyzed analogs (Fig. 1). Only a few surveys of hydrolyzed fumonisins in nixtamalized products have been reported. Amounts have been generally low (Dombrink-Kurtzman and Dvorak, 1999; Dvorak *et al*., 2008; Park *et al*., 2004; Seefelder *et al*., 2001); however, hydrolyzed fumonisins occasionally constituted a high percentage of the total fumonisins therein. When calculated on a micromolar basis, hydrolyzed fumonisin B_1 (HFB_1) accounted for $\geq$ 50% of the total FB_1 analogs in masa (Dombrink-Kurtzman *et al*., 2000; Voss *et al*., 2001) or tortillas (Dombrink-Kurtzman *et al*., 2000; Palencia *et al*., 2003). Human exposure to HFB_1 is therefore likely, especially in areas where nixtamalized maize is a diet staple (Missmer *et al*., 2006; Palencia *et al*., 2003).

HFB_1 ($\geq$ 100 μmol) induced anomalies, including NTD, in cultured rat embryos (Flynn *et al*., 1997), suggesting that it, like FB_1, might induce anomalies *in vivo* when tested in a sensitive animal model. HFB_1 is metabolized *in vivo* by rats to N-acylated metabolites that are structural analogs of ceramide (Seiferlein *et al*., 2007) and were more cytotoxic than HFB_1 *in vitro* (Abou-Karam *et al*., 2004; Seiferlein *et al*., 2007). The objectives of this study were to compare the NTD induction potential of HFB_1 and FB_1 and their ability to disrupt sphingolipid biosynthesis using the sensitive LM/Bc mouse model (Gelineau-van Waes *et al*., 2005). HFB_1 did not induce NTD at a dose level that was significantly higher than the reported lowest observed adverse effect level for NTD induction by FB_1 (Gelineau-van Waes *et al*., 2005). HFB_1 disrupted maternal sphingolipid metabolism, although much less potently than FB_1. Together, the findings indicate that, compared to FB_1, HFB_1 is not a significant risk factor for NTD.

MATERIALS AND METHODS

FB_1 and HFB_1. FB_1 was provided by Dr Robert Eppley, Center for Food Safety and Nutrition, U.S. Food and Drug Administration, College Park, MD. HFB_1 was prepared by hydrolysis of FB_1 in 2 N KOH (70°C overnight). After hydrolysis, the HFB_1 was cleaned up over a 35 cc C18 SepPak column (Waters, Milford, MA) (Voss *et al*., 1996) that was eluted in sequence with 30% aqueous acetonitrile, 70% acetonitrile, and actonitrile. The eluants were assayed by high-performance liquid chromatography (HPLC)-fluorescence detection after precolumn derivatization with *ortho*-phthalaldehyde (OPA) (Pierce, Rockford, IL) (Burns *et al*., 2008) to confirm elution of HFB_1 only in the 70% fraction. After removal of the solvent from the 70% fraction by rotoevaporation and freeze drying, the purity of the HFB_1 preparation (a slightly yellow oily material) was assessed by liquid chromatography-tandem electrospray ionization mass spectrometry as described in Zitomer *et al*. (2008).

Dosing solutions. The high-dose solution (2 mg/ml) was prepared by dissolving the HFB_1 preparation in 50 ml hypotonic (0.3%) saline, which also served as the dosing vehicle. HFB_1 concentration of the high-dose solution was determined by HPLC as described above (Burns *et al*., 2008), with quantification by comparison to an external standard of HFB_1 (generously provided by M. Busman, United States Department of Agriculture (USDA), Agricultural Research Service (ARS), Peoria, IL). The remaining dosing solutions (0.25, 0.5, and 1 mg/ml) were prepared by diluting aliquots of the high-dose solution with vehicle. The dosing solutions were sterile filtered (Millex-GV 0.22 μm filters; Millipore, Bedford, MA) into autoclaved bottles affixed with rubber caps and stored refrigerated. The dosing solution for the positive control group, 1 mg/ml FB_1 (determined gravimetrically), was prepared in the same manner.

Animals. The experimental protocol was approved by Institutional Animal Care and Use Committee, Richard B. Russell Agricultural Research Center, USDA, ARS. Acclimated male and female mice of the inbred LM/Bc strain (Gelineau-van Waes *et al*., 2005) were individually housed (except during mating) in stainless steel wire-mesh cages kept in an environmentally controlled room with a 12-h light/dark cycle. The animals had free access to feed (2019 Global Diet; Tecklad, Madison, WI) and fresh tap water.

Experimental design. Mating pairs were randomly selected and cohabited in the males' cages. Females were examined each morning, and the first day in which a vaginal plug was observed was designated embryonic day (E)0. On E0, the females were weighed and assigned to one of six groups by stratified randomization. They were observed daily and weighed again on E6, E9, E15, and before necropsy.

