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The serum concentrations of lupine alkaloids in orally-dosed Holstein cattle

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ABSTRACT

Teratogenic alkaloid-containing *Lupinus* spp. cause congenital defects known as crooked calf disease that is periodically economically devastating for the cattle industry. Previous research indicates that cattle breeds may eliminate plant toxins differently, potentially altering their susceptibility. The objective of this study was to describe the toxicokinetics in Holsteins of anagyrine, the teratogenic lupine alkaloid that produces crooked calf disease. Other alkaloids including lupanine, an unidentified alkaloid and 5,6-dehydrolupanine were also evaluated. Dried ground *Lupinus leucophyllus* was orally dosed to four Holstein steers and blood samples were collected for 96 h, analyzed for serum alkaloid concentrations and toxicokinetic parameters calculated. The serum elimination of anagyrine in Holstein steers was faster than those reported for beef breeds. This suggests that Holsteins may be less susceptible to lupine-induced crooked calf disease. Additional work is needed to confirm these findings and to verify if there is a breed difference in disease incidence or severity.

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1. Introduction

There are more than 500 species of lupine in the genus *Lupinus*. Some species contain quinolizidine and/or piperidine alkaloids, both of which can be teratogenic and/or acutely toxic to livestock species. These teratogenic alkaloid-containing lupines cause significant losses to the livestock industry in the western United States due to the morbidity and mortality of calves suffering from what is colloquially termed "crooked calf disease" (Panter et al., 1999). The pathogenesis of crooked calf disease has been identified as lupine alkaloid inhibition of fetal movement during specific, critical periods of embryogenesis (days 40–100 of gestation in cattle). Lupine-induced terata have been classified as palatoschisis (cleft palate), ankylosis and arthrogryposis (angular limb deformities including axial rotation and joint fusion), and scoliosis, torticollis, and kyphosis (vertebral and spinal deformities) (Green et al., 2013a). The severe changes can result in dystocia, inability to stand and nurse and these are often fatal. Less severe terata can impair the calf's ability to nurse resulting in retarded growth. Angular appendicular and axial lesions are permanent and many become progressively worse as the calf ages and arthritis develops as the joints and limbs are subjected to greater load-bearing stress. Palatial lesions impair the calf's ability to nurse and often result in inhalation pneumonia. The frequency of crooked calves in cattle herds can vary based on lupine abundance which is dependent on precipitation (Ralphs et al., 2011). During periods of high precipitation and the resulting high teratogenic lupine abundance, the frequency of crooked calf deformities can be up to 100% of a calf crop (Panter et al., 2012).

The plant alkaloids associated with crooked calf disease have been characterized and one prominent teratogenic alkaloid that has been identified is the quinolizidine alkaloid anagyrine. Various animal studies have indicated that lupines containing anagyrine at concentrations equal to or greater than 1.5 g/kg dry plant material have the potential to cause terata in cattle (Keeler, 1973, 1976). Fourteen North American lupine species have been identified by Davis and Stout (1986) that have anagyrine above the 1.5 g/kg threshold concentration that could be potentially teratogenic. At this laboratory, *in vitro* research has shown that anagyrine acts as a pharmacological agonist at fetal muscle-type nicotinic acetylcholine receptors (nAChR) (Green et al., 2010). In addition to anagyrine, the piperidine alkaloid ammodendrine is also found in some lupine species and it also acts at nAChR to inhibit fetal movement and cause crooked calf disease (Green et al., 2010, 2013a; Keeler and Panter, 1989). Other related but less well investigated teratogenic alkaloids found in lupine species include *N*-acetyl hystrine and *N*-methylammodendrine (Panter et al., 1998). These teratogenic alkaloids are quickly absorbed from plant material in the rumen, can be detected in blood plasma almost immediately after oral dosing, and reach maximum serum concentrations at approximately 24 h post exposure (Gardner and Panter, 1993, 1994). While much is known about the binding of teratogenic alkaloids to the receptor and the

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pathogenesis of crooked calf disease, little is known about the variation in individual animal susceptibility to these alkaloids or if there are biomarkers or genotypes that are associated with different responses to lupine-induced disease and if these markers might be used to select animals with greater resistance to disease.

Lupine spp. are native, long-lived perennials with extensive seed banks that can be viable for centuries. Population control in most situations is expensive or impossible. Consequently there is a need to develop animals or management strategies that might be safely used in lupine containing plant communities. Identification of plant toxin-specific biomarkers in livestock would increase the precision by which animals are selected for resistance to poisoning. This includes the quantification of individual animal and breed differences in response to specific toxic plants. For example, recent research at this laboratory has identified differences in larkspur (*Delphinium* spp.) alkaloid toxicokinetics between Angus and Holstein cattle with Holstein cattle eliminating larkspur toxins faster than Angus cattle (Green et al., 2012, 2013b).

The purpose of this research was to determine the serum toxicokinetics of the four most abundant alkaloids in the lupine species *Lupinus leucophyllus* in Holstein steers for comparison with other breeds of cattle. Holsteins were selected for this research because they are genetically uniform, and have distinct differences from beef breeds that have been used in past lupine experiments (Lee et al., 2008a, 2008b; MacEachern et al., 2009; VanRaden et al., 2011). Moreover, Holsteins have been shown to eliminate larkspur alkaloids from *Delphinium andersonii* faster than Angus cattle (Green et al., 2012, 2013b). Identifying resistant animals is the initial step to identify biomarkers that might be used to select resistant animals that might be more grazed in lupine-rich plant communities.

