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The low intestinal and hepatic toxicity of hydrolyzed fumonisin B_1 correlates with its inability to alter the metabolism of sphingolipids

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ABSTRACT

Fumonisins are mycotoxins frequently found as natural contaminants in maize, where they are produced by the plant pathogen *Fusarium verticillioides*. They are toxic to animals and exert their effects through mechanisms involving disruption of sphingolipid metabolism. Fumonisin B_1 (FB_1) is the predominant fumonisin in this family. FB_1 is converted to its hydrolyzed analogs HFB_1, by alkaline cooking (nixtamalization) or through enzymatic degradation. The toxicity of HFB_1 is poorly documented especially at the intestinal level. The objectives of this study were to compare the toxicity of HFB_1 and FB_1 and to assess the ability of these toxins to disrupt sphingolipids biosynthesis. HFB_1 was obtained by a deesterification of FB_1 with a carboxylesterase. Piglets, animals highly sensitive to FB_1, were exposed by gavage for 2 weeks to 2.8 μmol FB_1 or HFB_1/kg body weight/day. FB_1 induced hepatotoxicity as indicated by the lesion score, the level of several biochemical analytes and the expression of inflammatory cytokines. Similarly, FB_1 impaired the morphology of the different segments of the small intestine, reduced villi height and modified intestinal cytokine expression. By contrast, HFB_1 did not trigger hepatotoxicity, did not impair intestinal morphology and slightly modified the intestinal immune response. This low toxicity of HFB_1 correlates with a weak alteration of the sphinganine/sphingosine ratio in the liver and in the plasma. Taken together, these data demonstrate that HFB_1 does not cause intestinal or hepatic toxicity in the sensitive pig model and only slightly disrupts sphingolipids metabolism. This finding suggests that conversion to HFB_1 could be a good strategy to reduce FB_1 exposure.

1. Introduction

Mycotoxins are structurally low-molecular-weight metabolites produced by fungi. As secondary metabolites they are not essential to fungi growth but may contaminate animal feed and human food at all stages of the food chain. Their worldwide occurrence is considered to be a major risk factor affecting human and animal health, and in addition leads to considerable worldwide economic losses.

Among mycotoxins, fumonisins are of major concern. These metabolites produced by *Fusarium verticillioides* and other fungi are common fungal contaminants of corn and some other grains [1]. Indeed, as much as 59% of corn and corn-based products are contaminated worldwide with variable amounts of fumonisin B_1 (FB_1). Fumonisin B_2 (FB_2) and B_3 (FB_3) are simultaneously produced with FB_1 on grains, and the difference from FB_1 is related to the absence of hydroxyl group in FB_2 and FB_3 on the aminopentol backbone. However, FB_1 remains the most prevalent of the fumonisin subspecies, about 70–80% of the total fumonisins content [2]. Fumonisins exert complex biological effects. The toxic effects of fumonisins range from hepatotoxicity and renal toxicity to species-specific effects such as pulmonary edema in pigs and

Abbreviations: AP_1, aminopentol; b.w., body weight; CerS, ceramide Synthase; FB_1 FB_2 FB_3, fumonisin B_1 B_2 B_3; GGT, Gamma-glutamyl transferase; HE, hematoxylin-eosin; HFB_1 HFB_2 HFB_3, hydrolyzed fumonisin B_1 B_2 B_3; HPLC-FLD, high performance liquid chromatography with postcolumn fluorescence derivatization; IFN, interferon; IL, interleukin; IPEC-1, intestinal porcine epithelial cell line; LC–MS, liquid chromatography–mass spectrometry; MHC-II, major histocompatibility complex class II; NOAEL, no observable adverse effect level; OPA, ortho-phthalaldehyde; ppm, parts per million (or mg/kg of feed); RPL32, ribosomal protein L32; Sa, sphinganine; SEM, standard error of mean; SI, small intestine; So, sphingosine; TCA, tricarballylic acids; TNF, tumor necrosis factor.

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leukoencephalomalacia in horses [3,4]. In humans, exposure to fumonisins has been linked to esophageal cancer and neural tube defects [4,5]. The effect on the intestine has been less investigated but villous alterations, reduced expression of immune mediators and an increase in intestinal colonization by opportunistic pathogenic bacteria in piglets have been detected [6–8].

Fumonisins are structurally similar to sphingoid bases, and were identified as potent inhibitors of sphinganine N-acyl transferase (ceramide synthase) [9]. Toxicity and carcinogenicity of fumonisins are related to the disruption of sphingolipid metabolism that occurs as a result of inhibition of ceramide synthase [4,9].

Several strategies have been developed to reduce fumonisin exposure. The alkali treatment of FB_1-contaminated maize, named nixtamalization, is widely used in Latin America, to produce tortillas [10]. This alkaline hydrolysis cleaves the tricarballylic acid side chains of FB_1 leading to the formation of hydrolyzed fumonisin B_1 (HFB_1). Conversion of FB_1 to HFB_1 can also be obtained by microbial degradation, through the use of fumonisin carboxylesterases, an enzyme specific of the FB_1 catabolism [11,12]. Carboxylesterases are ubiquitous enzymes, which can be found in tissues of all animals, and are responsible for detoxification of numerous exogenous compounds. However, so far it has not been demonstrated inside of animals a degradation of FB_1 through these enzymes.