The dams were dosed in the mornings of E7 and E8 with 0 (vehicle control), 2.5, 5.0, 10, or 20 mg/kg body weight (bw) HFB_1 administered by ip injection at a volume rate of 10 ml/kg bw. The positive control group was treated with 10 mg/kg bw FB_1 as described. One half of the females were euthanized (CO_2) on E9 and the remainder on E16. At necropsy, the internal organs were examined and the maternal liver and kidneys weighed. Implantation sites were counted on E9. Gravid uteri were weighed on E16 and their contents examined (see below). Specimens of maternal liver were fixed in 10% neutral-buffered formalin at both E9 and E16. Additional liver samples were frozen (−80°C) for sphingolipid analyses.

Uteri from dams killed on E16 were excised and examined to determine the number of total implantation sites, early resorptions, late fetal deaths, and viable fetuses. Fetuses were excised, examined for NTD, and weighed. The livers and placenta from two fetuses per litter were collected, weighed, and stored frozen (−80°C) for biochemical evaluations.

Histopathology. The livers of five dams killed at E9 and five killed at E16 from the vehicle control, high-dose HFB_1 (20 mg/kg bw), and positive control (10 mg/kg bw FB_1) groups were processed, stained with hematoxylin and eosin, and microscopically examined.

Sphingolipid analysis. Free sphingoid bases and sphingoid base 1-phosphates were quantified by liquid chromatography-mass spectrometry

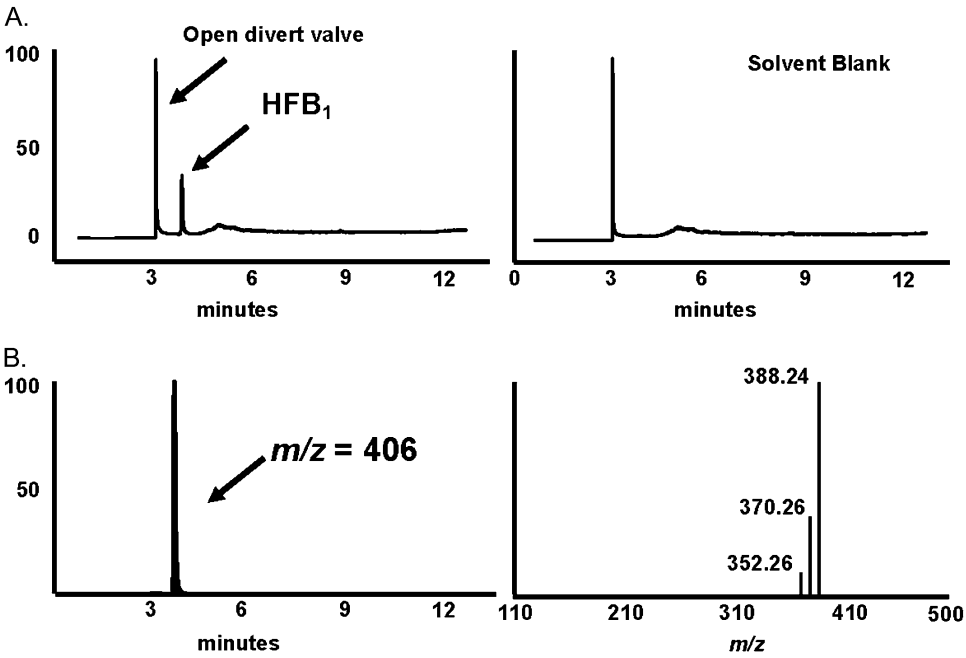

FIG. 2. Mass spectrometric confirmation of the total conversion of FB_1 to HFB_1. (A) Total ion chromatogram showing a single peak corresponding to HFB_1 ($m/z = 406$) in the preparation used to make the dosing solutions. No FB_1 ($m/z = 722$) was found therein. (B) Chromatogram of the single peak at $m/z = 406$ obtained by mass spectrometry/mass spectrometry showed the same fragmentation pattern as standard HFB_1. y-axes indicate relative abundance.

(LC-MS) as described by Zitomer *et al.* (2009). Complex sphingolipids (CSL) were extracted and acid hydrolyzed as described in Riley *et al.* (2000) and the sphingoid base backbones quantified by LC-MS (Zitomer *et al.*, 2009).

