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The effect of intermittent dosing of *Nicotiana glauca* on teratogenesis in goats

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ABSTRACT

Sustained inhibition of fetal movement in livestock species, induced by several poisonous plants, can result in numerous skeletal-contracture malformations. Lupines are responsible for a condition in cattle referred to as "crooked calf syndrome" that occurs when pregnant cattle graze teratogenic lupines. Similar malformations are also seen in animals poisoned by *Conium maculatum* (coniine) and *Nicotiana glauca* (anabasine). A proposed management strategy to limit these types of birth defects includes utilizing an intermittent grazing schedule to allow short durations of grazing lupine-infested areas interrupted by movement to a lupine-free pasture. The objective of this study was to use a goat model to determine if an intermittent schedule of five continuous days on treatment followed by two days off treatment would be sufficient to decrease, or prevent, the incidence of anabasine-induced malformations. The data from this study suggest that, for *N. glauca* in goats, the intermittent grazing program of five days exposure with two days of non-exposure is insufficient to prevent significant skeletal malformations from occurring. However, this study did demonstrate an inverse relationship between the amount of serum anabasine in the dam and the extent of fetal movement.

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1. Introduction

Lupines are responsible for a condition in cattle referred to as "crooked calf syndrome" that occurs when pregnant cattle graze lupines containing the quinolizidine alkaloid anagyrine, or the piperidine alkaloids ammodendrine, *N*-methyl-ammodendrine or *N*-acetyl hystrine (Panter et al., 1999). Similar malformations are also seen in animals poisoned by *Conium maculatum*, containing the alkaloids coniine, γ-coniceine, and *N*-methylconiine, and *Nicotiana glauca*, containing the alkaloid anabasine, with a similar mechanism of action (Panter et al., 1999). A goat model using *N. glauca* has been established to study the mechanism of action of lupine (and other plants with a similar mode of action)-induced cleft palate and crooked calf syndrome (Panter et al., 1990a, 1990b). In goats, the teratogenic insult period includes gestation days (GD) 30–60 (Keeler, 1984; Panter et al., 1990a, 1990b) with a narrow period for cleft palate induction that includes GD 35–41 (Panter and Keeler, 1992). The severity and type of the malformations are dependent on the amount of specific alkaloid(s) in the plants, the stage of pregnancy when the plants are ingested, and the length of time ingestion takes place (Panter et al., 2013). The current hypothesis as to the mode of action of lupine-induced teratogenesis entails sustained inhibition of fetal movement during the sensitive period of gestation.

Current management recommendations to reduce the incidence of lupine-induced teratogenesis include: 1) adjusting grazing to avoid the most toxic stage of plant growth (pod stage during late summer and early fall). 2) Changing breeding schedules such that the pregnant livestock are not in lupine-infested areas during the critical window (GD 40–100 in cattle) during the pod stage (e.g., change to fall calving). 3) Reduce the teratogenic plant (lupine) population density by herbicide treatments. 4) Manage rangeland grazing to maximize grass production.

Another potential management strategy could include utilizing an intermittent grazing schedule to allow short durations of grazing in lupine-infested areas followed by movement to a lupine-free pasture. The anticipated outcome of using an intermittent grazing schedule would be such that the sustained inhibition of fetal movement does not occur. Our hypothesis is that intermittent periods of normal movement will allow the fetus to develop normally within the sensitive developmental period. However, the details of

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such an intermittent grazing program need to be developed. Therefore, the objective of this study was to use a goat model to determine if an intermittent schedule of five continuous days on treatment followed by two days off treatment would be sufficient to decrease, or prevent, the incidence of anabasine-induced malformations.

2. Materials and methods

2.1. Plant

N. glauca was collected in July 2000 and 2004 near Kingman, Arizona. Green plant including leaves, stems, flowers, and woody parts were collected, chopped, and spread on tarps and allowed to air dry. The dry, coarsely chopped plant material was further ground to pass through a 2.4 mm mesh screen using a Gehl Mix-All model 55 (Gehl Company, West Bend, WI, USA). After processing, the ground plant material was stored in plastic bags away from direct light at ambient temperature in an enclosed building until use. Percent anabasine in the plant material was measured just prior to treatment using previously published methods (Keeler et al., 1981).

2.2. Animals

Fourteen female crossbred Spanish-type goats, weighing 40 ± 9 kg, were naturally bred to Spanish-type bucks. Gestation day (GD) 0 was considered to be the day of the last standing heat. The goats were randomly assigned into two groups (10 treated goats and four control goats). *N. glauca* was administered twice daily (7 am and 3 pm) at 5.5 mg anabasine/kg BW, while the control group was dosed twice daily with 1 g alfalfa/kg BW. The dried finely ground plant material was administered via oral gavage in approximately 1 L of tap water. The goats were treated for 5 consecutive days, then off treatment for 2 days, from GD 30 through GD 62. Thus, the intermittent cycle was repeated five times. All animals had free access to alfalfa hay and fresh water. Animals were observed throughout each day for clinical signs of toxicity.

