Ponderosa Pine Needle Induced Parturition in Cattle: Analysis for Presence of Mycotoxins

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Ponderosa pine (*Pinus ponderosa*) needles were fed to pregnant beef cows. Mean gestation lengths of 250.6 days for winter pine needles (PN) and 253.2 days for summer PN were reduced ($p < 0.001$) compared to controls (274.8 days). The levels of nine mycotoxins in fresh Ponderosa PN from trees, old PN from the ground, dry PN from trees, and PN collected from trees cut in the winter and summer were below detectable limits. The mycotoxins evaluated included aflatoxins B_1, B_2, and G_1, zearalenone, zearalenol, ochratoxin, T-2 toxin, diacetoxyscirpenol (DAS), and deoxynivalenol (DON). All of the collections of PN analyzed were either known to induce or believed to induce abortion in cattle. Inadequate cleanup prevented evaluation of zearalenone and zearalenol in winter-collected PN and dry PN from trees and T-2 toxin and DAS in winter-collected PN, fresh PN collected from trees, old PN from the ground, and dry PN collected from live trees. Pine needles collected from trees cut in the summer induced early parturition in 100% of the pregnant cows fed these PN and were successfully evaluated for all nine mycotoxins; however, levels were below detectable limits. The mycotoxins evaluated in this study do not appear to be involved in PN abortion.

INTRODUCTION

Pine needles (PN) from the Ponderosa pine (*Pinus ponderosa*) cause premature parturition or abortion in pregnant cows (Bruce, 1927; MacDonald, 1952; Stevenson et al., 1972; James et al., 1977). Pine needle induced parturition usually occurs in the fall, winter, and early spring, which generally corresponds to the last trimester of pregnancy; however, abortions may occur earlier. For a current review of the syndrome of PN-induced parturition in cattle, refer to James et al. (1989).

Chow et al. (1974) and Anderson and Lozano (1977) suggested a mycotoxin as the abortifacient factor inducing premature parturition in cattle ingesting Ponderosa PN. Reproductive failure occurred in mice after ingestion of lab chow mixed with a freeze-dried aqueous fraction pressed from a PN water slurry. Chow et al. (1974) suggested that mycotoxins rather than PN caused the reproductive failure. Therefore, ground PN were autoclaved and then reseeded with 1 g of ground PN and allowed to incubate for 2 weeks at room temperature. The mixture was filtered and the filtrate freeze-dried and mixed with lab chow. Subsequently, the same procedure was used except the mixture was autoclaved to inactivate any fungus that might be present. The inactivated mixture did not cause reproductive failure in mice, whereas the mixture believed to have active fungal growth did cause reproductive failure (Chow et al., 1974).

In mycotoxin-associated diseases, abortion alone occurs infrequently in the absence of other manifestations. Generally mycotoxicosis manifests a combination of the following syndromes: hepatotoxicity, nephrotoxicity, hemopoietic changes, dermonecrotic effects, reproductive or endocrine disturbances not associated with abortion, pulmonary dysfunction, CNS effects, gastrointestinal disturbance, decreased immune response, teratogenesis, and carcinogenesis.

There are several nonabortion disease entities in livestock associated with ingestion of mycotoxin-contaminated feeds (Hesseltine, 1979; Buck et al., 1976). However, there are toxigenic fungi which have been incriminated in bovine abortion such as *Aspergillus ochraceus* (ochratoxin), *Penicillium roquefortii*, and *Claviceps paspali* (ergot) (Hesseltine, 1979; Buck et al., 1976).

Observations in the field and research results provided information to suggest that PN are more potent inducers of early parturition in winter or early spring than summer. This suggestion has prompted some ranchers and researchers to speculate that perhaps a cool-season mycotoxin may be responsible. Therefore, this experiment was conducted as a first step to answer two questions: (1) Were any of the potent mycotoxins routinely screened in animal feeds present on or in the PN? (2) Is there a difference in the parturifacient potential between PN collected from trees in the winter versus the summer?