Free sphinganine (Sa) concentrations in maternal liver were quantified for five dams per group killed on E9. More detailed sphingolipid profiles were determined at E9 and E16 for randomly selected females from the vehicle control, high-dose HFB_1, and the positive control groups. The more extensive profiles included the concentrations of free Sa, sphingosine (So), sphingoid base 1-phosphates (Sa 1-P and So 1-P), 1-deoxy sphinganine (1-deoxy Sa) as well as CSL in maternal liver ($n = 5$ per group), fetal liver ($n = 4$–5 per group), and placentae ($n = 5$ per group) from the vehicle control, 20 mg/kg bw HFB_1, and 10 mg/kg bw FB_1 groups. The concentration of a novel compound tentatively identified as 1-deoxy sphingosine (1-deoxy So) (see "Discussion" section) was also estimated.

Statistics. Statistical analyses were done using Sigma Stat (Systat Software, San Jose, CA). Parametric data were analyzed by one-way ANOVA followed by Duncan's test to identify differences among groups. Non-parametric data were analyzed using the Kruskal-Wallis rank sum test followed by Tukey's test or, for sphingolipid profile results, by ANOVA after $\log_{10}$ transformation (Riley and Voss, 2006). Tests were two tailed, and significance was judged at $p < 0.05$.

RESULTS

HFB_1 Preparation

No FB_1 was detected in any of the cleanup fractions. HFB_1 eluted exclusively in 70% acetonitrile, and the HPLC chromatogram of this fraction did not reveal any other significant OPA-positive peaks (data not shown). LC-MS (total ion chromatogram, scan range = 100–2000 *m/z*, Fig. 2A) of this fraction revealed a single peak having the same retention time and *m/z* (406) as standard HFB_1. The material exhibited the same mass spectra as the HFB_1 standard when evaluated by mass spectrometry/mass spectrometry (Fig. 2B).

Maternal Clinical, Necropsy, and Histopathology

Neither HFB_1 nor FB_1 had any effect on the general appearance of the animals. No differences in maternal body weight between the vehicle control and other groups were found, although the weight of the dams given 10 mg/kg bw FB_1 (35.3 ± 2.6 [SD] g) was 8–12% lower than that of the other groups (38.4 ± 3.9 to 39.9 ± 1.6 g) on E15. Weight gains, E6 through E15, of the groups given HFB_1 ranged from 6.7 ± 2.8 (10 mg/kg bw) to 7.9 ± 2.2 g (20 mg/kg bw) and did not differ from the vehicle controls. Weight gain of the positive control group (4.0 ± 2.0 g) during gestation was significantly decreased compared to all groups (7.2 ± 1.1 to 7.9 ± 2.2) except the one given 10 mg/kg bw HFB_1 (6.7 ± 2.8).

Except for an increase in relative liver weight of the dams given 10 mg/kg bw FB_1 (6.7 ± 0.68% bw compared to 5.7 ± 2.1% bw in the vehicle controls), no differences in absolute or relative organ weights were found at E16. This difference reflected the slightly lower (~8–12% lower than other groups, not significantly different) body weight of the FB_1-treated dams.

No liver lesions attributable to HFB_1 were found at either E9 or E16. In contrast, 10 mg/kg bw FB_1 caused overt hepatic lesions in all dams examined on E9 that were consistent with

TABLE 1
Reproductive and Litter Data of Dams Given 0–20 mg/kg bw HFB_1 or 10 mg/kg bw FB_1 by ip Injection on E7 and E8[a]

Treatment	Total implants	Early resorptions	Late fetal deaths	Viable fetuses	Postimplant death index	Weights		Litters with NTD[d]
						Fetuses[b] (g)	Placentae[c] (mg)	
HFB_1 mg/kg bw								
0 (vehicle control) ($n = 8$ litters)	11.2 (1.0)	1.1[e,f] (1.0)	0.3[e] (0.5)	9.9[e] (1.6)	12.2[e] (11.6)	0.55[e] (0.05)	124.3[e] (10.2)	0[e]
2.5 ($n = 8$ litters)	11.8 (2.1)	1.3[e,f] (1.3)	0.5[e] (0.5)	10.0[e] (2.0)	14.5[e] (11.2)	0.54[e] (0.06)	112.9[e,f] (12.1)	0[e]
5.0 ($n = 8$ litters)	11.0 (1.9)	0.5[e] (0.8)	0.5[e] (0.8)	10.0[e] (1.1)	7.9[e] (9.4)	0.47[e] (0.09)	118.5[e,f] (17.2)	0[e]
10 ($n = 9$ litters)	9.9 (3.9)	0.9[e,f] (1.1)	0.4[e] (0.7)	8.6[e,f] (3.6)	12.6[e] (12.4)	0.54[e] (0.12)	123.7[e] (15.0)	0[e]
20 ($n = 9$ litters)	11.0 (1.9)	1.7[e,f] (2.0)	0.7[e] (1.0)	8.7[e,f] (3.5)	23.8[e,f] (25.5)	0.53[e] (0.07)	122.4[e] (15.9)	0[e]
FB_1 mg/kg bw								
10 (positive control) ($n = 10$ litters)	11.5 (2.1)	4.6[f] (3.0)	3.0[f] (0.9)	3.9[f] (2.1)	66.2[f] (18.4)	0.33[f] (0.07)	92.4[f] (14.1)	10[f] [4.7]

