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Abstract

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Keywords

Ames Laboratory, fumonisin; deoxynivalenol; zearalenone; mycotoxin binder; Grainsure E

Disciplines

Food Science | Human and Clinical Nutrition | Other Animal Sciences

Comments

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Efficacy of a Mycotoxin Binder against Dietary Fumonisin, Deoxynivalenol, and Zearalenone in Rats

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ABSTRACT: It was hypothesized that a mycotoxin binder, Grainsure E, would inhibit adverse effects of a mixture of fumonisin B1, deoxynivalenol, and zearalenone in rats. For 14 and 28 days, 8–10 Sprague–Dawley rats were fed control diet, Grainsure E (0.5%), toxins (7 μg fumonisin B1/g, 8 μg of deoxynivalenol/g and 0.2 μg of zearalenone/g), toxins (12 μg of fumonisin B1/g, 9 μg of deoxynivalenol/g, and 0.2 μg of zearalenone/g + Grainsure E), or pair-fed to control for food intake of toxin-fed rats. After 28 days, decreased body weight gain was prevented by Grainsure E in toxin-fed female rats, indicating partial protection against deoxynivalenol and fumonisin B1. Two effects of fumonisin B1 were partly prevented by Grainsure E in toxin-fed rats, increased plasma alanine transaminase (ALT) and urinary sphinganine/sphingosine, but sphinganine/sphingosine increase was not prevented in females at the latter time point. Grainsure E prevented some effects of fumonisin B1 and deoxynivalenol in rats.

KEYWORDS: fumonisin, deoxynivalenol, zearalenone, mycotoxin binder, Grainsure E

INTRODUCTION

Mycotoxins are produced by fungi such as *Aspergillus*, *Penicillium*, *Fusarium*, and *Alternaria* that contaminate at least 25% of the world's agricultural commodities.¹ The content of mycotoxins varies, depending on storage, environmental, and ecological conditions and temperature and moisture conditions in crops.

Deoxynivalenol or vomitoxin (Figure 1), derived from *Fusarium graminearum* and *Fusarium culmorum*, is associated with *Fusarium* head blight. Deoxynivalenol inhibits protein synthesis by binding to ribosomes,² and it suppresses peripheral blood leukocyte subsets, inhibits lymphocyte proliferation, and induces pro-inflammatory cytokine and chemokine expression in cell culture or animal models. Fumonisin B1 (Figure 1), produced by *Fusarium verticillioides* and *Fusarium proliferatum*, is neurotoxic, hepatotoxic, nephrotoxic, immunosuppressive, and carcinogenic in various animal species. Fumonisin B1 inhibits the enzyme ceramide synthase (sphinganine/sphingosine *N*-acyltransferase) and cause the accumulation of sphingoid bases in blood and urine.³ A greater ratio of sphinganine/sphingosine is a biomarker for fumonisin exposure in animal models. Zearalenone, a nonsteroidal estrogenic mycotoxin isolated from *F. graminearum*, is a potent estrogenic metabolite causing infertility, abortion, or other breeding problems in swine (Figure 1).⁴ Zearalenone and its metabolites resemble estradiol and bind estrogen receptors, causing mammary cancer, fetal skeleton anomalies, and uterotrophic and embryopathic activity. Several combinations of mycotoxins frequently occur, such as the co-occurrence of deoxynivalenol with zearalenone or of nivalenol or aflatoxin B1 with ochratoxin A or other *Fusarium* toxins in cereal grains, animal feeds, and forages.⁵

Several mycotoxin adsorbents prevented deoxynivalenol, fumonisin B1 (FB1), or zearalenone toxicity, including inorganic binders, such as zeolites, bentonites, clays, and organic adsorbents such as glucomannan, cholestyramine, and pectin. Zeolite and related clays partially prevented aflatoxin (2 mg/kg of bw) induced maternal and developmental toxicities in rats.⁶ Cholestyramine at 2% in the diet significantly decreased sphinganine/sphingosine

in kidney and urine in rats fed fumonisins at 20 $\mu\text{g}/\text{g}$.⁷ A carbon/aluminosilicate-based product (2%) significantly decreased mycotoxin absorption, that is, 88% for aflatoxin B1, 44% for zearalenone, and 29% for the fumonisins and ochratoxin, in an *in vitro* gastrointestinal model.⁸ However, the toxicity of the mixture of deoxynivalenol, fumonisin B1, and zearalenone in rats has not been reported, nor have protective factors been identified against all three toxins. Grainsure E is a product formulated with different clay minerals having mycotoxin adsorption capability, and it can be altered by acid activation to increase specific surface area, porosity, and surface acidity. A wide range of activated clay minerals were tested *in vitro* for their mycotoxin binding efficacy (difference between the amount adsorbed and desorbed). On the basis of the obtained results a combination of clay minerals was selected to obtain a wide range of binding capacity, further supplemented with butylated hydroxyanisole as a preservative to formulate Grainsure E. The proposed action of the mycotoxin binder, Grainsure E, is that mycotoxins that are adsorbed in the gut by the clay minerals in this binder will not be taken up and will thus be less toxic to animals.

