Toxicity of *Fusarium proliferatum*-Fermented Nixtamalized Corn-Based Diets Fed to Rats: Effect of Nutritional Status

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Male F344/N rats were fed corn, nixtamalized corn, *Fusarium proliferatum*-fermented (FP) corn to provide 50 mg/kg fumonisin B_1 (FB_1), or nixtamalized FP corn (NFP), with and without supplemented nutrients. Nixtamalization of FP corn almost completely hydrolyzed FB_1 and produced 7–10 mg/kg hydrolyzed fumonisin (HFB_1). Relative liver weight was significantly increased in nutrient-supplemented rats fed FP or NFP corn. Plasma cholesterol increased in rats fed FP or NFP corn. Plasma glutamate-pyruvate transaminase was elevated in all rats fed FP corn and in nutrient-supplemented rats fed NFP corn. All rats fed FP corn and nutrient-supplemented rats fed NFP corn exhibited hepatocellular adenomas, whereas unsupplemented rats fed NFP corn had few adenomas. HFB_1 and other uncharacterized *F. proliferatum* mycotoxins produced by nixtamalization were less toxic than were toxicants, including FB_1, present in FP corn. Improvement in nutritional status due to nixtamalization stimulated NFP corn toxicity.

INTRODUCTION

Fumonisins (FB) are recently discovered carcinogenic mycotoxins (Bezuidenhout et al., 1988; Gelderblom et al., 1991) produced by the commonly occurring corn fungi *Fusarium moniliforme* and *Fusarium proliferatum*. *F. moniliforme*-inoculated corn was hepatocarcinogenic in rats (Wilson et al., 1985). Isolated fumonisins were hepatocarcinogenic in rats (Gelderblom et al., 1988, 1991) and caused equine leukoencephalomalacia (Wilson et al., 1992; Kellerman et al., 1990) and porcine pulmonary edema (Harrison et al., 1990). The occurrence of *F. moniliforme* containing FB was associated with human esophageal cancer in southern Africa (Marasas et al., 1988; Sydenham et al., 1990).

Median fumonisin concentrations usually in corn-based human foods and animal feeds range from 0.1 to 3 μg/g (Hopmans and Murphy, 1993; Murphy et al., 1993; Ross et al., 1991; Sydenham et al., 1991). Fifty milligrams per kilogram of fumonisin B_1 fed to rats from 18 to 24 months was carcinogenic (Gelderblom et al., 1991). However, a no-effect level for fumonisins has not been determined. Human exposure to fumonisins may pose a cancer risk in regions where corn is a dietary staple. Therefore, detoxification of fumonisin-contaminated corn may be necessary. Detoxification of fumonisin-contaminated corn was not accomplished by treatment with 2% ammonia at low pressure for 4 days, a process that successfully decontaminates aflatoxin-contaminated corn (Norred et al., 1991). Although the fumonisin content of ammoniated corn decreased, the hepatotoxicity of the ammoniated corn culture material remained the same as that of nonammoniated fumonisin-contaminated corn. This suggests that other forms of fumonisins or other mycotoxins in the corn can contribute to the toxicity of *F. moniliforme*-contaminated corn. Park et al. (1992) showed decreases in FB_1 levels with high pressure/ambient temperature and low pressure/high temperature ammonia as well as no increases in mutagenic potential due to these processes.

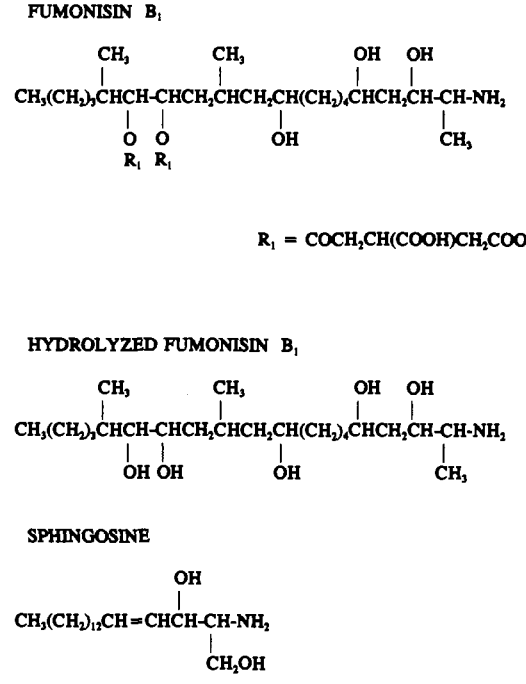

Figure 1. Structures of fumonisin B_1, hydrolyzed fumonisin B_1, and sphingosine.