Note. Values in columns not sharing superscript (e,f) are significantly different, $p < 0.05$.
[a]E0 is defined as the day on which a vaginal plug was first observed; dams were euthanized and litters examined on E16, 1 week after the last dose.
[b]Value is the average of the mean (SD) fetus weights for each litter.
[c]Value is the average mean (SD) of two randomly selected placentae/litter.
[d]Value indicates the number of litters having at least one NTD-positive fetus; number in brackets indicates the average number of affected fetuses/litter.

the effects of FB_1 (Bolger *et al*., 2001; Gelineau-van Waes *et al*., 2009; Voss *et al*., 2007b) and judged to be moderately severe. Specific features included scattered hepatocellular apoptosis, less frequently observed foci of necrosis involving single to a few hepatocytes, increased numbers of mitotic figures, and variability in cell and nucleus size. The hepatic lesions were readily reversible. The only evidence suggestive of FB_1 exposure remaining at E16 was a minimal number of isolated small foci of necrotic cells and, subjectively, slightly more mitotic figures than seen in the other groups.

Reproductive Data

No differences in the number of total implants/dam were found at E9 or E16. Counts ranged from 9.0 (5.0 mg/kg bw HFB_1) to 12.4 (20 mg/kg bw HFB_1) at E9 (data not shown) and from 9.9 (10 mg/kg bw HFB_1) to 11.8 (2.5 mg/kg bw HFB_1) at E16 (Table 1). No statistically significant differences were found among the vehicle control and HFB_1-treated groups at E16 for the following (Table 1): early resorptions/litter, late deaths/litter, or live fetuses/litter. HFB_1 also had no significant effect on (live) fetal or placental weight. In contrast, 10 mg/kg bw FB_1 was fetotoxic (Table 1) as demonstrated by the significant decrease in viable fetuses (group mean = 3.9 compared to 8.6–10.0 in the other groups) and increase in late fetal deaths (group mean = 3.0 compared to 0.3–0.7 in the other groups) found in the positive control litters. The number of early resorptions/positive control litter (mean = 4.6) was also higher than in the other groups (means ranged from 0.5 to 1.7), although a statistical difference from the vehicle control group was not demonstrated. Fetal and placental weights of the positive control group were also significantly decreased: their respective values were 40 and 26% less than those of the vehicle control group (Table 1).

No fetuses with NTD were found in the vehicle control or any HFB_1-treated group. At least one NTD-affected fetus was found in all litters from dams treated with 10 mg/kg bw FB_1, and in all cases, the anomaly presented as exencephaly. NTD were found not only in apparently viable fetuses but were also recognizable in some of the dead fetuses that had not yet undergone advanced autolysis. As a result, the number of NTD/positive control litter (mean = 4.7) was higher than that of viable fetuses/litter (mean = 3.9).

Tissue Sphingolipids

Multiple changes in the tissue sphingolipid profiles were found in the high-dose (20 mg/kg bw) HFB_1 group and, to a significantly greater extent, in the positive control (10 mg/kg bw) FB_1-treated group. Most effects were reversible, that is, with a few exceptions, the differences between the HFB_1- or FB_1-treated and vehicle control groups were more pronounced 1 day after the last dose (E9) than a week later (E16).

Dams

A dose-dependent increase in Sa (picomole per gram) of maternal liver was found in the HFB_1-treated groups on E9 (Fig. 3), and as a result, Sa in the high-dose group was significantly greater than in the vehicle control group. Liver Sa of dams treated with FB_1 (10 mg/kg bw) was markedly (Table 2) elevated and significantly increased compared to all other groups, including the high-dose HFB_1 group. Concentrations of 1-deoxy Sa were slightly higher (1.9-fold) in the high-dose HFB_1 group than in the vehicle controls on E9 and markedly increased in the FB_1-treated dams at that time. Concentrations of both Sa and 1-deoxy Sa in the high-dose HFB_1-treated and FB_1-treated groups decreased sharply after