This study was conducted to test the hypothesis that a mycotoxin binder would decrease hepatotoxicity, nephrotoxicity, and reproductive toxicity over 14 and 28 days in Sprague–Dawley rats fed deoxynivalenol, fumonisin B1, and zearalenone. Plasma clinical chemistry, urine sphingolipid concentrations, and organ morphology and histopathology analyses were analyzed to assess the hypothesis.

MATERIALS AND METHODS

Materials. The mycotoxin binder Grainsure E, a proprietary product of a specific blend of components designed to bind fungal toxins, was obtained from Kemin Europa N.V., Belgium. Zearalenone and standards

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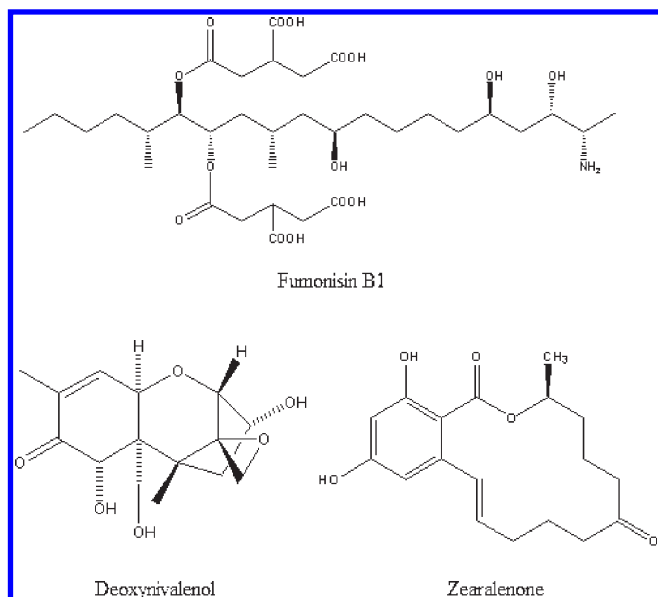


Figure 1. Mycotoxin structures.

of D-sphingosine, DL-erythro-dihydrosphingosine (sphinganine), O-phthalaldehyde, and 2-mercaptoethanol were obtained from Sigma-Aldrich (St. Louis, MO); D-erythro-C20-dihydrosphingosine (internal standard) was bought from Matreya LLC (Pleasant Gap, PA); CHCl_3 and CH_3OH (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). *F. verticillioides* MRC 826 corn culture material (after inoculation with 10–20 beads of fumonisin spores, in modified Myro medium (0.5 g/L of MgSO_4) plus 1% corn-hull-extract incubated for 69 days) containing 9807 μg of FB1/g was obtained from the Veterinary Medical Diagnostic Laboratory at the University of Missouri, Columbia, MO.⁹ Rice culture infected with *F. graminearum* R6575 (containing 640 μg of deoxynivalenol/g and 57 μg of 15-acetyldeoxynivalenol/g), after inoculation on potato dextrose agar plates and incubation in carboxymethylcellulose media (composed of carboxymethylcellulose, $\text{NH}_4\text{NO}_3 \cdot 7\text{H}_2\text{O}$, and yeast extract) mixed with enriched white rice, was prepared previously by Dr. Hendrich's laboratory according to the method of Clifford et al.¹⁰

Diet. Rats were divided into five groups: control (fed AIN93G diet obtained from Harlan Teklad, Madison, WI), Grainsure E (0.5% in AIN 93G), toxins (mixture of fumonisin B1, deoxynivalenol, and zearalenone in AIN93G), toxins + Grainsure E, and pair-fed controls (fed daily the mean food intake of toxin group as measured on the previous day). The intended concentrations of toxins were 12 μg of deoxynivalenol/g, 8 μg of fumonisin B1/g, and 0.5 μg of zearalenone/g, selected on the basis of realistic values of natural contamination observed in cereals worldwide.⁵ Contaminated culture materials (4.2 g of culture containing 9807 μg of FB1/g and 95.8 g of culture containing 640 μg of deoxynivalenol/g) were mixed with 1100 mL of water and then further mixed with 5 kg of AIN93G. Because zearalenone is insoluble in water, 2.55 mg of zearalenone was dissolved in 5 mL of ethanol/5 kg of diet, mixed with diet ingredients by a Hobart mixer; each diet, with or without Grainsure E, was mixed for 20 min with the same amount of water (Hobart Corp., Troy, OH). After 1–2 days drying, each diet was cut into bars and stored at -20°C until feeding. To check the homogeneity, three samples of each diet were taken randomly and analyzed for mycotoxins by Eurofins (Des Moines, IA) according to AOAC methods for the determination of fumonisin B1, deoxynivalenol, and zearalenone in corn, cereal, and maize products using HPLC.^{11–13} The limit of detection was 100 ng/g for fumonisin B1 and deoxynivalenol and 50 ng/g for zearalenone. Control diets with and without Grainsure E contained no detectable

