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Effectiveness of Cholestyramine in the Detoxification of Zearalenone as Determined in Mice

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Zearalenone (ZEN), a ß-resorcyclic acid lactone, is a naturally occurring estrogenic mycotoxin produced by several *Fusarium* fungi that frequently occur in cereal crops in temperate regions of the world (Joffe 1986). Concentrations of zearalenone observed under field conditions range from less than 1 to over 300 ppm, but rarely exceed 10 ppm (Edwards *et al.* 1987). The presence of zearalenone in feedstuffs disrupts reproductive function in domestic animals (Mirocha *et al.* 1977). Female swine are particularly sensitive to zearalenone (Friend *et al.* 1990), and toxic effects may include constant estrus, pseudopregnancy and infertility (Diekman and Green 1992). Zearalenone binds to uterine and oviduct estrogen receptors (Coulombe 1993).

One method to alleviate the toxicity of mycotoxins is to eliminate or reduce absorption from the gastrointestinal tract using dietary additives. A number of compounds have shown limited success depending on the mycotoxin involved. Aluminosilicates alleviated aflatoxin toxicity (Harvey *et al.* 1989), while alfalfa reduced the toxic effects of zearalenone (James and Smith 1982). The deleterious effects of 250 mg/kg dietary zearalenone were eliminated by addition of 5% dietary zeolite (Smith 1980). Bentonite was shown to bind aflatoxin *in vitro* (Masinmango *et al.* 1979) and protect against T-2 toxicosis *in vivo* (Carson and Smith 1983). Explanations of mechanisms of action of these compounds include ionic binding between the agent and toxin, alteration of enterohepatic bile acid circulation, and entrapment of the toxin molecule in the matrix of the binding agent.

Cholestyramine is an insoluble quaternary ammonium anion exchange resin used successfully as an antihypercholesteremic treatment (Kolata 1984). Anion exchange resins strongly bind bile acids (Tennent *et al.* 1960) and various other anionic compounds (Gallo *et al.* 1965) and may weakly adsorb neutral or cationic compounds by non-specific binding. It is possible that one or more of these binding mechanisms may be utilized to reduce mycotoxin toxicity. Cholestyramine has been shown to

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increase ochratoxin A excretion in the rat (Madhyastha *et al.* 1992) and to inhibit the enterohepatic circulation of this mycotoxin in mice (Roth *et al.* 1988). *In vitro* incubations of 0.1 μg ZEN/mL with increasing concentrations of cholestyramine from 0.001 to 1.00 mg/mL in our laboratory showed that partial (11%) and 100% binding occurred at 0.025 mg/mL and 1.00 mg/mL cholestyramine, respectively. To test this agent further, *in vivo* studies were conducted.

The prepuberal mouse uterine weight bioassay has been used extensively for detection of estrogenic compounds. Thigpen *et al.* (1987a) proposed a standard procedure for the bioassay, and determined the estrogenicity of various compounds relative to diethylstilbestrol (Thigpen *et al.* 1987b). Some work has been done with zearalenone using the uterine weight bioassay (Smith 1980), but doses of zearalenone were high (50 ppm-500 ppm), no purity of toxin was reported, and details of the assay were not described. The authors are not aware of any similar dose response research using pure zearalenone in the diets of prepuberal mice. We report here the adaptation of the prepuberal mouse uterine weight bioassay for dietary zearalenone, and the use of the assay to evaluate the efficacy of cholestyramine to reduce the toxicity of zearalenone. This assay could be used to detect zearalenone in grains or feeds, and to evaluate potential zearalenone detoxification treatments before proceeding to more expensive and time-consuming studies in domestic livestock.

MATERIALS AND METHODS

In three trials, 15 d old weanling female outbred mice (ICR) from the Centre for Food and Animal Research mouse colony were blocked by body wt and allocated randomly to treatments within each block. Animals were housed 4 per cage in transparent polypropylene cages. The feed used for preparation of all diets was Certified Rodent Diet 5002 (PMI Feeds, Inc., Missouri). There was no detectable (<0.10 mg/kg) zearalenone in the basal diet. Purified zearalenone (International Mineral and Chemical Corp, Indiana), and cholestyramine (Bristol-Myers, Inc.) were added to diets using the method described by Rotter *et al.* (1992). All experimental diets were analyzed for zearalenone to confirm the uniformity of mix. Feed and water were available ad libitum. Initial and 5 d body wt, and 5 d uterine wt were recorded for each mouse.

