Effects of Intravenous Fumonisin B₁ in Rabbits: Nephrotoxicity and Sphingolipid Alterations


Departments of Veterinary Pathobiology (L.A.G., A.M., R.M.W., W.M.H.) and Biosciences (V.R.B.), University of Illinois, Urbana, United States Department of Agriculture, Agricultural Research Service, National Center for Agricultural Utilization Research (R.F.V.), Peoria, Illinois; Toxicology and Mycotoxin Research Unit, Russell Research Center, United States Department of Agriculture, Agricultural Research Service, Athens, Georgia (R.T.R., J.L.S.)

ABSTRACT Fumonisin B₁ is hepatotoxic in all species, but nephrotoxicity has only been reported in rats. It is a specific inhibitor of sphinganine N-acyltransferase. Our objective was to determine the target organs for fumonisin toxicosis in the rabbit. We administered fumonisin B₁ (≥95% pure) intravenously to adult rabbits and examined selected clinical, biochemical, and histological parameters for up to 5 days. In a pilot study, rabbits were given fumonisin B₁ at 1, 0.5, 0.3, 0.15, or 0 mg/kg daily for 4 or 5 days and then euthanized. Additional rabbits were given a single dose of fumonisin B₁ at 1 mg/kg and euthanized on day 2 or 4. In the formal time-course study, rabbits were given a single dose of fumonisin B₁ at 0 or 1.25 mg/kg and euthanized on days 1, 3, or 5. Rabbits given multiple doses of fumonisin B₁ were lethargic and anorectic, and had decreased urine production. Liver- and renal-associated clinical chemistry parameters were elevated. Renal lesions consisted of severe proximal tubular necrosis. Liver lesions were variable and consisted of mild necrosis, hepatocyte vacuolation, and bile stasis. The sphinganine-to-sphingosine ratio, in both target and nontarget tissues, was markedly elevated in treated rabbits. A single dose of fumonisin B₁ induced renal but not hepatic injury. Therefore, the target organs for fumonisin B₁ toxicity in rabbits are kidney and liver, with the kidney being more sensitive.

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Key Words: Fusarium moniliforme, Mycotoxin, Sphinganine, Sphingosine, Liver, Kidney, Hepatotoxicity, Histopathology

INTRODUCTION

Fumonisins, mycotoxins produced by Fusarium moniliforme and other Fusarium species, are common contaminants of corn. Although hepatic injury has been induced experimentally in all vertebrate species studied to date [Jaskiewicz et al., 1987; Kellerman et al., 1990; Haschek et al., 1992; Ledoux et al., 1992; Voss et al., 1993], other target organs appear to be more species-specific. Ingestion of fumonisin-contaminated corn has been associated with spontaneous outbreaks of equine leukoencephalomalacia [Kellerman et al., 1990] and acute pulmonary edema in pigs [Harrison et al., 1990]. Other species-specific effects induced experimentally by fumonisin include renal injury in rats [Voss et al., 1993], potentiation of atherogenic plaque formation in primates [Fincham et al., 1992], and esophageal mucosal hyperplasia in chronically dosed pigs [Casteel et al., 1993]. Additional concerns related to human health are the ability of fumonisin to induce hepatocellular carcinomas in the rat [Gelderblom et al., 1991], and the epidemiologic association of consumption of fumonisin-containing foods with esophageal cancer in humans [Marasas et al., 1988; Norred and Voss, 1994].

The mechanism of fumonisin toxicity remains unknown, but may be related to altered sphingolipid biosynthesis. The fumonisin backbone strongly resembles that of the sphingoid bases, sphinganine and sphingosine, and fumonisin B₁ inhibits sphinganine N-acyltransferase, a critical enzyme in the biosynthesis of sphingolipids [Wang et al., 1991]. Elevations of the sphinganine-to-sphingosine ratio in serum and tissue obtained from horses, pigs, and rats have been correlated with ingestion of feed containing fumonisin B₁ [Riley et al., 1994b].