However, toxicity in animals was not examined. Nixtamalization, the traditional treatment of corn with $Ca(OH)_2$ and heat, used to produce masa (tortilla flour), has been suggested to decontaminate fumonisin-contaminated corn because almost no fumonisin could be detected in corn products made with masa (Sydenham et al., 1991).

Fumonisins are unusual mycotoxins in that they are relatively water-soluble. The metabolism of fumonisins and their modes of action as carcinogens remain to be fully determined. Fumonisins have been shown to inhibit sphingosine synthesis (Wang et al., 1991) and may promote carcinogenesis by acting as indirect or direct antagonists to sphingosine's cytostatic action (Merrill, 1991), given the partial structural similarity between fumonisins and sphingosine (Figure 1).

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The dependence of fumonisin toxicity on nutritional status has not been determined, but early studies of fumonisin toxicity and carcinogenicity have examined the effects of feeding *F. moniliforme*-fermented corn (Wilson et al., 1985). Corn has relatively poor nutritional quality. It is deficient in protein and especially lacking in calcium, riboflavin, and lysine (U.S. Department of Agriculture, 1992). The action of many carcinogens has been shown to depend on nutritional status. For example, increasing total caloric intake (Pariza and Boutwell, 1987), fat intake (Carroll and Khor, 1975), or protein intake (Youngman and Campbell, 1991) have been shown to promote experimental carcinogenesis of several types. Protein (Youngman and Campbell, 1991), calorie (Pariza and Boutwell, 1987), or linoleate (Ip et al., 1985) restriction suppresses carcinogenesis. Because nixtamalization improves the nutritive value of corn (Carter and Carpenter, 1982), the hydrolysis of the toxicant may interact with nutritional status to permit or enhance carcinogenic effects of *F. moniliforme* or *F. proliferatum* corn.

This study was performed to determine the effects of nixtamalization and/or nutrient supplementation of *F. proliferatum*-fermented (FP) corn, containing 50 mg/kg fumonisin B_1, on diethylnitrosamine-initiated hepatocarcinogenesis in male F344/N rats. This strain of *Fusarium* was chosen because it is a predominant producer of fumonisin B_1.

MATERIALS AND METHODS

Chemicals. All chemicals were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise specified.

Animals and Diets. Ten-day-old male F344/N rats were injected intraperitoneally with diethylnitrosamine (15 mg/kg of body weight) in corn oil to initiate carcinogenesis. Eight groups of six rats were fed and provided free access to water for 4 weeks in a room with a 12-h light/dark cycle maintained at 22 °C and 50% humidity. The rats were randomly assigned to one of the following diets: ground corn containing 70 g of dextrose/kg of corn; nixtamalized ground corn containing 70 g of dextrose/kg of corn; corn or nixtamalized corn supplemented with nutrients (per kilogram of corn: casein, 60 g; thiamin, 0.002 g; riboflavin, 0.005 g; pyridoxine, 0.002 g; niacin, 0.010 g; folacin, 0.002 g; $CaCO_3$, 1.6 g; NaCl, 1.0 g; methionine, 3.0 g; choline, 2.0 g) to approximate an AIN-76 diet (American Institute of Nutrition, 1980) based on the estimated nutrient content of whole-ground unbolted dry corn meal (U.S. Department of Agriculture, 1992). Four of the eight diets were formulated with corn fermented with *F. proliferatum* strain M5991, a strain producing predominantly FB_1 (donated by Dr. Paul Nelson, Pennsylvania State University, University Park, PA), to produce a concentration of approximately 50 mg of fumonisin B_1 (FB_1)/kg of diet.