EXPERIMENTAL PROCEDURES

Plant Preparation. Pine needles were collected in the vicinity of John Day, OR, where abortion in cattle due to ingestion of PN has occurred (Stevenson et al., 1972). Large collections of PN were made in 1983 from both winter-cut and summer-cut trees for feeding trials in cattle. The PN were stripped from the trees and allowed to air dry in a covered shed. Pine needle samples were collected from each of these larger collections of winter-cut and summer-cut pines, from the dead PN on the ground shed from living trees, from fresh PN from living trees, and from dead brown PN picked from living trees. All PN samples were bagged separately and sent to the Laboratories of Veterinary Diagnostic Medicine, Toxicology Section, University of Illinois, for mycotoxin analysis. Pine needle samples were analyzed for nine mycotoxins commonly screened in animal feeds: aflatoxins B_1, B_2, and G_1, zearalenone, zearalenol, ochratoxin, T-2 toxin, diacetoxyscirpenol (DAS), and deoxynivalenol (DON).

Sample Preparation for Analysis. The samples were prepared and analyzed according to methods based on an unpublished method (George Rottinghaus, Veterinary Diagnostic Laboratory, University of Missouri—Columbia). All solvents were of HPLC grade, and all chemicals were of analytical grade. Two 100-g portions of each sample were ground and extracted with 200 mL of acetonitrile in 4% aqueous potassium chloride solution (9:1; one portion to serve as the spike). Fifty milliliters of this extract was then filtered through Whatman No. 4 filter paper.

To prepare the sample for aflatoxins B_1, B_2, and G_1, zearalenone, zearalenol, ochratoxin A, T-2 toxin, and DAS analyses, 20 mL of the filtrate was mixed with 15 mL of distilled water and 100 μL of glacial acetic acid and then passed through an activated C-18 cartridge (Daigger Scientific, Chicago, IL). This was followed by a wash using additional acetonitrile/4% KCl solution. Samples visually appraised and determined to not be sufficiently cleaned up by the C-18 cartridge were next passed through a column packed with 1.5 g of Florisil (D-100, 60–100 mesh) (Fisher Scientific Co., Itasoa, IL) activated with 4 mL of methylene chloride and solvated with 4 mL of acetonitrile. This was followed by a rinse with 9:1 acetonitrile plus water. Ten milliliters of HPLC grade chloroform was added to the eluate which was then shaken and centrifuged at 2000 rpm for 3 min. The top layer was discarded. The remainder was concentrated to dryness with compressed air, transferred to small spotting vials by using chloroform (approximately 3 × 2 mL), and again concentrated to dryness. It was then redissolved with 100 μL of benzene/acetone (98:2). Four microliters was used in the analysis for the aflatoxins, zearalenone, zearalenol, and ochratoxin A as described below.

The remaining 96 μL of the above preparation, used for analysis for T-2 toxin and DAS, was air-dried, redissolved in 0.5 mL of chloroform, and added to a prewetted (2 mL of chloroform) column packed with 1 g of basic alumina (Brockman Activity Grade 1) (Sigma Chemical Co., St. Louis, MO) topped with a 4-mm layer of anhydrous sodium sulfate. The vial was rinsed with 1 mL of chloroform after the sample was added to the column. The material was eluted with 30 mL of chloroform, the first 5 mL being discarded. This eluate was concentrated to dryness and redissolved with 100 μL of benzene/acetone (98:2).

Ten milliliters of the original filtrate was treated as previously described for the other mycotoxins but was further cleaned up for DON analysis by using a Clin Elut column (Analytichem International, Harbor City, CA). Sample material was then concentrated to dryness by using compressed air, transferred to small spotting vials by using 3 mL of 2:1 HPLC grade acetone/methanol, and again concentrated to dryness prior to analysis for DON.

Mycotoxin Analyses. To analyze for aflatoxins B_1, B_2, and G_1, zearalenone, zearalenol, ochratoxin A, T-2 toxin, and DAS, 4 μL of each sample, redissolved in the benzene/acetone mixture, was spotted on Whatman LHPK TLC plates. Each sample was spotted together with standards at a level of 20 ng of aflatoxins B_1, B_2, and G_1, 1 μg of zearalenone and zearalenol, 50 ng of ochratoxin A, and 1 μg of T-2 and DAS. Standard quantities (0.5 μg/g) of each mycotoxin were spiked onto PN samples and subsequently extracted and analyzed as described for each mycotoxin. Recoveries from the same matrix were ≥80%. The plates were developed in toluene/ethyl acetate/acetone (3:2:1) containing 2% formic acid. Plates were evaluated under long-wavelength ultraviolet light for the aflatoxins, zearalenone, zearalenol, and ochratoxin. Plates were sprayed with chromotropic acid [98% disodium salt of 4,5-dihydroxynaphthalene-2,7-disulfonic acid (Sigma)] and heated for 4–5 min in a 110 °C oven and evaluated under normal light for T-2 and DAS.