mycotoxins (deoxynivalenol, T-2 tetraol, Fusarenone-X, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, T-2 triol, T-2 toxin, isoT-2 toxin, scirpentriol, nivalenol, 15-acetylscirpentriol, neosolaniol, HT-2 toxin, acetyl T-2, zearalenol, zearalenone, and FB1). Final dietary toxin concentrations were deoxynivalenol, $8.4 \pm 0.8 \mu\text{g/g}$ in toxins diet or $8.8 \pm 0.3 \mu\text{g/g}$ in toxins + Grainsure E diet; fumonisin B1, $7.3 \pm 0.6 \mu\text{g/g}$ in toxins diet or $11.7 \pm 0.8 \mu\text{g/g}$ in toxins + Grainsure E diet; zearalenone, $0.2 \pm 0.01 \mu\text{g/g}$ in toxins diet or $0.2 \pm 0.02 \mu\text{g/g}$ in toxins + Grainsure E diet. The homogeneity of the mycotoxins in the diets was satisfactory.

Animal Study Design. Animal use and related procedures were approved by the Iowa State University Committee on Animal Care. Eighty-four male (~ 106 g) and female (~ 93 g) Sprague–Dawley rats (21 days old) were purchased from Harlan Teklad (Madison, WI). After adapting to the control diet for 2 weeks, rats were randomly assigned to treatment diets so that mean initial body weights of the five groups were similar. The positions of the animal cages were changed once weekly. Rats were fed and food intake measured daily for 14 or 28 days. Forty animals (8 animals from each group) were euthanized on day 15, and the 44 remaining animals (8 animals from control, Grainsure E, and paired groups, and 10 animals from toxin and toxin + Grainsure E groups) were euthanized on day 29 (feed was withheld overnight prior to necropsy). All animals had free access to drinking water in a temperature-controlled (23°C) room with a light–dark cycle of 12 h.

Animal Health and Organ Pathology. Animal health was evaluated daily and body weight recorded weekly. Urine was collected in a metabolic cage on day 14 or 28. Blood was collected by cardiac puncture, heparinized, and centrifuged at 3000 rpm for 15 min to prepare plasma that was stored at -60°C until analysis. Liver, kidney, spleen, thymus, uterus, ovaries, and testes were collected, weighed, and fixed in 10% buffered formalin for histopathological analysis. After a week of fixation, tissues were trimmed, placed in cassettes, and subjected to routine processing and staining with hematoxylin and eosin. Liver, kidney, spleen, thymus, and testis or ovary, oviduct, and uterus were examined by Dr. Ronald K. Myers, Iowa State University Veterinary Pathology, board certified veterinary pathologist (without knowledge of the identity of the groups).

Determination of Clinical Chemistry in Rat Plasma. Plasma of each animal (500 μL) was tested by the Veterinary Pathology Laboratory, Iowa State University, with two replicates. Clinical chemical parameters including ion concentrations, blood urine nitrogen, creatinine, glucose, total protein, albumin, alkaline phosphatase (ALP), and alanine transaminase (ALT) were compared among treatments. A Boehringer Mannheim/Hitachi 912 System (Roche Diagnostics GmbH, Mannheim, Germany) was used, and end point assay was selected to indicate the component concentration. All reagent kits were obtained from Roche Diagnostics Corp. (Indianapolis, IN).

Determination of Sphingosine and Sphinganine in Rat Urine. High-performance liquid chromatography (HPLC) analysis of sphingosine and sphinganine was performed on a Varian instrument with a 9300 automatic injector (Palo Alto, CA). The model 470 fluorescence detector was from Waters Inc. (Milford, MA). The column used was a 15 cm \times 4.6 mm i.d., 5 μm , Supelcosil ABZ+Plus (Sigma-Aldrich, St. Louis, MO). The mobile phase was $\text{CH}_3\text{OH}/\text{H}_2\text{O}$ (9:1, v/v) and the flow rate was 1.5 mL/min. Two milliliters of urine from females or 10 mL of urine from males (due to lesser concentration of sphinganine and sphingosine in male urine) was extracted according to a published method.¹⁴ D-Erythro-C20-dihydrosphingosine was used as internal standard, and after extraction, samples were derivatized with o-phthalaldehyde for HPLC analysis using a fluorescence detector ($\lambda_{\text{ex}} = 334$ nm, $\lambda_{\text{em}} = 440$ nm). Standards were prepared by adding 1, 5, 10, 20, or 50 μL of 1 μM solutions of sphingosine and sphinganine, 10 μL of 1 μM C20, and 100 μL of o-phthalaldehyde to the mobile phase. Samples were left for at least 0.5 h at room temperature after the addition