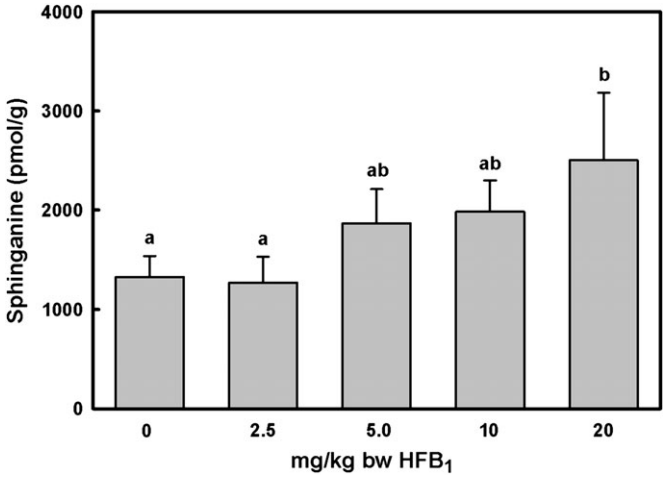

FIG. 3. Concentrations of sphinganine (Sa) in maternal liver were increased in a dose-dependent manner on E9. The dams were given up to 20 mg/kg bw HFB_1 on E7 and E8 by ip injection and euthanized 24 h later for hepatic sphingolipid analysis. Liver sphinganine concentrations of dams given FB_1 by the same protocol were significantly higher than those given the high dose of HFB_1 (Table 2). Values indicate the group mean; error bars denote SD; $n = 5$ per group.

E9 and, with the exception of 1-deoxy Sa in positive control dams, no significant differences were found at E16 (Table 2). Concentrations of Sa 1-P were increased (63-fold relative to the vehicle controls) on E9 only in the FB_1-treated positive controls (Table 2). Thereafter, Sa 1-P concentrations in this group decreased so that the difference (significant at $p < 0.05$) between this group and the vehicle controls was 2.7-fold by E16. Sa 1-P was also significantly elevated (about threefold) in the high-dose HFB_1-treated group but only at E16.

On E9, So concentration was slightly elevated in both the high-dose HFB_1-treated and the FB_1-treated groups, although statistical significance was demonstrated only for the latter. No differences in So concentration were found at E16 and no differences in So 1-P levels were found at either time. Concentrations of the putative 1-deoxy So were significantly elevated at E9 in the high-dose HFB_1-treated group and to a significantly greater extent in the positive control group. Amounts in the vehicle control group at E9 and E16, as well as in the high-dose HFB_1 and positive control groups at E16, were negligible

The treatments also affected maternal CSL, although the pattern of response found in the HFB_1- and FB_1-exposed groups differed (Table 3). FB_1 treatment significantly decreased all four classes of CSL examined at E9. Like other sphingolipid changes, the effect was reversible: no significant differences were found between the vehicle and positive control groups on E16. In contrast, in dams treated with 20 mg/kg bw HFB_1, CSL incorporating So or 1-deoxy Sa were not decreased and CSL incorporating Sa as well as those incorporating 1-deoxy So were significantly increased on E9. Concentrations of the latter two CSL classes decreased after E9 so that there were no significant differences at E16.

Placentae

Placentae were only collected for analysis on E16. While no differences between the 20 mg/kg bw HFB_1-treated and the vehicle control groups were found (data not shown), there were several differences between the positive control and the other two groups. These included the following: increased Sa (9.2 ± 2.3 nmol/g compared to a range of 6.0 ± 1.1 to 6.2 ± 1.0 nmol/g in the other groups), increased 1-deoxy Sa (9.0 ± 2.9 nmol/g compared to 1.4 ± 0.2 to 2.5 ± 1.9 nmol/g in the other groups), and increased 1-deoxy So (8.7 ± 5.1 nmol/g compared to

TABLE 2

Liver Concentrations of Selected Sphingoid Bases and Their Metabolites in Pregnant LM/Bc Mice 1 Day (E9) and 8 Days (E16) after Treatment with 0 (Vehicle Control Group) or 20 mg/kg bw HFB_1 or 10 mg/kg bw FB_1 (Positive Control Group) on E7 and E8[a]

	Free sphingoid bases and metabolites (pmol/g)[b]					
Day and treatment	Sa	1-deoxy Sa	Sa 1-P	So	1-deoxy So[c]	So 1-P
E9						
Vehicle	1330[d] (210)	287[d] (72)	8[d] (1)	2406[d] (466)	ND	127 (37)
HFB_1 20 mg/kg bw	2501[e] (683)	509[e] (135)	12[d] (5)	5834[d,e] (4621)	28[d] (8)	150 (33)
FB_1 10 mg/kg bw	73,388[f] (16811)	4105[f] (649)	504[e] (85)	7616[e] (1102)	246[e] (57)	162 (53)
E16						
Vehicle	1843 (181)	214[d] (31)	10[d] (5)	2203 (778)	ND	92 (20)
HFB_1 20 mg/kg bw	1482 (197)	197[d] (23)	32[e] (14)	1882 (192)	ND	124 (33)
FB_1 10 mg/kg bw	3487 (1116)	343[e] (77)	27[e] (8)	2831 (778)	ND	99 (25)

Note. 1-Deoxy-Sa, 1-deoxysphinganine; 1-Deoxy So, 1-deoxy sphingosine (putative); ND, not detected; Sa 1-P, sphinganine 1-phosphate; So 1-P, sphingosine 1-phosphate.