The bioassay was conducted as described by Thigpen *et al.* (1987a). On the fifth day of treatment, mice were euthanized using carbon dioxide, the reproductive tract was excised, oviducts and ovaries were removed, the uterus was trimmed and blotted. Interuterine fluid was expressed by gentle pressure on the tissue from the blunt edge of a scalpel blade. Uterine wt was measured using an analytical balance (Sartorius A200S, Germany).

In each of the three trials, a randomized block design was used, with four mice in a cage considered sub-sampling. In Trial 1, there were six replicates of four dietary zearalenone levels (0, 2, 4, 8 mg/kg diet). The second trial included a control diet

and four diets with increasing levels of cholestyramine (0, 1.25, 2.5, and 5 g/kg), each supplemented with 6 mg/kg zearalenone. The five dietary treatments were included in each of three replicates. The results of Trials 1 and 2 were then used to determine the doses of zearalenone and cholestyramine that might serve as controls for future bioassays of contaminated (or suspect) feedstuffs, and potential binding agents. These doses were tested using a larger number of mice in the third trial, with five replicates of a 2 x 2 factorial involving two concentrations of zearalenone (0, 6 mg/kg diet) and 2 concentrations of cholestyramine (0, 2.5 g/kg diet).

Analyses of variance were applied to wt gain (Day 5 body wt-Day 1 body wt), uterine wt, and relative uterine wt (uterine wt/body wt x 100). Orthogonal polynomials (Snedecor and Cochran 1980) were used to examine the response to increasing level of cholestyramine in the diet. The t-test was used to compare diets with and without added zearalenone.

RESULTS AND DISCUSSION

In Trial 1, a generally linear dose response of uterine wt and relative uterine wt to dietary zearalenone was observed ($p<0.0001$) (Table 1). The quadratic component of the dose response was not significant, but as the concentration of ZEN increased from 4 to 8 mg/kg, a levelling off (12% increase) of the dose response was observed, as compared with the increase in uterine wt observed between 2 mg and 4 mg ZEN/kg diet (21%), or between 4 and 8 mg ZEN/kg diet (18%). Previous studies have shown that positive estrogenic stimulation for this age of prepuberal mouse is indicated by an increase in uterine weight to approximately 25 mg (Bickoff *et al.* 1962).

Table 1. Body weight gain, uterine weight and relative uterine weight of prepuberal mice consuming dietary zearalenone (ZEN) (Trial 1)[1]

n[2]	ZEN (mg/kg diet)	Wt gain (g)	Uterus wt (g)	Relative uterus wt (%)
6	2	2.20	0.0178	0.164
6	4	2.16	0.0208	0.194
6	8	2.21	0.0236	0.217
	SEM	0.27	0.0012	0.008
	Linear trend	$p>0.9800$	$p<0.0001$	$p<0.0001$

[1] Initial average body weight ($\pm$ SD) of mice in Trial 1 was 8.57 ± 0.29,
[2] Number of cage replicates per treatment. Each replicate had 4 mice per cage.

Cochran and Cox (1957) describe a procedure for estimating the probability of obtaining significant differences between treatments in future experiments of a given sample size when the underlying population parameters are known. Assuming the results for relative uterine wt in Trial 1 accurately reflect these parameter values it was estimated that the probability of obtaining a significant ($P<0.05$) difference between controls and 4, 6 and 8 mg ZEN/kg diet was 80, 90 and 96%, respectively, if 6 replicates of 4 mice are used. Since the purpose of future experiments will be to determine the reduction in toxicity by various detoxification treatments, it is important that a significant difference between control and toxin treatment be obtained. Thus it was decided to opt for 6 mg ZEN/kg diet, that is, a level where a significant difference could be expected in 9 out of each 10 trials.