Table 1. Comparison of Body Weight Gain and Food Intake of Different Groups after 14 and 28 Days of Feeding^a

treatment	14 days		28 days	
	male	female	male	female
Body Weight Gain (g)				
control	75.5 ± 6.7 a	41.3 ± 4.1 a	160.1 ± 14.0 a	80.3 ± 5.5 a
Grainsure E	81.1 ± 3.2 a	41.8 ± 5.2 a	127.7 ± 5.9 ab	85.1 ± 11.2 a
toxins	61.3 ± 4.5 b	37.7 ± 1.6 b	120.0 ± 12.2 b	58.1 ± 4.9 b
toxins + Grainsure E	64.7 ± 12.3 ab	41.3 ± 8.0 ab	120.7 ± 10.5 b	78.0 ± 6.1 a
pair-fed ^b	78.3 ± 2.1 a	40.1 ± 1.7 a	123.7 ± 18.5 b	75.1 ± 5.9 a
Food Intake (g)				
control	150.0 ± 28.4 a	137.7 ± 14.6 a	397.4 ± 52.8 a	403.7 ± 38.6 a
Grainsure E	144.7 ± 31.6 a	126.7 ± 38.6 a	403.7 ± 73.5 a	384.7 ± 87.5 a
toxins	87.3 ± 16.2 b	93.3 ± 17.6 b	248.3 ± 50.2 b	275.3 ± 59.5 b
toxins + Grainsure E	98.6 ± 28.0 ab	98.0 ± 21.0 ab	277.7 ± 59.5 ab	310.3 ± 70.1 ab

^aDifferent letters in a column indicate significant differences ($p < 0.05$). ^bThe food intake of the pair-fed group was given according to the average amount consumed by the animals in the toxin-fed group 1 day before.

of *o*-phthalaldehyde before injection to HPLC. Urine concentrations of sphingosine and sphinganine were calculated by establishment of calibration curves using the ratio of peak area of sphinganine or sphingosine to peak area of internal standard.

Statistical Analysis. Data are given as the mean ± SD. Differences in body weights, food intakes, organs weights relative to the body weights, clinical chemical parameters, and sphinganine and sphingosine concentrations in urine among treatment groups were evaluated statistically using a two-sample *t* test, ANOVA, and Tukey's multiple-comparison test by SAS 9.1. Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Body Weight Gain and Food Intake. After 14 days, the body weight gain was significantly less in rats fed toxins compared with control, Grainsure E, and pair-fed groups ($p = 0.036$), but rats fed toxin + Grainsure E did not differ compared with rats fed toxins or control diets in both males and females (Table 1). After 28 days, pair-fed male rats or those fed with toxins and toxins + Grainsure E showed significantly less body weight gain than controls, and females fed toxins showed significantly less body weight than controls (Table 1). No difference was seen in females fed toxins + Grainsure E compared with controls. Food intake was partly restored by Grainsure E in toxin-fed rats because the intake of this group did not differ from controls or toxin-fed rats after either time point in males or females.

The decreases of both body weight gain and food intake were important signs of mycotoxicoses in rats.¹⁵ Food intake reduction can be due to the anorexic effects of deoxynivalenol or the toxic effects of fumonisin B1.¹⁶ In our study, Grainsure E protected body weight gain only in females fed toxins after 28 days, indicating a sex difference in the mechanism of action of Grainsure E possibly related to interactions between the mycotoxin binder and endogenous estrogens and/or zearalenone.

Organ Weight Measurement. The relative kidney weight in toxin-fed males decreased 5.1% ($p = 0.036$) and thymus increased 7.4% ($p = 0.044$) compared with controls after day 14 (Table 2), whereas differences were not seen in toxins + Grainsure E fed males compared with control, Grainsure E, or pair-fed males. After day 28, the relative kidney weight decreased

significantly in males fed toxins or toxins + Grainsure E compared with control, Grainsure E, and pair-fed males, and males fed toxins showed 5.9% less kidney weight than did males fed toxins + Grainsure E ($p = 0.041$), indicating that Grainsure E counteracted nephrotoxic effects of the mycotoxins. No differences were found between the relative kidney and thymus weight in pair-fed and control males on day 15, but the relative thymus weight was less in the other four groups compared with controls on day 29. Because the relative liver or kidney weight was decreased by fumonisin B1,¹⁶ not affected by deoxynivalenol,¹⁷ or increased by zearalenone,¹⁸ relative organ weight changes may be complicated in indicating the combined effects of mycotoxins with different mechanisms and target organs. The significant effects of Grainsure E on the relative weights of spleen, testes, and thymus only after day 28 needs more study. In female rats, it is interesting that there were no significant differences in relative organ weights among treatments for females, except for increases of relative uterus and ovary weights in the other four groups compared with controls after day 14. Heneweer et al.¹⁹ studied different doses of zearalenone in rats and found that only 1 and 10 mg/kg bw (equivalent to 10 and 100 $\mu\text{g/g}$ in diet) significantly increased relative uterus weight compared with controls, but not 0.03, 0.1, and 0.3 mg/kg bw (equivalent to 0.3, 1, and 3 $\mu\text{g/g}$ in diet). The amount of zearalenone in our study was 0.2 $\mu\text{g/g}$ diet; therefore, zearalenone may not have caused uterine hypertrophy in our study. The reason we saw significantly greater weight of uterus and ovaries after day 14 might be related to some interaction between the treatments and estrus, which begins at 6–7 weeks after birth; this would be worth further investigation.