Values in columns at each time (E9 or E16) not sharing superscript (d,e,f) are significantly different, $p < 0.05$.

[a]The first day in which mating was confirmed by the presence of a vaginal plug is defined as E0.

[b]Values are the group mean; the SD is given in parentheses, $n = 4$–5 per group.

[c]Due to unavailability of a standard, provisional identification of this compound as 1-deoxy So is based on mass spectral properties and tissue concentration data (see text).

TABLE 3
Liver Concentrations of Selected Complex Sphingolipids in Pregnant LM/Bc Mice 1 Day (E9) and 8 Days (E16) after Treatment with 0 (Vehicle Control Group) or 20 mg/kg bw HFB_1 or 10 mg/kg bw FB_1 (Positive Control Group) on E7 and E8[a]

	Complex sphingolipid (pmol/g)[b]			
Day and treatment	CSL-Sa	CSL-1-deoxy Sa	CSL-So	CSL-1-deoxy So[c]
E9				
Vehicle	4974[d] (665)	999[e] (262)	115,665[e] (21,361)	216[e] (37)
HFB_1 20 mg/kg bw	7744[e] (558)	1209[e] (140)	135,692[e] (24,336)	330[d] (49)
FB_1 10 mg/kg bw	535[f] (776)	149[d] (221)	20,136[d] (8184)	39[f] (59)
E16				
Vehicle	3985 (783)	1071 (344)	137,003 (28,071)	165[d,e] (37)
HFB_1 20 mg/kg bw	3830 (246)	1030 (465)	132,380 (34,397)	136[d] (39)
FB_1 10 mg/kg bw	4966 (706)	1200 (516)	157,136 (50,528)	235[e] (57)

Note. CSL-1-deoxy Sa, complex sphingolipids incorporating 1-deoxy sphinganine; CSL-1-deoxy So, complex sphingolipids incorporating (putative) 1-deoxy sphingosine; CSL-Sa, complex sphingolipids incorporating sphinganine; CSL-So, complex sphingolipids incorporating sphingosine; ND, not detected.

Values in columns at each time (E9 or E16) not sharing superscript (d,e,f) are significantly different, $p < 0.05$.

[a]The first day in which mating was confirmed by the presence of a vaginal plug is defined as E0.

[b]Values are the group mean; the SD is given in parentheses, $n = 4$–5 per group.

[c]Due to unavailability of a standard, provisional identification of the sphingoid base 1-deoxy So is based on mass spectral properties and tissue concentration data (see text).

1.3 ± 0.4 to 2.7 ± 1.7 nmol/g in the other groups). The 1-phosphate metabolites of Sa and So were both decreased in the placentae of dams given 10 mg/kg FB_1: the respective results for Sa 1-P and So 1-P were 64 ± 13 and 238 ± 61 pmol/g. Sa 1-P averaged 108 ± 19 to 121 ± 42 and So 1-P averaged 304 ± 58 to 352 ± 69 pmol/g in the vehicle control and HFB_1-treated groups, respectively. No statistically significant differences in placental CSL were found. However, CSL incorporating Sa or So in the 20 mg/kg bw HFB_1 and 10 mg/kg bw FB_1 groups averaged 20–30% less than their corresponding vehicle control values. Conversely, CSL incorporating the compound tentatively identified as 1-deoxy So averaged 27 and 65% higher in the groups treated with 20 mg/kg bw HFB_1 or 10 mg/kg bw FB_1, respectively. A similar pattern was apparent for CSL incorporating Sa.

Fetal Liver

There was a slight increase in 1-deoxy Sa and a slight decrease in So 1-P in fetal livers of the positive control group at E16 (data not shown). No other significant differences in free sphingoid base or complex sphingolipid concentrations were found.

DISCUSSION

The etiology of NTD is complex and involves genetic, environmental, and nutritional components (Gelineau-van Waes *et al*., 2009; Green and Copp, 2005). Epidemiological (Missmer *et al*., 2006) and experimental (Gelineau-van Waes *et al*., 2005, 2009) evidence suggest that fumonisins are a potential NTD risk factor in populations dependent upon maize as a diet staple. Consumption of tortillas, especially homemade tortillas likely containing fumonisins, during the first trimester of pregnancy was identified as an NTD risk factor (Missmer *et al*., 2006).