To analyze for DON, the samples were redissolved in 100 μL of acetone/methanol (2:1). Four microliters of samples and spikes with 1 μg of DON standard were spotted on Whatman LHPK TLC plates. These were developed in toluene/acetone (1:1). The plates were then sprayed with aluminum chloride solution (Mallinckrodt Inc., St. Louis, MO) (20 g of aluminum chloride in 100 mL of methanol) followed by heating in an oven at 110 °C for 4–5 min. Plates were evaluated under ultraviolet light.

FEEDING TRIALS

Fifteen pregnant cows of mixed breeding (Hereford × Charolais × Tarentaise) with known breeding dates were assigned to three treatment groups. Group 1 cows were controls and were group-fed 8.4 kg/cow per day chopped grass/alfalfa hay mixed and divided into a.m. and p.m. feedings. Group 2 cows were group-fed chopped PN at 3.0 kg/cow per day from the winter collection mixed with 5.7 kg/cow per day of chopped grass/alfalfa hay, and group 3 cows were group-fed 2.6 kg/cow per day of chopped PN from the summer collection mixed with 5.8 kg/cow per day of chopped grass/alfalfa hay. The average daily amount fed was divided into a.m. and p.m. feed. Frequently, throughout each day, the PN hay mixture was hand-mixed to entice cows to eat the mixture. All cows had free access to fresh water and trace mineralized salt. The quantity of feed given to treatment cows equaled the amount given the control groups.

Statistical Analysis. The mean gestation lengths and calf birth weights between treatment groups and controls were compared by a one-way analysis of variance. Means were separated by Fisher's least significant difference (LSD).

Table I. Results of Mycotoxin Analyses on Pine Needle Samples

mycotoxin	sample[a] A	B	C	D	E	DL[b]
aflatoxin B_1	–	–	–	–	–	10 ppb
aflatoxin B_2	–	–	–	–	–	10 ppb
aflatoxin G_1	–	–	–	–	–	10 ppb
zearalenone	*	–	–	–	*	0.5 ppm
zearalenol	*	–	–	–	*	0.5 ppm
ochratoxin	–	–	–	–	–	0.5 ppm
T-2 toxin	*	–	*	*	*	0.5 ppm
diacetoxyscirpenol	*	–	*	*	*	0.5 ppm
deoxynivalenol	–	–	–	–	–	0.5 ppm

[a] A, Winter-collected pine needles; B, summer-collected pine needles; C, old pine needles collected off the ground; D, fresh pine needles collected from live trees; E, dead dry pine needles collected from live trees. –, Below detectable limits; *, inadequate clean up and could not complete the analysis. [b] DL, Detectable limits in ppb (parts per billion) or ppm (parts per million). Detection limits of established laboratory protocol were used and verified by spiking the PN matrix with each mycotoxin at the DL and subsequently extracting spiked PN matrix. TLC plates from spiked PN matrix were visually compared with TLC plates spotted with each mycotoxin standard at the DL.

Table II. Effects of Winter and Summer Pine Needles (PN) on Pregnant Cows

no. of calves born	treatment	PN fed/cow per day, kg	mean days of gest	calf wt,[b] kg	no. of calves that died/no.
5	control	0	274.8 ± 8.0	29.9 ● 8.1	0/5
5	winter PN	3.0	250.6[a] ± 14.3	26.2 ± 6.5	4/5
5	summer PN	2.6	253.2[a] ± 9.2	24.7 ± 4.2	3/5

[a] Mean day of gestation when parturition occurred was significantly earlier ($p < 0.001$) in the pine needle fed groups compared to controls (LSD = 16.07). [b] Mean calf weights were not significantly different between groups ($p > 0.05$).