Toxicity of HFB_1 is poorly documented. *In vitro* and *in vivo* data indicate that HFB_1 has a limited ability to inhibit ceramide synthase [13–15]. Hepatic and renal lesions were reported in rats fed nixtamalized material containing HFB_1 [16,17]. By contrast, in mice fed purified HFB_1, no hepatotoxicity or pathological changes were detected [14]. Similarly, HFB_1 was not carcinogenic in rats [18] and did not affect fetal development through the formation of neural tube defects in either mice or rats, whether intraperitoneally or orally administered [15,19]. Thus, conflicting data exist on the toxicity of HFB_1.

Pig is highly sensitive to FB_1 and, upon short term exposure, adverse effects are observed in this animal species at lower levels than in rodents [8,20,21]. Pig is potentially exposed to high level of FB_1. In addition pig can be regarded as the most relevant animal model for extrapolating to humans, with a digestive physiology very similar to that of human [22,23].

The objectives of this study were to compare the toxicity of HFB_1 and FB_1 and to assess the ability of these toxins to disrupt sphingolipid biosynthesis using the sensitive pig model [8,21]. HFB_1 did not induce intestinal and hepatic toxicity at a dose level that was significantly higher than the reported NOAEL for FB_1 [19]. HFB_1 slightly disrupted sphingolipid metabolism, although much less potently than FB_1. Together, the findings indicate that HFB_1 is less toxic than FB_1, and that conversion of FB_1 to HFB_1 could be a good strategy to reduce fumonisin exposure.

2. Material and methods

2.1. Animals

Eighteen, 4-week-old weaned female piglets (Pietrain/Duroc/Large-white) were obtained locally in France. Animals were acclimatized for 1 week in the animal facility of the INRA ToxAlim Unit (Toulouse, France), prior to being used in experimental protocols. Six pigs were allocated to each treatment on the basis of body weight; control group: 10.98 ± 0.16; FB_1 group: 10.92 ± 0.51; HFB_1 group: 10.98 ± 0.46. During the 14-day experimental period, animals were given free access to water and were fed with a basal diet *ad libitum*, as previously described [21]. All animal experimentation procedures were carried out in accordance with the European Guidelines for the Care and Use of Animals for Research Purposes (Directive 86/609/EEC). Three of the authors have an official agreement from the French Veterinary Services for animal experimentation.

2.2. Mycotoxins

Lyophilized culture material of *F. verticillioides*, containing 13.7 g/kg FB_1, 5.2 g/kg FB_2, and 1.7 g/kg FB_3, was obtained from Biopure – Romer Labs Diagnostic GmbH, Tulln, Austria. Aliquots of 24.25 g culture material were resuspended in 250 mL 10 mM sodium phosphate buffer pH 7.0, homogenized with an ultra-turrax, incubated at 22 °C for 30 min, and centrifuged for 10 min at 8000 rpm in a Beckman JA10 rotor. This extraction was repeated twice. The supernatants from all three extractions of all aliquots were combined, the pH was re-adjusted to 7.0, and this extract was separated into two equal halves in 5 L bottles. Fumonisin carboxylesterase FumD was prepared by fermentation of recombinant *Pichia pastoris* as previously described [11]. Lyophilized fermentation supernatant was dissolved in water at a concentration of 100 mg/mL, and 774 μL of this solution was added per liter to one of the two aliquots of extract. Both aliquots were incubated overnight at 22 °C with shaking at 60 rpm, and both were heat-inactivated by boiling in a microwave oven for one minute in aliquots of about 650 mL. A portion of the FumD solution was heat-inactivated for 40 min in a boiling water bath, and the same amount of enzyme that was used for preparation of the hydrolyzed fumonisin extract was added, in heat-inactivated form, to the intact fumonisin extract. Samples of both extracts from before, during, and after incubation were analyzed by LC–MS using a previously described method [11] to confirm that the fumonisins were hydrolyzed and intact, respectively, in the two extracts. Since more material was required, the extracts were combined with equal amounts of extracts which were prepared from the same culture material in the same way, except that water instead of 10 mM phosphate buffer was used for extraction. The final extract of intact fumonisins was analyzed by Quantas – Romer Labs Diagnostic GmbH, Tulln, Austria, using a certified LC–MS method, and found to contain 530.85 mg/L FB_1, 133.30 mg/L FB_2, and 35.60 mg/L FB_3. Extracts were stored at −20 °C. For the no fumonisins control group, 5 mM sodium phosphate containing the same concentration of heat-inactivated FumD as the two extracts was made.

2.3. Experimental design and tissue/blood sampling

Animals received daily by gavage the different solutions (control, FB_1, HFB_1) at the concentration of 2.8 μmol FB_1/kg b.w./day. At weekly time intervals, blood samples were aseptically collected from the left jugular vein. Blood was collected in tubes containing sodium heparin for biochemistry, or citrate for fibrinogen measurement (Vacutainer®, Becton-Dickinson, USA). Plasma samples were obtained after centrifugation of blood and stored at −20 °C for later analysis. Upon termination of the experiment, corresponding to 14 days of dietary exposure to treatments, immediately after electrical stunning, pigs were killed by exsanguinations, and liver and the entire gastrointestinal tract were removed. The mesentery was cut using scissors, and the small intestine (SI) was aligned on a table and measured without applying tension. The SI was divided into three parts of equal length, and 15 cm tissue segments were collected from the middle of each part (named in the present study as Proximal SI, Mid SI and Distal SI). Subsamples of the SI segments were either fixed in 10% buffered formalin (Sigma, Saint-Quentin Fallavier, France) for histopathological analysis or flash-frozen in liquid nitrogen and stored at −80 °C until RNA extraction. Liver and mesenteric nodes were similarly prepared, and for liver an additional piece of hepatic