To our knowledge, the effects of fumonisins in rabbits have not been reported previously. The primary objective of
this study was to elucidate the target organs of fumonisin toxicosis in the rabbit using clinicopathologic, histopathologic, and sphingolipid endpoints. A secondary objective was to determine if the rabbit could serve as a laboratory animal model for fumonisin toxicoses in large domestic species such as the pig or horse.

**MATERIALS AND METHODS**

**Animals and Feed**

Adult New Zealand White rabbits were obtained from Myrtle's Rabbitry (Thompson Station, TN) and housed individually. All rabbits were acclimated for at least 5 days prior to the study. Rabbits were offered Laboratory Rabbit Diet HF5326 (PMI Feeds, Inc., St. Louis, MO) and water ad libitum. The diet used in the pilot studies contained 0.62 ppm total fumonisins, while that for the formal time-course study contained 1.4 ppm, when analyzed by high performance liquid chromatography (HPLC) at the Laboratories of Veterinary Diagnostic Medicine, University of Illinois, Urbana, IL.

**Dosing and Monitoring**

Rabbits were tranquilized with 0.1 mg/kg of a 1:1 mixture of acepromazine maleate (10 mg/ml; Fort Dodge, Inc., Fort Dodge, IA) and butorphanol tartrate (10 mg/ml; Torbugesic; Fort Dodge, Inc.), given intramuscularly prior to dosing and bleeding. Fumonisin B₁ (purified to >95% by R.F.V.) was dissolved in sterile phosphate-buffered saline (PBS). Fumonisin B₁ or an equivalent amount of PBS (vehicle) was administered intravenously (i.v.) through the marginal ear vein or the central auricular artery. Animals were observed at least daily for clinical signs such as inappetence, inactivity, and decreased urine and fecal output.

**Experimental Protocols**

**Pilot studies**

**Daily dosing.** One female (T1) and 1 male (T2) rabbit were given fumonisin B₁ i.v. daily at 1 mg/kg (0.5–1 ml total volume) for 5 days, beginning on day 0. In addition, male rabbits (1 per dose) were given fumonisin B₁ at 0.5, 0.3, or 0.15 mg/kg/day (0.5–1 ml total volume) for 4 or 5 days. One control male (C3) was untreated, while the second control female rabbit (C4) was given the vehicle i.v. for 5 days.

**Single-dose study.** Two male rabbits were given a single i.v. dose of fumonisin B₁ at 1 mg/kg (0.5 ml total volume) on day 0; 1 rabbit was euthanized on day 2 and the other on day 4. The control rabbit was given vehicle only and was euthanized on day 2.

**Pathology**

Rabbits were euthanized with an intraperitoneal overdose of pentobarbital (Euthanasia-5 Solution; Henry Schein, Inc., Port Washington, NY), and a gross necropsy was immediately performed. For all experiments, tissues previously reported to be targets of fumonisin in other species, including kidney, liver, lung, pancreas, and brain, were fixed in 10% neutral buffered formalin, routinely processed, sectioned at 3–4 μm, stained with hematoxylin and eosin, and examined by light microscopy.

**Sphingolipid Analysis**

The free sphinganine and sphingosine concentrations in base-treated chloroform-methanol extracts of tissues and fluids were determined by HPLC with C:20 sphinganine as an internal standard [Riley et al., 1994b].

**RESULTS**

**Pilot Studies**

**Clinical signs and total dose**

Rabbits given fumonisin B₁ daily exhibited progressively decreased activity and decreased urine and fecal output from
The most striking alterations were observed in the kidneys of rabbits given fumonisin B₁, and consisted of proximal tubular necrosis. In general, lesions occurred multifocally within the cortex and were more extensive in the outer stripe of the medulla. Mitotic figures and individual cell necrosis were frequently seen in the proximal tubular epithelium. Hepatic alterations in fumonisin B₁-treated rabbits were much more inconsistent. Both treated and control rabbits had mild-to-moderate lymphocytic periportal infiltrates with mild fibrosis. Lesions were not observed in the kidneys of control rabbits or in other organs of treated or control rabbits.