RESULTS AND DISCUSSION

All PN samples evaluated analytically were negative or below detectable limits for subject mycotoxins (Table I). Because of the nature of some of the PN samples, the pigments could not be adequately removed for quantitation of some of the spots on the TLC plates. Therefore, we were unable to evaluate winter PN for zearalenone, zearalenol, T-2 toxin, or DAS, and we were unable to evaluate PN picked from the ground, fresh PN, or dry PN from live trees for T-2 toxin or DAS. Because we were able to adequately clean up the summer-collected PN and analyze for all nine mycotoxins (these PN induced early parturition in all cows fed), we determined that we had sufficient data for this early study.

All cows fed winter-cut or summer-cut PN delivered calves early (Table II). The mean stage of gestation at delivery was 274.8 days for controls, 250.6 days for winter-cut needles, and 253.2 days for summer-cut needles. The treatment cows delivered calves significantly earlier ($p < 0.001$) in both PN-fed groups compared to controls, but there was no significant difference ($p > 0.05$) between the two PN groups. Seven of 10 calves in the PN groups died

from respiratory complications due to the premature parturition. These calves appeared normal considering the stage of gestation when parturition occurred. All calves received individual care which contributed to the survival of three calves from PN-fed cows.

The compounds responsible for abortions from PN are not known. Concentration may change in Ponderosa pine as a function of temperature, moisture, soil types, and location, and even between individual trees, and that may account for some of the variation observed in the incidence of PN-induced abortions from year to year. In 1983, when the PN in this study were collected, the precipitation was higher than normal (19.13 in.) compared to an annual average of 13.60 in. from 1957 to 1985 or 15.55 in. from 1977 to 1985. (Annual precipitation records for the John Day, OR, vicinity were provided by the city of John Day, OR.) Until we know the abortifacient involved, we will not be able to measure the variability between trees in different locations, years, types of trees, etc.

Some workers have suggested that mycotoxins are responsible for PN abortion (Chow et al., 1974; Anderson and Lozano, 1977). But results of this experiment suggest that none of nine mycotoxins analyzed were responsible for the abortifacient action of the PN. Experiments by other workers suggesting fungal components of PN as the cause of the abortions used mice as the animal model, and their results are regarded as inconclusive as far as the effects on cattle are concerned (Chow et al., 1974; Anderson and Lozano, 1977). Cattle and mice are, of course, much different in their mode of placentation as well as in their digestive systems.

In the experiment reported here, fungal growth was not observed on the needles and all needle material was kept dry and in a cool place. The experiment does not eliminate the possibility that an unknown mycotoxin may be involved. However, in a mycotoxin-induced condition, one might expect other pathological or clinical manifestations of toxicosis besides early parturition, such as hepatotoxicity, hemopoietic changes, dermatitis, pulmonary dysfunction, CNS effects, gastrointestinal disturbances, or decreased immune response. Hematological, serological, and pathological findings from previous experimentation in which parturition was induced with PN were nonspecific (Stuart et al., 1989).

The physiological mechanism of abortion in cattle from PN is not known. Normally the processes of pregnancy maintenance and parturition are under the control of complex hormonally balanced events influenced by stress, nutrition, etc. Present information suggests that the abortifacient action of PN involves the hormones of pregnancy. The abortifacient action appears to be specific to cows because sheep (Call and James, 1976) and goats (Panter et al., 1987) cannot be induced to abort by using PN.

Even though this study does not eliminate mycotoxins as a primary cause of PN-induced parturition in cattle, it has provided important evidence and a first step in elucidation of this question. These data suggest that the nine mycotoxins evaluated are probably not involved in the PN-induced parturition; however, other mycotoxins may be. Because of the amount of information known about the tested mycotoxins and their potency, it is important that they be eliminated as contaminants of PN. Further research is needed to elucidate the toxins on or in PN and to understand the mechanism of action in the cow.

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Registry No. DAS, 2270-40-8; DON, 51481-10-8; aflatoxin B_1, 1162-65-8; aflatoxin B_2, 7220-81-7; aflatoxin G_1, 1165-39-5; zearalenone, 17924-92-4; zearalenol, 36455-72-8; ochratoxin A, 303-47-9; T_2 toxin, 21259-20-1.