Corn for fermentation was sterilized by autoclaving 1-kg batches for 40 min at 132 °C. The corn (Variety 636, Dekalb Co.) was determined to be aflatoxin-free by P. F. Ross (Osheim and Ross, 1982), National Veterinary Services Laboratory, Ames, IA. Each batch of sterile corn was inoculated with *F. proliferatum* culture material reconstituted in sterile phosphate buffered saline according to the procedure of Ross et al. (1990). The FP corn was cultured for 3 weeks at 22 °C in the dark, when the corn was frozen and analyzed for FB content (Ross et al., 1991). This fermented corn was dried and mixed with fumonisin-free corn to give a final concentration of 50 mg/kg FB_1 in the diet.

Clean ground corn or FP corn containing 50 mg/kg FB_1 was nixtamalized by heating 300 g of corn with 900 mL water containing 1.2% $Ca(OH)_2$ at 80–100 °C for 1 h. The mixture was steeped overnight. The nixtamalized [$Ca(OH)_2$-treated] corn (NFP) was washed with approximately 3 volumes of water and air-dried before nutrient supplementation. Each diet was analyzed in duplicate for FB_1 and hydrolyzed FB_1 (HFB_1) content by analytical HPLC (Wilson et al., 1990).

Animals were weighed and food intakes measured weekly. After euthanasia by CO_2 anesthesia and decapitation, 5 mL of rat blood was collected in tubes containing 0.25 mL of Na_2EDTA (40 mg/mL). Plasma was collected after centrifugation of blood for 15 min at 2000 rpm in an IEC low-speed floor-model centrifuge (International Equipment Co., Boston, MA) at room temperature. Liver weight was measured, and a portion of the largest lobe was fixed in formalin, embedded in paraffin, sectioned by microtome to a thickness of 10 μm, stained by standard methods with hematoxylin and eosin (Sheehan and Hrapchak, 1980), and examined microscopically.

Plasma was analyzed for glutamate-pyruvate transaminase (GPT) (Reitman and Frankel, 1957) and total cholesterol (Allain et al., 1981) by colorimetric diagnostic kits which included standards (Sigma). The analyses were performed with a variable-wavelength Gilford 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH). Cholesterol concentration and GPT activity were determined by extrapolation from standard curves.

All data were analyzed by one-way ANOVA ($p < 0.05$), and least significant differences between treatment means were determined by Duncan's multiple-range tests ($p < 0.05$), using the Statistical Analysis System (Cary, NC) on the Iowa State University mainframe computer.

Table I. Fumonisin B_1 and Hydrolyzed Fumoisin B_1 Contents of Experimental Diets

dietary treatment				
nixtamalized	nutrient-supplemented	*F. proliferatum*-fermented	FB_1, μg/g	HFB_1, μg/g
–	–	–	0.3 ± 0.0	ND[a]
–	–	+	48.0 ± 1.3	ND
–	+	–	0.2 ± 0.0	ND
–	+	+	45.2 ± 4.4	ND
+	–	–	0.1 ± 0.1	ND
+	–	+	0.0 ± 0.0	10.7 ± 0.4
+	+	–	0.2 ± 0.0	ND
+	+	+	0.8 ± 0.4	7.6 ± 0.2

[a] Not detected at a limit of detection of 25 ng/g. Duplicate samples of each diet were extracted, and each extract was analyzed twice by HPLC with fluorescence detection of the OPA derivative.

RESULTS

The *F. proliferatum*-fermented (FP) corn diets contained 45–48 mg/kg FB_1 and no HFB_1 (Table I). Very small amounts of FB_2 and FB_3 were found in the FP corn. Only 0.1–0.3 mg/kg FB_1 and no HFB_1 were detected in uninoculated corn diets. The nixtamalized corn diets fermented with *F. proliferatum* (NFP) contained 0–0.8 mg/kg FB_1 and 8–11 mg/kg HFB_1.

Body weights were greatest in rats fed nixtamalized nutrient-supplemented corn. Nutrient-supplemented rats had significantly greater body weights than did rats fed corn alone (Table II). Body weight was significantly suppressed in rats fed FP corn; however, body weight was most suppressed in rats fed unsupplemented unnixtamalized FP corn. Food intakes did not differ significantly among groups (Table II). Relative liver weight (liver weight as a percentage of body weight) was significantly increased by FP corn only in nutrient-supplemented rats, and especially in rats fed nixtamalized nutrient-supplemented corn (Table II). Rats fed unsupplemented FP corn did not show increased liver weight.