Determination of Clinical Chemistry in Rat Plasma. Grainsure E protected rats from nephrotoxicity and hepatotoxicity as shown by clinical chemical parameters. After day 14, females fed toxins differed from controls in increased chloride by 2.2% ($p = 0.042$) and decreased bicarbonate by 21.2% ($p = 0.021$, Table 3). The concentrations of phosphorus, magnesium, and ALP decreased, whereas chloride and glucose concentration increased in males fed toxins compared with controls, which were not seen in males fed toxins + Grainsure E. Similarly, the above clinical parameters of females fed toxins + Grainsure E did not differ compared with those of controls after day 14.

Table 2. Comparison of Relative Organ Weight^a of Different Groups after 14 and 28 Days of Feeding^b

treatment	no. per group	male					female
		kidney (%)	liver (%)	spleen (%)	testes (%)	thymus (%)	uterus and ovaries (%)
Day 14							
control	8	0.968 ± 0.026 a	4.570 ± 0.250 a	0.307 ± 0.024 a	1.341 ± 0.111 a	0.406 ± 0.017 a	0.307 ± 0.046 b
Grainsure E	8	1.066 ± 0.043 a	4.496 ± 0.086 a	0.313 ± 0.033 a	1.389 ± 0.086 a	0.377 ± 0.065 a	0.371 ± 0.108 a
toxins	9	0.919 ± 0.066 b	4.476 ± 0.697 a	0.284 ± 0.023 a	1.467 ± 0.092 a	0.436 ± 0.004 b	0.449 ± 0.071 a
toxins + Grainsure E	9	0.980 ± 0.033 a	4.348 ± 0.251 a	0.305 ± 0.006 a	1.357 ± 0.118 a	0.421 ± 0.023 a	0.468 ± 0.026 a
pair-fed	8	0.984 ± 0.047 a	4.451 ± 0.489 a	0.299 ± 0.056 a	1.353 ± 0.063 a	0.422 ± 0.040 a	0.509 ± 0.063 a
Day 28							
control	8	0.867 ± 0.055 A	3.816 ± 0.380 AB	0.243 ± 0.013 A	1.166 ± 0.065 C	0.372 ± 0.045 A	0.382 ± 0.063 A
Grainsure E	8	0.917 ± 0.055 A	3.714 ± 0.186 BC	0.220 ± 0.023 B	1.239 ± 0.108 B	0.284 ± 0.033 C	0.393 ± 0.240 A
toxins	9	0.754 ± 0.027 C	3.613 ± 0.284 BC	0.235 ± 0.020 AB	1.370 ± 0.138 A	0.325 ± 0.039 B	0.395 ± 0.153 A
toxins + Grainsure E	9	0.801 ± 0.012 B	3.366 ± 0.406 C	0.232 ± 0.010 AB	1.367 ± 0.105 A	0.311 ± 0.034 BC	0.369 ± 0.045 A
pair-fed	8	0.959 ± 0.079 A	4.239 ± 0.692 A	0.273 ± 0.042 A	1.406 ± 0.159 A	0.353 ± 0.045 B	0.399 ± 0.133 A

^aThe weight of every organ was relative to the body weight of each animal (%). ^bDifferent letters in individual columns indicate significant differences at the same time point (lower case letter for day 14 and capital letter for day 28, $p < 0.05$).

Table 3. Clinical Chemistry across Treatment Groups after 14 and 28 Days of Feeding^a