**Daily dosing study.** All treated rabbits had severe necrosis of the straight or distal portion of the proximal tubule, with extensive denudation of the basement membrane and prominent eosinophilic granular and hyaline casts. In addition, tubular regeneration was observed in rabbits given fumonisin B₁ at 0.15 or 0.3 mg/kg daily. Livers from rabbits dosed daily with fumonisin B₁ at 1 mg/kg had mild centrilobular hepatocellular necrosis, midzonal cord disorganization, and hepatocellular swelling with vacuolization. Many hepatocytes contained bile pigment (bile stasis). Hepatic lesions in rabbits dosed at 0.5, 0.3, or 0.15 mg/kg were dose-dependent and characterized by hepatocellular swelling and hepatic cord disorganization. Occasionally, ballooning degeneration of individual cells and mitotic figures were present, as well as perportal hepatocellular vacuolation and mild hepatocellular bile stasis. Individual cell necrosis of hepatocytes was frequently observed in the rabbit given fumonisin B₁ at 0.3 mg/kg. In the rabbit given fumonisin B₁ at 0.15 mg/kg, the changes were similar but much milder.

**Clinical pathology**

Serum and urinary parameters are presented in Table I. By day 5, creatinine and serum urea nitrogen concentrations in rabbits treated daily with fumonisin B₁ were elevated over control values. Urinary glucose and protein concentrations were also markedly elevated in rabbits treated daily with fumonisin B₁. Single-dosed rabbits showed mild elevations in serum urea nitrogen and creatinine.

Liver-associated parameters (ALP, ALT, AST, and GGT activities, as well as total bilirubin) were markedly increased over control values in daily-dosed rabbits. One rabbit given a single dose showed an increase in liver-related chemistry parameters on day 2.

**Pathology**

The sphinganine-to-sphingosine ratios are shown in Figures 1A,B. Sphinganine concentration (data not shown) was elevated in fumonisin B₁-treated rabbits, as compared to controls, for all samples analyzed. Tissue elevation was greatest for kidney > liver, lung > pancreas > muscle. Sphinganine was also markedly elevated in serum and urine. Results were more variable for sphingosine concentration, with elevated concentrations occurring only in some tissues (data not shown). Both treated rabbits had higher sphingosine concentrations than controls in kidney, serum, pancreas, muscle, and urine. Liver and lung sphingosine concentrations were similar for treated and control rabbits. The sphinganine-to-sphingosine ratio was markedly elevated in treated rabbits as compared to controls for all samples except brain. The order of sphinganine-to-sphingosine

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Fig. 1. Effect of fumonisin B₁ at 1 mg/kg/day on sphinganine-to-sphingosine ratio (Sa/Sø). Pilot study. A: Values for both target (liver and kidney) and nontarget (muscle) tissues from treated rabbits are greatly increased over control. B: Similar elevations are observed for serum and urine.

| TABLE II. Formal Time-Course Study* |
|----------------|----------------|
| Day 1 after treatment | Day 3 after treatment | Day 5 after treatment |
| Control | Treated | Control | Treated | Control | Treated |
| ALP (U/I) | 290 ± 63* | 355 ± 13 | 357 ± 36 | 425 ± 79 | 305 ± 44 | 285 ± 25 |
| ALT (U/I) | 33 ± 6 | 50 ± 8 | 35 ± 4 | 35 ± 4 | 40 ± 19 | 70 ± 14 |
| GGT (U/I) | 2.7 ± 3 | 5 ± 1 | 4 ± 2 | 5 ± 1 | 8 ± 1 | 13 ± 1 |
| Total bilirubin (mg/dl) | 0.7 ± 0.3 | 0.1 ± 0.03 | 0.2 ± 0.1 | 0.4 ± 0.2 | 0.1 ± 0.03 | 0.2 ± 0.03 |
| Urinary glucose (g/dl) | 0 | 0 | 0* | 700 ± 300 | 0* | 500 ± 0 |
| Urinary protein (mg/dl) | 18.3 ± 5.8 | 10 ± 0.6 | 2 ± 0.6 | 503.3 ± 90.2 | 0.3 ± 0.3 | 243.3 ± 60.1 |

*Effects of fumonisin B₁ given as a single i.v. dose at 1.25 mg/kg on selected liver- and renal-associated clinical chemistry parameters in rabbits.