All rats fed FP corn had significantly greater plasma GPT activities than the control groups, with the exception of rats fed unsupplemented nixtamalized FP corn (Table II). In groups fed FP corn, plasma GPT was increased to a greater extent in rats fed nutrient-supplemented corn than in rats fed unsupplemented corn. Compared with groups fed control corn, plasma cholesterol was significantly greater in all groups fed FP corn. The greatest

Table II. Effects of Feeding *F. proliferatum*-Fermented, Nixtamalized, and/or Nutrient-Supplemented Corn on Body Weight, Food Intake, Relative Liver Weight, Plasma Glutamate-Pyruvate Transaminase, and Plasma Cholesterol

dietary treatment							
FP[a]	nutrient supplement[b]	nixtamal[c]	body wt,[d] g	food intake,[e] g	rel liver wt, %	plasma GPT,[f] SF/mL	plasma cholesterol, mmol/L
–	–	–	74 ± 5[c]	692 ± 82	3.9 ± 0.5[cd]	55 ± 9[c]	1.72 ± 0.17[c]
+	–	–	49 ± 6[e]	852 ± 182	3.7 ± 0.5[cd]	117 ± 31[b]	4.57 ± 0.61[a]
–	+	–	127 ± 10[b]	811 ± 48	4.0 ± 0.2[c]	57 ± 7[c]	2.14 ± 0.20[c]
+	+	–	75 ± 7[c]	681 ± 209	5.2 ± 0.7[b]	139 ± 26[a]	3.58 ± 0.79[b]
–	–	+	75 ± 5[c]	701 ± 59	3.6 ± 0.2[cd]	47 ± 8[c]	1.74 ± 0.34[c]
+	–	+	61 ± 4[d]	726 ± 280	3.3 ± 0.3[d]	67 ± 5[c]	4.00 ± 0.57[ab]
–	+	+	162 ± 18[a]	911 ± 87	4.0 ± 0.3[c]	51 ± 13[c]	1.68 ± 0.41[c]
+	+	+	119 ± 13[b]	784 ± 215	5.8 ± 0.7[a]	127 ± 18[ab]	4.61 ± 0.65[a]

[a] *F. proliferatum*-fermented corn. [b] Diets supplemented with adequate protein, vitamins, and minerals to match AIN-76 recommendations. [c] Nixtamalized corn. [d] Body weight gain over 30 days. [e] Food intake over 30 days. [f] Plasma glutamate-pyruvate transaminase in Sigma–Frankel units. Data are expressed as mean ± standard deviations. Values in a column bearing different superscripts are significantly different, $p < 0.05$.

Table III. Incidence of Hepatic Histopathology[a] of Rats Fed Control or *F. proliferatum*-Fermented Diets

dietary treatment				% incidence	
nixtamalized	nutrient-supplemented	*F. proliferatum*-fermented	*n*	hepatic adenomas	hepatic cholangiomas
±	±	–	24	0[a]	0
–	–	+	6	83[b]	33
–	+	+	6	100[b]	50
+	–	+	6	14[a]	33
+	+	+	6	67[b]	17

[a] The microscopic lesions were classified according to the descriptors of Eustis et al. (1990). Each group bearing a letter different from that of the control group is significantly different from the combined controls, as analyzed by chi-square tests using a two-way contingency plan, $p < 0.05$.

increases in plasma cholesterol occurred in rats fed unsupplemented FP corn and in rats fed nutrient-supplemented NFP corn (Table II).

Rats fed nixtamalized or unnixtamalized FP corn developed hepatocellular adenomas, clear cell and hyperplastic foci, and cholangiomas, whereas rats fed clean corn did not (Table III; Figure 2). The photomicrographs are representative of the hepatic histopathological findings from each of the groups fed FP corn. The rats fed FP corn had diffuse mild to severe hyperplastic nodules (Figure 2A). The supplemented FP corn-fed group showed diffuse severe hyperplastic nodules (Figure 2B). The group fed NFP corn showed multiple clear cell foci (Figure 2C), whereas rats fed supplemented NFP corn had severe diffuse hyperplastic nodules (Figure 2D). The rats fed corn without nutrient supplementation showed fatty infiltration of the liver. Rats fed nixtamalized FP corn without nutrient supplementation developed less severe hepatic lesions than did the other groups fed FP diets. The rats fed unsupplemented FP corn also showed less development of neoplasia than the nutrient-supplemented rats fed FP corn.