treatment	chloride (mequiv/L)	bicarbonate (mequiv/L)	calcium (mg/dL)	phosphorus (mg/dL)	magnesium (mg/dL)	creatinine (mg/dL)	glucose (mg/dL)	ALP ^b (IU/L)	ALT ^c (IU/L)
Day 14									
control (M ^d)	99.4 ± 1.1 b	23.5 ± 4.3 a	12.7 ± 0.5 a	18.6 ± 2.1 a	4.3 ± 0.5 a	0.4 ± 0.0 b	35.5 ± 20.5 b	181.5 ± 11.4 a	51.0 ± 14.3 a
Grainsure E (M)	97.6 ± 1.3 b	26.1 ± 3.1 a	12.6 ± 0.2 a	17.6 ± 1.8 ab	4.5 ± 0.4 a	0.4 ± 0.0 b	30.2 ± 14.1 b	189.0 ± 2.3 a	61.0 ± 7.3 a
toxins (M)	102.5 ± 1.2 a	26.6 ± 3.3 a	12.6 ± 0.6 a	14.6 ± 1.8 b	3.4 ± 0.3 b	0.4 ± 0.0 b	60.2 ± 13.5 a	157.0 ± 9.8 b	55.0 ± 12.5 a
toxins + Grainsure E (M)	101.4 ± 2.8 ab	27.3 ± 6.3 a	13.6 ± 1 a	15.4 ± 1.8 ab	3.8 ± 0.7 ab	0.5 ± 0.0 a	35.5 ± 58.1 b	177.5 ± 16.8 a	74.0 ± 22.9 a
pair-fed (M)	99.2 ± 1.2 b	26.6 ± 3.0 a	12.7 ± 0.4 a	15.6 ± 1.9 ab	3.9 ± 0.2 a	0.4 ± 0.1 b	46.3 ± 8.5 b	171.5 ± 7.1 a	67.5 ± 10.6 a
Day 28									
control (F)	101.5 ± 2.8 B	27.7 ± 2.3 A	12.1 ± 0.2 A	16.6 ± 2.2 AB	4.2 ± 5.0 A	0.4 ± 0.0 A	52.7 ± 14.4 A	114.2 ± 19.6 A	44.5 ± 6.2 A
Grainsure E (F)	103.9 ± 1.6 AB	23.0 ± 5.5 AB	12.0 ± 0.4 A	15.8 ± 0.8 A	4.2 ± 0.2 A	0.4 ± 0.0 A	81.7 ± 52.1 A	111.0 ± 19.2 A	42.5 ± 17.9 A
toxins (F)	106.7 ± 1.7 A	21.9 ± 1.9 B	12.1 ± 0.6 A	13.6 ± 1.4 B	3.4 ± 0.7 A	0.3 ± 0.1 A	57.7 ± 9.2 A	113.5 ± 19.3 A	52.0 ± 7.7 A
toxins + Grainsure E (F)	103.8 ± 1.0 AB	24.6 ± 2.4 AB	12.2 ± 0.5 A	15.3 ± 1.3 AB	3.7 ± 0.5 A	0.3 ± 0.0 A	68.5 ± 17.1 A	126.5 ± 10.9 A	40.7 ± 2.6 A
pair-fed (F)	102.0 ± 2.4 AB	27.0 ± 3.8 AB	12.3 ± 0.4 A	14.2 ± 1.2 AB	4.1 ± 0.6 A	0.3 ± 0.0 A	51.7 ± 7.3 A	91.5 ± 17.4 B	41.0 ± 4.7 A
Day 28									
control (M)	97.2 ± 1.6 b	37.2 ± 1.8 a	12.3 ± 0.2 a	15.9 ± 1.0 a	3.7 ± 0.3 a	0.3 ± 0.1 a	44.2 ± 17.0 b	117.5 ± 11.1 b	33.5 ± 4.4 b
Grainsure E (M)	99.4 ± 1.7 b	32.8 ± 2.5 a	12.2 ± 0.5 a	16.3 ± 1.6 a	3.9 ± 0.4 a	0.4 ± 0.1 a	65.6 ± 41.8 b	131.0 ± 9.3 a	30.5 ± 1.7 b
toxins (M)	101.1 ± 2.3 a	30.7 ± 4.8 b	12.2 ± 0.6 a	14.4 ± 0.7 b	3.7 ± 0.5 a	0.4 ± 0.1 a	88.5 ± 38.9 a	123.2 ± 9.9 a	65.2 ± 20.2 a
toxins + Grainsure E (M)	98.8 ± 1.6 b	35.3 ± 2.2 a	12.3 ± 0.7 a	15.0 ± 2.1 a	3.3 ± 0.4 a	0.3 ± 0.1 a	72.0 ± 30.6 b	116.0 ± 12.2 b	33.4 ± 3.1 b
pair-fed (M)	99.8 ± 3.0 b	34.2 ± 2.7 a	12.2 ± 0.3 a	14.1 ± 1.2 b	3.6 ± 0.1 a	0.4 ± 0.1 a	62.5 ± 50.7 b	111.5 ± 19.5 b	33.7 ± 3.6 b
Day 28									
control (F)	96.4 ± 1.2 A	33.9 ± 4.7 A	12.2 ± 0.1 A	15.3 ± 2.3 A	3.6 ± 0.1 A	0.4 ± 0.1 A	43.5 ± 21.1 B	83.2 ± 13.8 A	29.0 ± 3.3 B
Grainsure E (F)	99.3 ± 1.5 A	36.2 ± 1.4 A	12.3 ± 0.2 A	15.4 ± 0.9 A	3.6 ± 0.1 A	0.4 ± 0.0 A	63.0 ± 8.1 B	90.0 ± 11.6 A	30.0 ± 5.0 B
toxins (F)	100.7 ± 1.1 A	34.8 ± 2.7 A	11.8 ± 0.2 B	12.4 ± 0.8 B	3.4 ± 0.1 A	0.4 ± 0.1 A	70.0 ± 23.9 A	89.6 ± 19.4 A	40.2 ± 7.7 A
toxins + Grainsure E (F)	101.6 ± 2.4 A	32.3 ± 3.6 A	11.6 ± 0.3 B	11.7 ± 0.6 B	3.3 ± 0.4 A	0.4 ± 0.1 A	50.8 ± 26.8 B	85.2 ± 6.9 A	31.4 ± 10.8 B
pair-fed (F)	98.3 ± 1.5 A	30.0 ± 1.1 B	12.5 ± 0.2 A	15.7 ± 1.4 A	3.9 ± 0.2 B	0.4 ± 0.1 A	60.5 ± 34.9 B	89.7 ± 17.5 A	27.3 ± 10.3 B

^aDifferent letters in individual columns indicate significant difference for different sex (lower case letter for male and capital letter for female, $p < 0.05$). ^bALP, alkaline phosphatase. ^cALT, alanine transaminase. ^dM, male; F, female; 8 animals per sex in control, Grainsure E, and pair-fed groups, and 9 animals per sex in toxin and toxin + Grainsure E groups.