# Results

Liver and kidney ratio, from greatest to least, was T₁ kidney > T₁ and T₂ liver > T₂ kidney > T₁ muscle > T₁ and T₂ lung, T₂ pancreas > T₂ muscle > T₁ pancreas.

**Formal Time-Course Study (Single-Dose)**

**Clinical signs**

On day 5, the fumonisin B₁-treated rabbits showed mild inappetence and moderate decreases in urinary and fecal outputs.

**Clinical pathology**

Renal-associated chemistry parameters were elevated on days 3 and 5 in treated rabbits. Serum urea nitrogen and creatinine were elevated by day 3 and increased further by day 5. Urinary glucose and protein peaked at day 3 (Table II). Multiple regression analysis showed a significant treatment-by-time interaction for creatinine (t = 7.46; P < 0.001; see Fig. 2A), and this interaction approached significance for serum urea nitrogen (t = 2.00; P = 0.07; see Fig. 2B). After day 1, fumonisin B₁ was associated with increased creatinine and serum urea nitrogen, and these effects increased over time.

Liver-associated parameters were highly variable throughout the study. Although some individual values in treated rabbits were elevated on days 3 and 5, only GGT showed a consistent, though very slight, elevation on day 5 (Table II).

**Histopathology**

On day 1, very mild renal changes were present in the fumonisin B₁-treated rabbits, consisting of proximal tubular epithelial cell swelling in the outer stripe of the medulla. There were occasional individual cell necrosis and mitoses in the proximal tubular epithelium. Renal damage was more severe on day 3, with moderate-to-marked individual tubular epithelial cell necrosis and sloughing in the cortex and the outer stripe of the medulla. Dilated tubules contained sloughed cells, protein, or granular casts. Proximal tubules...
in the outer stripe of the medulla were most severely affected. On day 5, affected tubules had denuded basement membranes (Fig. 3); however, fewer necrotic cells were observed overall. The denuded membranes occurred as a result of continued loss of epithelial cells affected at an earlier time point. In addition, there was evidence of regeneration. Kidneys from both treated and control rabbits had a few scattered intratubular foci of multinucleated giant cells.

Liver lesions in all fumonisin B₁-treated rabbits were very mild, consisting of mild centrilobular hepatocellular swelling (Fig. 4). On day 5, an occasional degenerated hepatocyte was observed. Both treated and control rabbits had mild-to-moderate periportal lymphocytic infiltrates with mild fibrosis. There were no lesions in any other organs in either treated or control animals.

**DISCUSSION**

Previous studies have shown that fumonisin is hepatotoxic to all species studied, and also that it induces species-specific target organ toxicity [Diaz and Boermans, 1994]. Our studies demonstrate that, as in other species, fumonisin is hepatotoxic in rabbits. It is also nephrotoxic, as reported in rats [Voss et al., 1993].
Based on our studies, the kidney is the major target organ of fumonisin B₁ toxicosis in the rabbit, with toxin-induced renal failure being the cause of death in 1 rabbit. Serum urea nitrogen and creatinine increased significantly over time in rabbits given a single dose of fumonisin B₁ (formal time-course study). These parameters were also markedly increased in all rabbits given multiple doses of fumonisin B₁ in the pilot study. Urinary glucose and protein concentrations were altered in a similar manner, except that the greatest elevation was observed on day 3 in the formal time-course study. This suggests that damage may have peaked prior to day 3. Histologically, proximal tubular damage was induced by fumonisin B₁, regardless of whether it had been given in a single dose or multiple doses. The most severe damage was observed with the multiple dosing regimen, even when the total dose was lower. Regardless of regimen, higher doses damaged the entire length of the proximal tubule, while lower doses affected primarily the straight, or S₁, segment. The formal time-course study demonstrated that damage induced by a single dose peaked by day 3, and was followed by tubular regeneration. Tubular regeneration was also observed in pilot rabbits given lower multiple doses. The observation of tubular regeneration suggests that not all cells are equally susceptible to fumonisin B₁. Thus, a more
resistant cell population remains for regeneration and repair. Interestingly, fumonisin B₁ was shown to inhibit replication in a primary culture of rabbit renal proximal tubular epithelial cells [Counts et al., 1994].