DISCUSSION

This study demonstrated the hydrolysis of FB_1 during nixtamalization (Figure 1). The hydrolysis of FB_1 by treatment with $Ca(OH)_2$ and heat did not detoxify FP corn, but rather produced a more toxic product or products; this finding is based on the fact that feeding NFP corn containing 8–11 mg/kg HFB_1 produced some toxicity symptoms as severe as feeding FP corn containing 45–48 mg/kg FB_1, in terms of effects on body weight, relative liver weight, plasma GPT and plasma cholesterol (Table II), and incidence of hepatic adenomas (Table III). The major toxic product of fumonisin hydrolysis during nixtamalization appeared to be HFB_1, but it is possible that other breakdown products, such as calcium–FB complexes and other products more difficult to identify than HFB_1, also played a role in the toxicity of nixtamalized FP corn. Further studies comparing purified HFB_1 and FB_1 toxicity are needed to prove HFB_1 toxicity.

How likely is it that toxicants in FP-fermented corn, other than FB_1 and its breakdown products, might be responsible for the toxicity and neoplastic responses observed in rats fed FP-fermented corn? Purified FB_1 has been shown to cause male rat hepatocellular carcinomas, when fed at 50 mg/kg in the diet for 18–24 months (Gelderblom et al., 1991), and purified FB_1 has been shown to reproduce the neoplastic and weight-suppressive effects of feeding crude *F. moniliforme* culture to rats. Purified FB_1 has also been shown to cause equine leukoencephalomalacia (Wilson et al., 1992; Kellerman et al., 1990), a disease first associated with feeding *F. moniliforme*-contaminated corn (Wilson and Maronpot, 1971). Although a no-effect level has yet to be established for FB_1, human foods commonly contain 0.2–3.0 mg/kg of FB_1 (Hopmans and Murphy, 1993; Sydenham et al., 1991). The small amounts of FB_1 remaining in NFP corn seem unlikely to be responsible for the severe toxicity signs observed. Fumonisin production by *Fusarium* species was associated with less production of other *Fusarium* mycotoxins (Sydenham et al., 1990). Because crude contamination of human foods by *F. moniliforme* and *F. proliferatum* would be the usual means of exposure to FB_1, identification of potential synergistic agents in *F. moniliforme* may be important. It seems likely that the predominant toxic factor in FP corn was FB_1 (Gelderblom et al., 1988).

The knowledge regarding fumonisin's mechanism of action as a toxicant and carcinogen is limited. Fumonisins are sphingosine synthesis inhibitors (Wang et al., 1991). Sphingolipids have been postulated to be anticarcinogens because they are negative regulators of protein kinase C (Merrill, 1991), a metabolic control point which is activated by numerous carcinogens (Weinstein, 1987). The present results suggested that FB_1 and HFB_1 act in similar ways, because their toxicity signs were similar. Whether these toxicity signs were associated with altered sphingolipid

