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# Effect of fumonisin-containing diet on the myenteric plexus of the jejunum in rats☆,☆☆

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ABSTRACT

Fumonisins are mycotoxins that naturally occur as contaminants in grains that are destined for animal and human consumption. These mycotoxins cause hepatotoxic, nephrotoxic, carcinogenic, teratogenic, immunotoxic, and neurotoxic effects in different intensities based on dose, time of exposure, and animal species. In the present study, male Wistar rats were fed between postnatal days 21 and 63 with diets that contained fumonisins B1 + B2 at concentrations of 1 and 3 mg/kg. The objective of the present study was to evaluate the effects of fumonisins on food intake, growth, weight gain, serum activity of the alanine aminotransferase and aspartate aminotransferase enzymes, and quantitative and morphometric parameters of myenteric neurons in the jejunum that are immunoreactive to HuC/D protein and neuronal nitric oxide synthase enzyme (nNOS). Diets that contained fumonisins did not significantly alter food intake or body and blood parameters. We did not observe significant differences in the neuronal density and proportion of nitrergic neurons but found a significant reduction of cell body areas in both neuronal populations. This study is the first to report the effects of fumonisins in the enteric nervous system. The possible mechanisms by which fumonisins impair neuronal development and the use of the enteric nervous system as a tool for the study of the neurotoxic effects of fumonisins are discussed. In conclusion, fumonisin-containing food negatively affected the growth of myenteric neurons.



## 1. Introduction

Fumonisins are mycotoxins that are produced by several fungus species, especially *Fusarium verticillioides* and *Fusarium proliferatum*, which produce large amounts of these mycotoxins and are present in several places in the world as contaminants in corn plantations (Voss et al., 2007). Streit et al. (2013) estimated that 50–63% of all grains produced for livestock feeding in the world are contaminated with fumonisins with a worldwide average of 0.914 mg/kg. In positive samples, the average level of fumonisins was 1.689 mg/kg, and the maximal level was approximately 77.502 mg/kg. Contamination with mycotoxins in grain plantations has attracted economic and scientific interest because these plantations are widely used for livestock and human feeding. Several countries have implemented laws that limit the maximal levels of fumonisins and other mycotoxins that are allowed in foods for human and animal consumption. The limits established by law are generally obtained by extrapolating experimental data, and a revision of permissible levels may be necessary based on new data that become available (Voss et al., 1996).

Fumonisins were isolated and chemically characterized for the first time in 1988. Since then, 28 analogs have been described. Fumonisin $B_1$ (FB1) is the most abundant analog representing 70–80% of the total amount. Fumonisin $B_2$ (FB2) represents 15–25%, and the remaining analogs occur in low amounts (Rheeder et al., 2002). Despite almost 30 years of research, the mechanism of action of fumonisins is not yet fully understood. Wang et al. (1991) reported that FB1 is a potent inhibitor of ceramide synthase, a key enzyme in the *de novo* synthesis and turnover of complex sphingolipids, such as sphingomyelin and glycosphingolipids, which participates in the composition of cellular membranes. In cells exposed to fumonisins, the synthesis of ceramide is reduced with a consequent decrease in the amount of sphingolipids and increase in their precursors, sphinganine and sphingosine. In addition to their structural role in cellular membranes, sphingolipids and their metabolic precursors participate in cellular signaling (Soriano et al., 2005). Thus, alterations caused by fumonisins in the metabolism of sphingolipids impact other metabolic pathways, thereby leading to alterations in development, morphology, and cell survival (Merril et al., 1997).

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☆☆ All of the procedures used in this study that involve animals are in accordance with the Brazilian law and the Guide for the Care and Use of Laboratory Animals.
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The consumption of foods that are naturally contaminated with fumonisins is related to the development of porcine lung edema and equine leukoencephalomalacia (Waes et al., 2005), which is a disease characterized by necrosis of the brain hemispheres. In humans, epidemiological studies indicate a relationship between the ingestion of fumonisins and an increased incidence of defects in neural tubes (Waes et al., 2005). The intake of fumonisins may also be related to neurodegenerative diseases, such as multiple sclerosis, Alzheimer's disease, and Parkinson's disease (Kovačić et al., 2009; Purzycki and Shain, 2010). Several studies have been conducted with the objective of clarifying the mechanisms by which fumonisins induce toxicity in the nervous system (Domijan, 2012). However, until now, such studies have focused on the effects on the central nervous system and rarely investigated the effects on the peripheral nervous system. Despite evidence that fumonisins affect the digestive system (Bouhet and Oswald, 2007) leading to alterations in intestinal absorption, increased susceptibility to infections, and alterations in the intestinal immune response, no study of which we are aware of has investigated the effect of these mycotoxins on the enteric nervous system, which is responsible for the intrinsic innervation of this system.

The enteric nervous system is the more complex portion of the peripheral nervous system. It is indispensable for maintaining homeostasis in the digestive system and an excellent model for the study of the nervous system as a whole (Anderson et al., 2006). Considering that exposure to neurotoxic substances during childhood can cause neurological problems later in development (Kovačić et al., 2009), the present study evaluated the effects of a fumonisin-contaminated diet (1 and 3 mg/kg fumonisin) on the myenteric plexus of the jejunum in developing rats.

## 2. Material and methods

The Ethical Committee on Animal Experimentation of the Universidade Estadual de Maringá (protocol no. 044/2010) approved the procedures in this study that involved the use of animals.