Tortillas are made from masa produced by the alkaline cooking process known as nixtamalization. The process differs somewhat among households and commercial operations so that, depending on cooking conditions, recipe, and quality of the maize, the amount of fumonisins remaining in the masa is variable (De La Campa *et al*., 2004). Surveys for hydrolyzed fumonisins in nixtamalized products are limited but suggest that, while concentrations are generally low, their contribution to total fumonisin content can be significant. HFB_1 constituted 25% (micromolar basis) of the FB_1 analogs in masa, made from low-quality maize (Voss *et al*., 2001). Equimolar amounts of HFB_1 and FB_1 were found in tortillas made using the process as practiced in Guatemalan households (Palencia *et al*., 2003) and the micromolar ratio of HFB_1:FB_1 in a survey of nixtamalized products from Sonora, Mexico, averaged from 0.37 to 0.62 (Cortez-Rocha *et al*., 2005). HFB_1:FB_1 ratios in alkaline-cooked snacks from the German market ranged from 0.2 to 3.7 (Seefelder *et al*., 2001). Dvorak *et al*. (2008) found HFB_1 in 22 of 38 tortilla samples and micromolar ratios of HFB_1:$FB_1 \geq 0.5$ were found in 22% of the positive samples. Ratios from 0.94 to 1.48 were found in masa, tortillas, and tortilla chips prepared in the laboratory from contaminated corn (Dombrink-Kurtzman *et al*., 2000). Together, these surveys indicate that pregnant women, especially those consuming large amounts of alkaline-cooked maize, are exposed at least on occasion to HFB_1.

The LM/Bc mouse was selected as the test strain because, using the ip dosing protocol of Gelineau-van Waes *et al*. (2005, 2009), it is the most sensitive animal model for NTD induction

by FB_1. While the no observed adverse effect level in this model has not been rigorously established, the experiments of Gelineau-van Waes *et al.* (2005) suggest that it is below 5 mg/kg bw. At the latter dose, they found that 40% of the litters (at least one fetus with NTD found in the litter) and 5% of the total fetuses were NTD positive. Both variables increased in a dose-related manner so that 100% of the litters were affected at $\geq$ 15 mg/kg bw and the maximum incidence of affected fetuses, about 80%, was found at the highest dose tested, 20 mg/kg bw FB_1. In this experiment, HFB_1 was clearly less potent than FB_1 as it did not elicit NTD when given at 20 mg/kg bw, a dose that equals 49 μmol/kg bw. This is 3.5-fold higher than the positive control dose of FB_1 (10 mg/kg bw = 14 μmol/kg bw) and sevenfold higher than 5 mg/kg bw FB_1 (7 μmol/kg bw), the lowest dose tested by Gelineau-van Waes *et al.* (2005).

A lowest adverse affect level for HFB_1-induced fetotoxicity was not established and some data suggest that overt fetotoxicity would appear at higher doses. First, late deaths were twice as frequent in high dose as in the vehicle control litters. The number of live fetuses/litter at 10 or 20 mg/kg bw tended to be lower than in the other HFB_1-treated and vehicle control groups. While not significantly different from those groups, the late fetal death rate in the high-dose group was also not significantly different from the positive control group. Accordingly, the postimplant death index of the high-dose group was twice that of the vehicle controls and was not significantly different from the positive control group.

FB_1 was moderately hepatotoxic to the dams at E9. As previously observed in rats (Voss *et al.*, 1998), hepatotoxicity was reversible as 1 week later, at E16, only a few isolated apoptotic, necrotic, or mitotic cells were present. HFB_1 was in contrast not hepatotoxic as judged by morphological criteria, a finding that is in agreement with Howard *et al.* (2002), who found microscopic lesions in female mice fed $\geq$ 50 ppm FB_1 for 4 weeks, but not in groups fed micromolar equivalents of HFB_1. Similarly, oral doses of 120 mg/kg bw HFB_1 were not toxic to pregnant rats (Collins *et al.*, 2006), whereas in a separate study, $\geq$ 25 mg/kg FB_1 bw caused significant nephrotoxicity and heptotoxicity (Collins *et al.*, 1998).

Fumonisins disrupt sphingolipid metabolism by inhibiting ceramide synthases, enzymes catalyzing the *de novo* synthesis of ceramide from fatty acids and sphingoid bases (Riley *et al.*, 2001; Zitomer *et al.*, 2009). The ensuing disruption of sphingolipid-dependent signaling and physiological processes is the key event mediating fumonisin's diverse biological effects (Bolger *et al.*, 2001; Gelineau-van Waes *et al.*, 2009; Riley *et al.*, 2001; Voss *et al.*, 2007b), which include inhibition of folate utilization (see below) (Gelineau-van Waes *et al.*, 2005; Sadler *et al.*, 2002; Stevens and Tang, 1997). HFB_1 disrupts ceramide synthase *in vitro* 3–10 times less potently than FB_1 (Norred *et al.*, 1997; Seefelder *et al.*, 2002). Nixtamalized *Fusarium* culture materials increased kidney and liver Sa and So concentrations when fed to rats (Burns *et al.*, 2008; Voss *et al.*, 1998), although the possibility remains that these effects were mediated by residual or "hidden" FB_1 (matrix-bound forms not detected by HPLC) (Kim *et al.*, 2003; Park *et al.*, 2004) remaining in the nixtamalized preparations rather than HFB_1. In this regard, it is noteworthy that liver Sa of female mice fed diets amended with 143 μmol/kg HFB_1 (150 ppm FB_1 equivalents) was unaffected (Howard *et al.*, 2002).