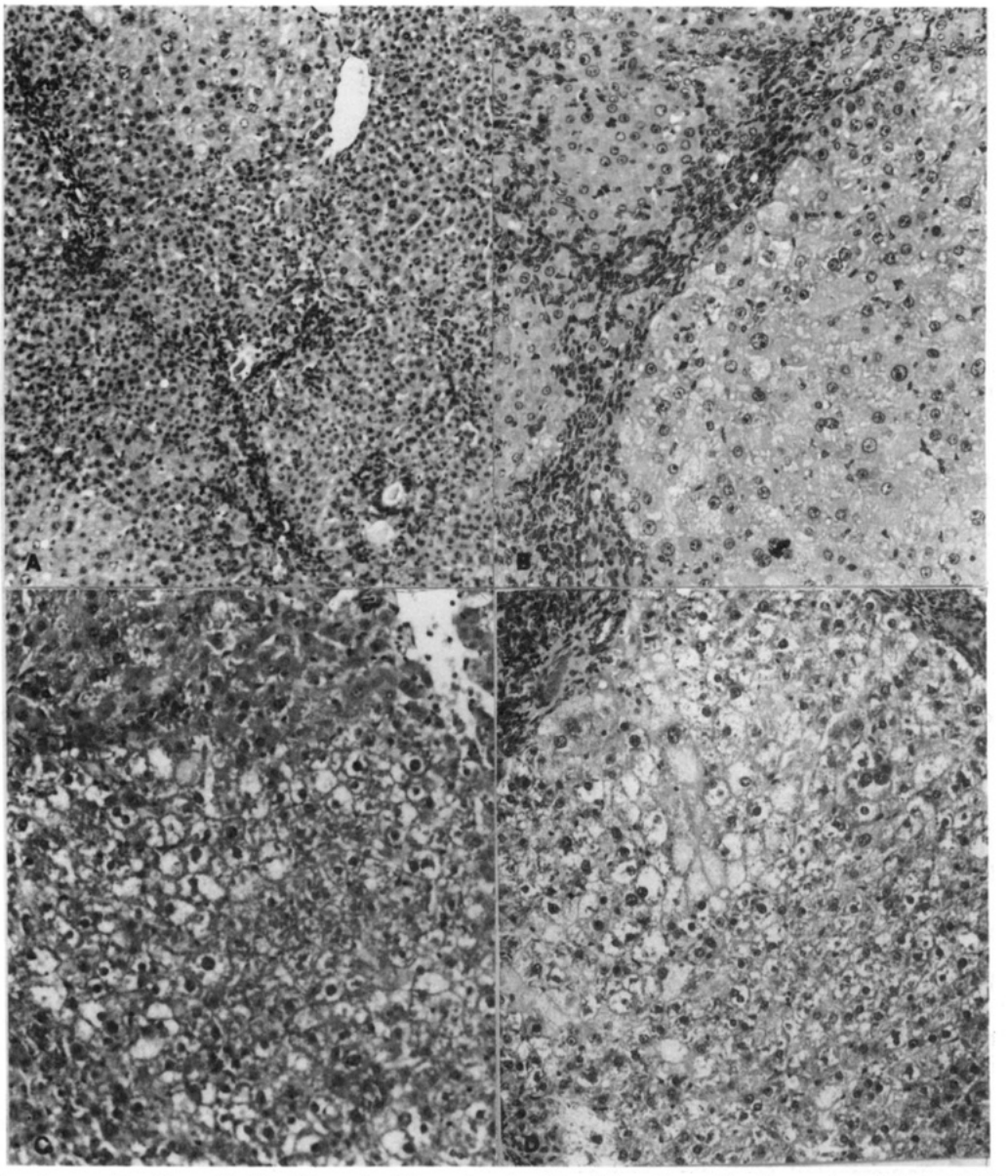

Figure 2. Photomicrographs of formalin-fixed hematoxylin- and eosin-stained hepatic sections from rats fed *F. proliferatum*-fermented (FP) corn: (A) FP corn only; (B) supplemented FP corn; (C) nixtamalized FP corn (NFP); (D) supplemented nixtamalized FP corn.

metabolism remains to be seen. It seemed reasonable that HFB_1 would also be a sphingosine synthesis inhibitor, bearing more structural similarity to sphingosine than fumonisin itself (Figure 1).

The present study suggested that some fumonisin toxicity signs may be more directly related to carcinogenesis than are other toxicity signs. Plasma GPT levels were significantly elevated in the same groups which show the greatest development of neoplasia (Tables II and III): all rats fed FP corn and rats fed supplemented NFP corn. Hepatomegaly is a common effect of tumor-promoting agents. It has been associated with the most severe neoplasia, which occurred in nutrient-supplemented rats fed FP corn or NFP corn (Table II), but rats fed unsupplemented FP corn also developed neoplasia, without hepatomegaly.