2. Materials and methods

2.1. Plant material

Flowering *Lupinus leucophyllus* was collected August 11, 2011 near Soda Springs, Idaho at latitude 42°50.346′ N, longitude 111°19.117′ W in a sagebrush steppe ecosystem (Poisonous Plant Research Laboratory collections No. 2011-07). A voucher specimen was deposited at the Poisonous Plant Research Laboratory Herbarium (#3245). The collection was processed and stored as previously described (Green et al., 2011).

2.2. Alkaloid extraction from plant material and quantitative analysis

Plant material was dried at ambient temperature, ground to pass through a 2 mm screen, and 100 mg of plant material was weighed into a 16 ml screw-top glass test tube and extracted using a previously reported procedure (Lee et al., 2007a).

2.3. GC/MS plant analysis

Gas chromatography–mass spectrometry analysis was performed as previously reported (Lee et al., 2007b). In brief, plant material was analyzed by gas chromatography–mass spectrometry using a Finnigan MAT GCQ equipped with a split/splitless injector and a DB-5MS (30 m × 0.25 mm; J&W Scientific) column. Injection port temperature was 250 °C and operated in the splitless mode. Split vent flow rate was 50 ml/min and purged after 0.80 min. Oven temperature was 100 °C for 1 min; 100–200 °C at 40 °C/min; 200–275 °C at 5 °C/min; and held at 275 °C for 1.5 min. Electron impact ionization (EI) at 70 eV was used with an ion source temperature of 200 °C.

2.4. GC/MS alkaloid identifications

Lupanine and anagyrine were identified from a commercially obtained standard (Biomedical Research Co., Los Angeles, CA) and an authenticated (mass spectrometry and nuclear magnetic resonance) sample of anagyrine, respectively. 5,6-Dehydrolupanine was identified by retention indices (RI) calculated by linear extrapolation from RI values generated from known standards and assigned RI numbers from the literature and their EI mass spectra (Wink et al., 1995), relative retention time (RR_t) to lupanine, and EI mass spectra to that reported in the literature (Kinghorn and Balandrin, 1984).

2.5. HPLC–MS sera analysis

Frozen sera samples were allowed to thaw at room temperature. A 0.5 ml aliquot was placed into a 1.5 ml snap cap microcentrifuge tube and 0.5 ml of acetonitrile was added. The samples were vortexed for 10–20 s and centrifuged for 10 min at 3000 RPM at room temperature. Supernatant, 100 µl was transferred to a 1.5 ml autosample vial containing 900 µl deionized distilled water and vortexed for 10–20 s. Samples were injected (5 µl) onto a Betasil Phenyl reversed phase column (100 × 2.1 mm i.d.) (Keystone Scientific, Inc., Bellefonte, PA, USA) protected by a guard column of the same phase. The alkaloids were eluted from the column with a gradient flow consisting of 5 mM ammonium formate, 0.1% formic acid and acetonitrile at a flow rate of 0.500 ml/min. The mobile phase program was 5 mM ammonium formate, 0.1% formic acid–acetonitrile, 97:3, v:v for 1 min followed by a linear gradient to a composition of 50% acetonitrile at 10 min. Flow from the column was connected directly to a Thermo Scientific (San Jose, CA, USA) VELOS PRO linear ion trap mass spectrometer via a heated electrospray ionization (HESI) source. From 0–3 min and from 7–10 min the flow from the column was diverted to waste, from 3–7 min the flow was directed to the ion source. Full scan mass data were acquired over a mass range of 200–500 amu. Under these conditions anagyrine, lupanine, an unidentified alkaloid with an MH^+ $m/z = 249$, and 5,6-dehydrolupanine eluted at 4.3, 4.4, 4.6, and 5.1 min, respectively.

Peak areas of anagyrine, lupanine, unidentified 248, and 5,6-dehydrolupanine were determined from reconstructed ion chromatograms of the MH^+ ions of $m/z = 245$, 249, and 247, respectively and were quantified against a seven point anagyrine standard curve over the range of 0.00244 µg/ml–0.625 µg/ml in water:acetonitrile (95:5). The limit of quantitation (LOQ) was estimated to be 10 ng/ml (in sera) and limit of detection (LOD) was estimated to be 4 ng/ml in sera using the method discussed earlier.

2.6. Cattle

Four yearling Holstein steers (289 ± 13 kg body weight) maintained on alfalfa hay were used in this study conducted under veterinary supervision with the approval of the Utah State University Institutional Animal Care and Use Committee. A sixteen gauge indwelling catheter was placed in the jugular vein of each steer as previously described (Green et al., 2011). The catheters were used to sample venous blood at time 0 just prior to oral dosing, and at 0.25, 0.5, 0.75, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 10.0, 12.0, 18.0, 24.0, 36.0, 48.0, 60.0, 72.0, and 96.0 h post-treatment with minimal stress. The lupine was orally dosed at 2.5 g dried, ground plant material per kg body weight.

2.7. Data analysis

Body weight, plant dosage and serum alkaloid concentrations are expressed as the mean ± standard deviation (SD). Kinetic profiles were analyzed using standard pharmacokinetic software as previously described (Green et al., 2011). Briefly, kinetic evaluations

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