Previous studies showed increased serum chloride in rats given 0.25–1.0 mg/kg bw deoxynivalenol (2.5–10 $\mu\text{g/g}$ in diet),¹⁷ and lower serum calcium and phosphorus in pigs fed 10 μg of deoxynivalenol/g in diet compared with controls,²⁰ but elevated serum calcium and magnesium were found in rats fed fumonisin

B1 (7.5 $\mu\text{g/g}$), whereas serum chloride, phosphate, and ALP were not altered by fumonisin B1.¹⁶ In our experiment, significant increases of serum chloride and decreases of serum phosphorus and magnesium were found in rats fed the mixture of three mycotoxins, which may be a sign of nephrotoxicity, because

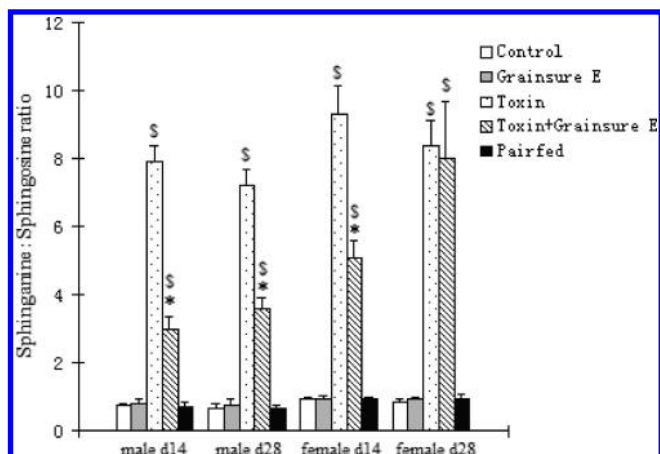


Figure 2. Ratio of free sphinganine/sphingosine in urine of male and female rats on days 14 and 28. \$ and * are representative of significant differences compared with control and toxin groups, respectively ($p < 0.05$). Each bar represents the mean \pm SD (8 animals per sex in control, Grainsure E, and pair-fed groups, and 9 animals per sex in groups fed toxins).

the concentrations of these ions in serum depend partly on the rate of renal excretion.²¹

After day 28, the concentrations of chloride and ALP increased in males fed toxins, but bicarbonate and phosphorus decreased compared with controls, whereas these changes did not occur in males fed toxins + Grainsure E. Phosphorus was also significantly decreased in pair-fed male rats, with no significant differences between pair-fed and toxin-fed males. This implies that the change of phosphorus was not due to toxins but to lesser food intake. Calcium and phosphorus both decreased in females fed toxins and toxins + Grainsure E. More interestingly, glucose was significantly elevated in toxin-fed animals but not in rats fed toxins + Grainsure E compared with controls. Deoxynivalenol increased blood insulin, glucose, and free fatty acid after subcutaneous treatment at 1 mg/kg bw (10 μ g/g in diet) in rats for 3 days, which might be due to harmful effects of deoxynivalenol on the pancreas.²² Therefore, Grainsure E acted against nephrotoxicity in terms of ion absorption and excretion as well as glucose elevation, probably induced by deoxynivalenol and fumonisin B1, and Grainsure E alone has no adverse effects on chloride, bicarbonate, calcium, phosphorus, and magnesium in either males or females, further indicating the protection of Grainsure E without affecting utilization and metabolism of essential minerals.

Grainsure E counteracted increased plasma ALT in toxin-fed male and female rats after day 28. ALT in males and females fed toxins increased by 95% ($p = 0.018$) and 39% ($p = 0.031$), but not in rats fed toxins + Grainsure E (Table 3). The increase of ALT, an indicator of hepatotoxicity, was also shown in fumonisin B1-¹⁶ or zearalenone-treated rats,²³ although at much greater zearalenone doses than in our study (>20 μ g/g of diet). Although deoxynivalenol is a protein synthesis inhibitor, no significant changes of the concentrations of cholesterol or protein were found between toxin-fed and control groups. This might be because fumonisin B1 induced increased serum total protein and cholesterol,¹⁶ whereas deoxynivalenol suppressed protein or cholesterol synthesis in mice.²⁴

Sphingosine and Sphinganine in Rat Urine. Fumonisin B1 inhibits ceramide synthase, resulting in an interruption of de

novo sphingolipid synthesis. Sphinganine or sphinganine/sphingosine was elevated in kidney and urine of rats fed fumonisin B1.²⁵ In our study, urinary concentrations of sphingosine and sphinganine and sphinganine/sphingosine did not differ significantly among males and females fed control, Grainsure E, and pair-fed diets. Sphinganine/sphingosine significantly increased by \sim 11-fold for males and by \sim 10-fold for females fed toxins ($p = 0.007$) compared with controls on day 14 (Figure 2), but male rats fed toxins + Grainsure E had less of an increase in sphinganine/sphingosine than those fed toxins alone ($p = 0.031$) on both days 14 and 28, whereas this was observed only at day 14 for females.