Dramatically increased plasma or serum cholesterol was a sign of *F. moniliforme* toxicity seen in primates (Fincham et al., 1992). In vervets, an African primate species, low-fat diets containing *F. moniliforme* culture material that provided increasing doses of FBs caused a dose/response in plasma cholesterol. This strongly suggested that FBs increased plasma cholesterol in primates. Therefore, fumonisins were probably responsible for increasing plasma cholesterol in rats in the present study. Other tumor-promoting agents, such as phenobarbital, increased serum cholesterol (Katayama et al., 1991). Perhaps the negative effect of fumonisin on sphingolipids also had the effect of up-regulating cholesterol production, as one of many effects of disrupting basic cell signaling. Increased plasma cholesterol was related to *F. proliferatum*-promoted carcinogenesis, because increased plasma cholesterol occurred in all FP- and NFP-fed rats (Table II). But plasma cholesterol was lower in nutrient-supplemented rats fed FP corn than in rats fed unsupplemented FP corn (Table II). This suggested that when carcinogenesis was more severe (Table III), plasma cholesterol declined. Thus, greater plasma cholesterol may be an indicator of the earlier stages of carcinogenesis. In the rats fed nixtamalized FP corn, unsupplemented and supplemented rats showed similar plasma cholesterol. This may mean either that HFB_1 acted in part by a different mechanism than does FB_1 or that NFP corn containing 11 mg/kg HFB_1 was not quite as potent a carcinogen as FP corn containing 45 mg/kg FB_1, i.e., that hepatocarcinogenesis in the NFP corn-fed rats was not quite as advanced as in the FP corn-fed

rats. The lack of development of neoplastic nodules in unsupplemented rats fed HFB_1 may support either of these two viewpoints (Table III).

This study indicated that the production of hydrolyzed fumonisins in the processing of foods for human consumption may be of significant concern. The further possibility that hydrolyzed fumonisins might be produced *in vivo* seems to be worthy of investigation because of the potential toxicity of such products.

The role of nutritional status in carcinogenesis was also of fundamental concern. The action of fumonisin and its metabolites may depend to some extent on nutritional status. Carcinogenesis by many agents has been shown to be suppressed by nutritional deprivation of varying sorts, including deprivation of calories, protein, and essential fatty acids (Pariza and Boutwell, 1987; Youngman and Campbell, 1991; Ip et al., 1985). For example, diets containing less than 9% protein totally suppressed the promotion of aflatoxin B_1-induced rat hepatocarcinogenesis (Youngman and Campbell, 1991). The corn diet fed in the present study was low in protein and in some other nutrients such as thiamin and niacin for which relative deprivation has not been studied with respect to hepatocarcinogenesis. Rats fed corn alone showed fatty infiltration of the liver (Figure 2), which was a clear sign of malnutrition, and especially of protein malnutrition, due to a lack of the protein needed to synthesize lipoproteins to export fat from the liver (Latham, 1990). Perhaps FB_1 carcinogenesis does not depend on protein status as much as aflatoxin carcinogenesis does. In our study, increased body weight (Table II), due to nutrient supplementation and nixtamalization, was associated with increased plasma GPT and severity of neoplasia (Tables II and III; Figure 2). This was seen in comparing liver from an unsupplemented FP corn-fed rat (Figure 2A) with liver from a supplemented FP corn-fed rat (Figure 2B). Although rats fed NFP corn had about 20% lower body weights than did rats fed supplemented FP corn, only one rat fed NFP corn developed hepatic adenomas, whereas all supplemented FP corn-fed rats had hepatic adenomas (Table III). This suggested that NFP corn containing 11 mg/kg HFB_1 was not as potent a promoter as was FP corn containing 45 mg/kg FB_1. Nixtamalization combined with supplementation of FP corn significantly increased body weights (Table II) and adenomas (Table III). Body weights in this group fed 8 mg/kg HFB_1 were greater than in supplemented FP corn-fed rats (Table II), but the extents of development of neoplasia were similar in both groups (Table III). This suggested that although NFP corn containing 8 mg/kg HFB_1 was not as potent a promoter as was FP corn containing 45 mg/kg FB_1, the better nutritive value of nixtamalized corn (Carter and Carpenter, 1982) made NFP corn almost as hepatocarcinogenic as FP corn. Thus, nixtamalization is probably not a useful strategy for detoxification of *F. moniliforme*- or *F. proliferatum*-contaminated corn in populations of good nutritional status. If fumonisins are nutrient-independent carcinogens, they might be especially significant to carcinogenesis in undernourished populations as well.

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