The results of this experiment are to our knowledge the first unequivocal demonstration of sphingolipid metabolism disruption *in vivo* by HFB_1. HFB_1 is acylated by ceramide synthase *in vitro* and by rats *in vivo* (Seiferlein *et al.*, 2007). The N-acylated metabolites are structural analogs of ceramide, inhibited ceramide synthase, and were more potent cytotoxins than HFB_1 when tested in HT29 human colorectal (Seiferlein *et al.*, 2007) or other mammalian cell lines (Abou-Karam *et al.*, 2004). Studies are needed to determine if the metabolic effects in the HFB_1-treated mice were mediated directly by HFB_1, indirectly by N-acyl metabolites, or both. Regardless, HFB_1 was a significantly less potent modulator of sphingolipid metabolism than FB_1.

The identity of a novel metabolite previously observed to accumulate in cells exposed to FB_1 has been recently identified as the novel sphingoid base, 1-deoxy Sa that, like Sa, was cytotoxic to LLC-PK_1 and DU-145 cells (Zitomer *et al.*, 2009). The increase, albeit transient, of 1-deoxy Sa in liver of the dams dosed with 10 mg/kg bw FB_1 or 20 mg/kg bw HFB_1 in this study independently confirms that it accumulates in the liver of fumonisin-exposed mice as reported by Zitomer *et al.* (2009). Liver concentration of another compound tentatively identified as 1-deoxy So was also transiently elevated by both fumonisins. Structural confirmation was not possible as a standard is currently unavailable. Identification as 1-deoxy So was inferred from the mass ion (m/z = 284.2) and the similarity of the compound's mass spectra to that of 1-deoxy Sa. The concurrent elevation of putative 1-deoxy So and depletion of its CSL by FB_1 inhibition of ceramide synthase is further evidence that the compound is a sphingoid base. Structural confirmation and *in vivo* toxicological significance of the putative 1-deoxy So remains to be determined.

There is evidence both against and for fumonisins being NTD risk factors. FB_1 was not teratogenic to rabbits (LaBorde *et al.*, 1997), rats (Collins *et al.*, 1998), or CD1 mice (Reddy *et al.*, 1996) when given orally at maternally toxic doses. However, folate deficiency increases NTD risk (Green and Copp, 2005; reviewed by Gelineau-van Waes *et al.*, 2009; Missmer *et al.*, 2006) and FB_1 inhibited 5-tetramethylfolate uptake by CaCo2 cells through a mechanism involving complex sphingolipid depletion (Stevens and Tang, 1997). Accordingly, folate supplementation partially protected cultured mouse embryos from NTD induction by FB_1 (Sadler *et al.*, 2002), while both folate and, more effectively, the complex sphingolipid GM_1 protected LM/Bc mouse embryos *in utero* (Gelineau-van Waes *et al.*, 2005). Our results are in conformity with the latter findings insofar as CSL were

depleted only on E9 and only by the treatment (20 mg/kg bw FB_1) eliciting NTD. It can be supposed that a threshold for NTD induction by fumonisins, perhaps including hydrolyzed fumonisins, would be lower in animals that have a preexisting vulnerability because of nutritional deficiencies, environmental conditions, or genetic predisposition (Gelineau-van Waes *et al*., 2009). Other consequences of ceramide synthase inhibition potentially contributing to NTD induction should also be considered. These include imbalances in pro-apoptotic (Sa) and "pro-survival" (So 1-phosphate) metabolites, inappropriate intercellular or intracellular signal transduction by So (or Sa) 1-phosphates or cytokines, as well as nonspecific events such as oxidative damage (Bolger *et al*., 2001; Gelineau-van Waes *et al*., 2009).

In summary, HFB_1 did not induce NTD when tested using the sensitive LM/Bc mouse model at 20 mg/kg bw, a dose which disrupted sphingolipid metabolism, albeit slightly. At the lesser dose level of 10 mg/kg bw, FB_1 markedly disrupted sphingolipid metabolism, caused moderate apoptotic liver lesions, and induced NTD. The results provide further evidence that hydrolyzed fumonisins are less toxic *in vivo* than their corresponding parent fumonisins and, specifically, that they are not a significant risk factor for NTD.

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