In Trial 2, the relative uterine wt was higher in mice consuming dietary zearalenone than in control mice ($p<0.01$) and it decreased ($p<0.0001$) with increasing level of cholestyramine (Table 2). Because there was a slight indication of decreased wt gains at the 5.00 g CHOL/kg diet, a concentration of 2.50 g CHOL/kg diet was selected for future trials. In addition, the mean relative uterus wt of control mice was higher than in the previous trial, due mainly to three mice with enlarged uteri.

In Trial 3, a significant zearalenone x cholestyramine interaction was observed for both uterine weight ($p=0.002$) and relative uterine weight ($p=0.003$). The addition of 2.50 g cholestyramine/kg diet reduced the effect of 6 mg/kg dietary zearalenone

Table 2. The effect of three levels of cholestyramine (CHOL) on weight gain, uterine weight and relative uterine weight of mice consuming 0 or 6 mg/kg dietary zearalenone (Trial 2)[1]

n	ZEN (mg/kg diet)	CHOL (g/kg diet)	Wt gain (g)	Uterus wt (g)	Relative uterus wt (%)
3	0	0	0.99	0.0165	0.170
3	6	0	1.36	0.0221	0.222
3	6	1.25	1.15	0.0159	0.165
3	6	2.50	1.39	0.0157	0.157
3	6	5.00	0.71	0.0119	0.127
		SEM	0.22	0.0012	0.011
		Linear trend[2]	$p<0.04$	$p<0.0001$	$p<0.0001$

[1] Initial average body weight ($\pm$ SD) of mice was 8.57 ± 0.26.
[2] Among zearalenone-containing diets only.

in prepuberal mice (Table 3). Relative uterine wt of zearalenone-fed mice was 44% higher than control and it decreased by 19% when cholestyramine was added.

As a general observation, considerable variation among animals was noted as previously observed by Thigpen et al (1987b). Ueno et al (1974) studied the effect on uterine wt of a daily oral dose of 2 mg/kg body wt zearalenone for 8 days in 3 wk old mice. Initial body weight in these mice was 12.4 ± 0.9. They reported that uterine wt increased for the first 5 days of the trial (from 15 to 35 mg), then decreased for the next 10 days to the starting level, then increased rapidly to 50 mg. Preliminary trials to characterize age at puberty of the strain of mice used for the present study revealed that rapid uterine growth occurs by 22 days of age. It is therefore essential that this strain of mice be younger than 22 d at the end of the bioassay. Individual bioassays should be conducted using the same strain, age, body weight and diet type to obtain consistent results. Housing conditions are also important; during preliminary trials it was determined that 15 d old weanling mice should be housed in groups rather than individually (as they consume more feed and gain more weight). Variation in health status, growth rate and maturity will also influence results. Preliminary trials must be conducted in each laboratory to determine the number of animals required to provide the precision necessary to make detection of a significant difference between control and toxin diets probable.

The present study has shown that under conditions described in Trial 1, there would be about an 80% chance of observing a significant difference in relative uterine weight between mice fed control and 4 mg ZEN/kg in a commercial rodent diet. While this probability might be considered high enough to detect a ZEN effect, the concentration of ZEN in the diet should be increased if the assay is intended to test detoxification treatments. Dietary cholestyramine (2.5 g/kg diet) will reduce the toxic effects of 6 mg ZEN/kg diet. Cholestyramine can be used as a positive control in the evaluation of other ZEN detoxification methods. Future studies will focus on

Table 3. Body weight gain, uterine weight, and relative uterine weight in prepuberal mice consuming dietary zearalenone and cholestyramine (Trial 3)[1]

n	ZEN (mg/kg diet)	CHOL (g/kg diet)	Wt gain (g)	Uterus wt (g)	Relative uterus wt (%)
5	0	0	1.68	0.0128	0.128
5	0	2.5	2.13	0.0140	0.135
5	6	0	1.85	0.0186	0.184
5	6	2.5	1.72	0.0149	0.149
		SEM	0.18	0.0006	0.006

[1] Initial average body weight (± SD) of mice was 8.25±0.28.

further optimization of cholestyramine concentration in the diet and testing of cholestyramine in naturally contaminated feedstuffs.

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