Grainsure E concentration (0.5%) was seemingly insufficient to protect against the toxic effects of fumonisin B1 when fed to female rats for the longer time. Females have been shown to be more sensitive than males to fumonisin B1 hepatotoxicity and males more sensitive than females to fumonisin B1 nephrotoxicity.²⁶ The effect of fumonisin B1 on sphingolipids may be more indicative of hepatotoxicity, which might explain the lesser efficacy of Grainsure E in females according to this measure. Neither glucomannan nor activated carbon was effective in mitigating effects of a mycotoxin mixture containing fumonisin B1²⁷ or fumonisin B1 alone²⁵ on sphingolipids. Grainsure E was at least partly protective in both males and females in preventing the inhibition of sphingolipid biosynthesis by fumonisin B1.

Histopathological Analysis of Organs. No consistent trends or important differences of size, amount, and location of cytoplasmic vacuoles in liver were noted across the groups or between sexes. No convincing morphologic evidence of hepatocellular toxic injury was found. However, after day 28, toxin-fed males (4 of 5 animals) had mild degenerative changes of kidney epithelial cells (hyaline/pyknotic cells in tubules with basophilic tubular clumps). This indicates that the kidney damage was mainly due to fumonisin B1 toxicity. This change was not seen in males fed toxins + Grainsure E after day 28, correlated with the finding that kidney weight was partly protected by Grainsure E after day 28 (Table 2). In spleen, all treatments showed normal extramedullary hematopoiesis, normal-appearing white and red pulp with normal periarteriolar lymphocytes at both times. Uteri of some rats had vacuoles and apoptotic cells in the endometrium and upper lamina propria, but the changes were present in all groups and were likely due to estrus, paralleled with the findings of greater relative weight of uterus and ovaries (Table 2). All testes appeared similar with variable but normal numbers of supporting cells, development stages, and mature spermatids within seminiferous tubules of the same tissue. No discernible differences were present among the thymuses; thus, the relative thymus weight differences were likely incidental (Table 2).

Several studies showed histopathological changes in mycotoxin-treated rats. Morsy et al.²⁸ reported that after treating rats with fumonisin (100 μ g/g diet) for 1 month, vacuolar degeneration in the epithelial cells of the renal tubules, hyaline casts scattered in the tubular lumina, and pyknotic nuclei were noticed. Hepatocyte death due to necrosis and apoptosis with swollen mitochondria and disintegrated cristae were consistently reported when rats were exposed to 50–250 μ g of FB1/g of diet.¹⁶ Male rats treated with 5 μ g of deoxynivalenol/g of bw (\sim 50 μ g/g in diet) for 28 days showed glandular stomach and pancreatic lesions, thymic lymphoid depletion, germ cell degeneration, and failure of sperm release.¹⁵ However, the toxicity was less severe when rats received less fumonisin B1 (10 or 1 μ g/g of diet) for 24 months compared with a higher dose (25 μ g/g of diet).²⁶

Therefore, the observation of discernible histopathological and morphological differences depends on the doses of mycotoxins.²⁹

In our study, no other differences between groups were seen in livers, thymuses, spleens, testes, ovaries, oviducts, and uteri, which might be due to the relatively low doses of fumonisin B1 (7–11 $\mu\text{g/g}$), deoxynivalenol (8 $\mu\text{g/g}$), and zearalenone (0.2 $\mu\text{g/g}$) fed.

In conclusion, Grainsure E prevented the mycotoxin (fumonisin B1 and deoxynivalenol) induced loss of body weight gain and food intake, hepatotoxicity, and nephrotoxicity in terms of decreased relative kidney weight, increased plasma ALT, elevated urinary sphinganine/sphingosine, and damaged kidney morphology, which supported partial protective effects of Grainsure E against these mycotoxins in rat. No evidence of reduced toxicity was found for zearalenone, which was probably due to the low dietary content of zearalenone. To our knowledge, the interactive effects of the mixture of deoxynivalenol, zearalenone, and fumonisin B1 have been studied in one other paper and only in vitro. Fumonisin B1 displayed antiestrogenic properties that antagonized effects of zearalenone. Addition of deoxynivalenol to zearalenone and fumonisin B1 was more effective in reduction of human colon cancer cell (Caco-2) viability and the apparent antiestrogenic effect of fumonisin B1 was less evident. The combination of the three toxins was not more effective in lipid peroxidation or in inhibiting DNA synthesis and methylation as compared to individual toxins, but the toxin combination was more effective than individual toxins in damaging DNA, suggesting an important aspect for further study.³⁰ We did not investigate the individual toxins in our study; antagonistic, additive, or synergistic effects of the combination of three toxins are possible. In light of these results, Grainsure E may be beneficial in reducing adverse effects of fumonisin B1 and deoxynivalenol in animals. Future research might focus on the study of immune responses and hormone status corresponding to deoxynivalenol and zearalenone. Additional end points, such as metabolites corresponding to the dysfunction of target organs of the toxins, should be included and analyzed. Ultrastructural analysis or morphometrics could also be utilized for further detailed histopathology.

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