### 2.1. Animals and experimental design

Thirty male Wistar rats (*Rattus novergicus*), which were 21 days of age and with approximately 50 ± 5 g body weight, were used. The animals were maintained in plastic cages with five animals per cage under controlled light (12 h/12 h light/dark cycle) and temperature (22 ± 2 °C).

The animals were randomly separated into three experimental groups: F0 (fed a diet without fumonisins), F1 (fed a diet with 1 mg/kg fumonisins [FB1 + FB2]), and F3 (fed a diet with 3 mg/kg fumonisins [FB1 + FB2]). The animals had ad libitum access to food and water. Each experimental group was composed of 10 animals. Five of the animals in each group were euthanized 15 days after the experiment began (36 days old), and the other five were euthanized 42 days after the experiment began (63 days old).

### 2.2. Experimental diets

For the preparation of the base diet (Table 1), corn, wheat bran, and soybeans were ground in a knife mill with a sieve (0.50 mm diameter particle size) and mixed by hand with the other ingredients. For the production of the diets with fumonisins, we added a sterilized culture medium of the fungus *F. verticillioides*, strain MRC 826, to the base diet, which was mixed again and homogenized. The Laboratório de Análises Micotoxicológicas (LAMIC) of the Universidade Federal de Santa Maria (UFSM), Rio Grande do Sul, Brazil, provided the culture medium. The food was then pelleted and dried in an oven with forced ventilation (55 °C) for 24 h.

Samples of each type of diet were sent to LAMIC for qualitative and quantitative analyses of fumonisins using liquid chromatography coupled with sequential mass spectrometry (LC–MS/MS). The analysis of the diet of the F0 group revealed very low levels of FB1 (0.159 mg/kg) and the absence of FB2. The analysis of the diet of the F1 group revealed a mean level of fumonisins of 0.996 mg/kg (0.729 mg/kg FB1 and 0.267 mg/kg FB2). The analysis of the diet of the F3 group revealed a mean level of fumonisins of 2.819 mg/kg (2.100 mg/kg FB1 and 0.719 mg/kg FB2).

**Table 1**
Composition of the base diet provided to rats during the experimental period.

| Ingredient | (%) |
|---|---|
| Corn | 33.63 |
| Soybean meal | 33.10 |
| Wheat bran | 27.20 |
| Soybean oil | 2.30 |
| Calcitic limestone | 2.07 |
| Bicalcic phosphate | 0.73 |
| Salt (iodized) | 0.50 |
| Vitamin premix[a] | 0.10 |
| Mineral premix[b] | 0.35 |
| BHT[c] | 0.02 |
| Dry matter (%)[d] | 93.60 |
| Raw energy (kcal/kg)[d] | 4120.46 |
| Raw protein (%)[d] | 22.37 |

[a] Vitamin supplement, composition per kg: 200 mg folic acid; 3000 mg nicotinic acid; 20 mg biotin; 1600 mg calcium pantotenate; 700 mg pyridoxine HCl; 600 mg riboflavin; 600 mg thiamin HCl; 4,000,000 UI vitamin A; 2500 mg vitamin B12; 100,000 UI vitamin D3; 100,000 UI vitamin E; and 75 mg vitamin K1.
[b] Mineral supplement, composition per kg: 14.26 mg boron; 142.94 g calcium; 44.9 g chloride; 72.41 mg copper; 28.65 mg chrome; 8.6 g sulfur; 1000 mg iron; 28.72 mg fluoride; 5.9 g phosphorus; 5.95 mg iodine; 2.85 mg lithium; 14.48 g magnesium; 300 mg manganese; 4.32 mg molybdenum; 14.31 mg nickel; 102.86 g potassium; 4.28 mg selenium; 143.26 mg silicon; 29.38 mg sodium; 2.87 mg vanadium; and 860 mg zinc.
[c] Botuil hydroxy toluene.
[d] According to the chemical analysis.

The animals' body weight and food intake were measured weekly. Food intake was evaluated by subtracting the remaining food from the total food given to the animals. We calculated the average food intake per 100 g of body weight based on these data. We calculated fumonisin intake per kg of body weight per day based on this average food intake and the average fumonisin concentrations in the foods. Lee's index, which was an indicator of the relationship between size and body mass (similar to the body mass index in humans), was calculated using the following formula: [(body weight in g)$^{1/3}$ / nasoanal distance in cm] × 1000 (Stephens, 1980).

### 2.3. Euthanasia and collection of biological samples

Fifteen days after the experiment began, five animals in each group were euthanized with an intravenous injection of sodium thiopental (120 mg/kg of body weight). Blood samples were collected to analyze the serum activity of the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes using standardized commercial kits (Gold Analisa, Belo Horizonte, Brazil). Forty-two days after the experiment began, the other five animals in each group underwent the same procedures for euthanasia, blood collection, and analysis. Additionally, the small intestine was removed, and its area was calculated by multiplying the width by the length. Jejunum samples from each animal were collected from the proximal portion of the organ and utilized for the study of myenteric neurons.

### 2.4. Immunohistochemistry

The jejunum samples were washed with 0.1 M phosphate-buffered saline (pH 7.4), filled with fixative (4% paraformaldehyde in 0.1 M PBS, pH 7.4), tied at the extremities, immersed in the same fixative, and maintained under refrigeration at 4 °C for 2 h. The extremities were then opened. The samples were washed twice with 0.1 M PBS (pH 7.4) and stored under refrigeration at 4 °C in 0.1 M PBS (pH 7.40) with the addition of 0.08% sodium azide (Sigma-Aldrich, St. Louis, MO,