These findings suggest that in the rabbit kidney, fumonisin B₁ targets the proximal tubular epithelium, especially at the corticomedullary junction. Damage extends proximally with high or multiple doses. The remarkable levels of protein in the urine could be explained by the extensive tubular epithelial necrosis. The loss of these cells would result in glucose being lost in the urine instead of being resorbed. Although it is possible that the glomeruli could be affected, allowing for leakage of serum proteins into the urine, the serum chemistry data did not show a concomitant decrease in total protein (data not shown), nor was there histologic alteration of glomeruli.

There are only a few other reports of renal damage associated with fumonisin toxicosis. Two sheep fed *F. moniliforme* (isolate MRC826)-contaminated culture material for 8 or 10 days developed acute nephrosis [Kriek et al., 1981]. Hydropic degeneration of the proximal tubular epithelium was reported in a colt fed fumonisin B₁ at 42.1 mg/kg (total dose) over 29 days, but the possibility of a fumonisin-induced lesion was not addressed [Kellerman et al., 1990]. Renal toxicity occurred in male rats fed fumonisin B₁ at ≥15 ppm and female rats fed fumonisin B₁ at ≥50 ppm for 4
weeks [Voss et al., 1993]. Lesions consisted of individual cell necrosis, tubular basophilia, and epithelial hyperplasia in the proximal tubules, with more severe changes at the corticomedullary junction. Ultrastructural changes included cytoplasmic vacuoles that sometimes contained multilamellar membranous whorls, and disorganization and distention of basilar membrane folds [Riley et al., 1994a]. In a study in which fumonisin B\textsubscript{1} was fed at 0–81 ppm to rats for 13 weeks, nephrosis was induced in males fed ≥9 ppm and females fed 81 ppm [Voss et al., 1995]. Differences between the rat and other species in lesion severity may be related to the dosing route or to a species difference in sensitivity to fumonisin B\textsubscript{1}-induced renal damage. Pigs and rats dosed with radiolabeled fumonisin B\textsubscript{1} intragastrically absorbed very little of the fumonisin B\textsubscript{1}, most of which was excreted unchanged in the feces [Shephard et al., 1992; Norred et al., 1993; Prelusky et al., 1994]. Bioavailability was determined to be <5% in pigs [Prelusky et al., 1994]. If bioavailability is similar in rabbits, the lowest dose of fumonisin B\textsubscript{1} given i.v. that caused renal damage (0.15 mg/kg/day) would be roughly equivalent to 40–80 mg/kg total fumonisin B\textsubscript{1} given orally, or 2 mg/kg/day in the pilot study. Renal lesions were not apparent in short-term studies with pigs [Haschek et al., 1992; Motelin et al., 1994] or in a subchronic study with mice [Voss et al., 1995].

The liver is also a target organ of fumonisin B\textsubscript{1} exposure in the rabbit, but is less severely affected than the kidney. As observed in the kidney, daily dosing induced more severe hepatic lesions and associated clinical chemistry alterations than a single dose. Experimentally, fumonisin B\textsubscript{1}-induced hepatotoxicity has been reported in other species, such as the horse and pig. Pigs are quite susceptible to fumonisin-induced hepatotoxicity, irrespective of whether dosed orally or i.v. [Harrison et al., 1990; Osweiler et al., 1992; Haschek et al., 1992; Colvin et al., 1993; Motelin et al., 1994]. Changes in the pig following short-term exposure consist of individual hepatocyte necrosis, hepatic cord disorganization, hepatocellular swelling, and increased mitoses. Chronic exposure can result in moderate hepatic necrosis with the development of hyperplastic nodules [Casteel et al., 1993].

As found in rabbits, rats appear to be less susceptible to hepatotoxicity than to nephrotoxicity following oral exposure to fumonisin B\textsubscript{1}. Voss et al. [1993] reported mild hepatic changes consisting of single-cell necrosis and cytoplasmic vacuolation in male and female rats fed fumonisin B\textsubscript{1} at 150 ppm for 4 weeks. However, ultrastructural changes were seen in male rats fed fumonisin B\textsubscript{1} at ≥15 ppm and in female rats at ≥50 ppm [Riley et al., 1994a]. Changes consisted of multilamellar membranous whorls in the bile canaliculus and hepatocyte cytoplasm, loss of microvilli from the bile canaliculus, and increased numbers of lysosomes. In a subchronic study in mice, only females fed the high-dose diet (81 ppm) developed hepatic lesions [Voss et al., 1995]. No renal lesions were observed in these studies in mice.

Rabbits given fumonisin B\textsubscript{1} at 1 mg/kg i.v. daily had increased concentrations of free sphinganine and free sphingosine, as well as elevated sphinganine-to-sphingosine ratios, for all tissues and fluids examined, except brain. Similar alterations in sphinganine-to-sphingosine ratio have been observed in the rat, pig, and horse given fumonisin B\textsubscript{1}, and these alterations are dose-dependent [Wang et al., 1992; Riley et al., 1993, 1994a]. Alterations have been reported not only in tissues that are injured by fumonisin (target organs), but also in those that do not appear to be affected. The kidney appears to be the most sensitive organ to fumonisin-induced sphingolipid alteration, irrespective of whether renal injury occurs.

Rats fed fumonisin B\textsubscript{1} at 15–150 \(\mu\)g/g diet showed dose-dependent elevations of sphinganine-to-sphingosine ratio in the liver, serum, kidneys, and urine [Riley et al., 1994a]. Increases in kidney sphinganine-to-sphingosine ratio were present at all doses, while increases in liver were present only at 150 \(\mu\)g/g in males and at ≥50 \(\mu\)g/g in females. In swine, marked alterations in sphinganine-to-sphingosine ratio occurred in the kidney in response to fumonisin B\textsubscript{1}, even though it is not a target organ in this species. In pigs fed fumonisin-contaminated feed containing 0–175 ppm total fumonisin for 14 days, pulmonary edema occurred at 175 ppm, and histologic liver injury at ≥23 ppm [Motelin et al., 1994]. In that study, a dose-dependent increase in sphinganine-to-sphingosine ratio occurred in kidney, liver, and lung, beginning at a dietary concentration of 23 ppm, with the greatest elevation in sphinganine-to-sphingosine ratio occurring in the kidney, a nontarget organ in swine [Riley et al., 1993]. Additionally, significant increases in serum sphinganine-to-sphingosine ratio were seen at the lowest dose of 5 ppm after 14 days, and at 39 ppm after only 5 days [Riley et al., 1993]. Fumonisin did not induce any other observable change at 5 ppm, indicating that the sphinganine-to-sphingosine ratio serves as a sensitive biomarker of exposure. This is also supported by findings in pigs fed either pure fumonisin B\textsubscript{1} (1.5 mg/kg daily, 64 mg/pig total) or feed contaminated with fumonisin B\textsubscript{1} at 20 ppm and fumonisin B\textsubscript{1} at 7 ppm (34 and 44 mg/kg total dose); these pigs had markedly elevated sphinganine-to-sphingosine ratios in the kidney, liver, and lung after only 5 days, with the greatest elevation in the kidney [Haschek et al., 1993].

Although fumonisin B\textsubscript{1} alters sphingolipid biosynthesis, the mechanistic implications of these observations in relation to fumonisin toxicity are still not fully understood. Fumonisins structurally resemble sphinganine and sphingosine, the sphingoid base backbone of complex sphingolipids. Fumonisin specifically inhibits sphinganine N-acyltransferase. Fumonisin B\textsubscript{1} exposure disrupts sphingolipid metabolism, resulting, for example, in elevation in free sphinganine and decreases in complex sphingolipids [Wang et al., 1991]. Disruption of sphingolipid metabolism has been correlated with inhibition of cell proliferation and cell death in LLC-PK\textsubscript{1} cells [Yoo et al., 1992]. Based on fumonisin B\textsubscript{1}-induced ultrastructural renal changes in rats, Riley et al.
ALTERS RABBIT KIDNEYS AND SPHINGOLIPIDS