All newborn goats were weighed, sex was determined, and each kid was examined carefully for cleft palate, facial symmetry, and skeletal defects (arthrogryposis, torticollis, kyphosis, lordosis, scoliosis, and rib cage anomalies) immediately after birth (GD 150 ± 1). All procedures were conducted under veterinary supervision and were approved by the Utah State University Institutional Animal Care and Use Committee.

2.3. Serum anabasine analysis

To monitor the serum concentration of anabasine, blood was collected via jugular venipuncture immediately prior to the morning dose (0 h), 1 h after the morning dose on GD 51, and 16 h after the afternoon dose on GD 51, which corresponded to 0 h on GD 52. The serum fraction of the blood samples was collected and stored at −20 °C until analysis. After thawing, the sera were thoroughly mixed and an aliquot (0.5 mL) transferred to a 1.5 mL microcentrifuge tube. Acetonitrile (0.5 mL) was then added to the microcentrifuge tube containing the aliquot of sera (0.5 mL) and mixed thoroughly. The microcentrifuge tubes containing the sera and acetonitrile mixture were then centrifuged (10,000 × *g* for 10 min). An aliquot (200 μL) of the supernatant from the microcentrifuge tube was transferred to a 300 μl conical autosample vial for HPLC-MS/MS analysis.

Analysis of anabasine in sera samples was accomplished using an Agilent Technologies 1260 Infinity solvent delivery and autosampler system (Agilent Technologies, Santa Clara, CA) coupled to a Thermo Scientific LTQ Velos Pro mass spectrometer (Thermo Scientific, San Jose, CA). Samples (20 μL) were injected onto an Aquasil C-18 reversed phase column (100 × 2.1 mm i.d., 3 μm particle size) (Thermo Electron Corporation, Waltham, MA USA) protected by a guard column of the same adsorbent. Anabasine was eluted from the column with a gradient (0.2 mL/min) consisting of 20 mM ammonium acetate in water (solvent A), 0.1% formic acid in water (solvent B) and acetonitrile (Solvent C). Initial mobile phase conditions were 95:5 (A:C) for 2 min. From 2 to 3 min the mobile phase composition was changed from 95:5 (A:C) to 95:5 (B:C) using a linear gradient. The mobile phase was then held constant at 95:5 (B:C) for the next 7 min for at total run time of 10 min. The HPLC system was re-equilibrated for 5 min at the initial mobile phase composition of 95:5 (A:C) prior to the next injection. The solvent flow rate was 0.200 mL/min from 0 to 3 min then linearly increased to 0.400 mL/min from 3 to 10 min and remained at 0.400 mL/min through the end of the analysis and re-equilibration of the column. The flow from the HPLC column was connected directly to the heated electrospray ionization (HESI) source of the mass spectrometer and was operated with a vaporizer temperature of 300 °C and capillary inlet temperature of 275 °C. Other operational parameters of the ion source and mass spectrometer were optimized using the standard "auto tune" procedure using a solution of reserpine added to the LC solvent flow 50:50 (A:C) via syringe pump to obtain maximum ion current of the respective MH^+ ion. For detection of anabasine the mass spectrometer was operated in an MS/MS mode, scanning product ions from a mass range of 50–200 amu after fragmentation of the protonated anabasine ($MH^+ = 163.2$) using a relative collision energy setting of 32%. Reconstructed ion chromatograms using the selected ions at 94, 120, 134, 146 and 163 *m/z* were used for detection and ultimately for peak area measurements and quantitation of anabasine. Anabasine eluted at 6.7 min. Anabasine in sera samples were quantified against a 6 point standard curve over the range of 8–250 ng/mL anabasine in 50:50 water:acetonitrile. The anabasine used for the standard curve had been previously isolated from *N. glauca* according to published methods (Keeler et al., 1984) and was determined to be greater than 95% pure (Welch et al., 2013). Fig. 1 depicts the chromatogram of a 62.5 ng/mL standard of anabasine (Fig. 1A) and the chromatogram of sera from goat 8230 1 h after *N. glauca* dosing, diluted 1:1 with acetonitrile (Fig. 1B).

2.4. Ultrasound

Pregnancy was confirmed in each goat via ultrasound imaging on GD 30. The number of fetal movements were determined by ultrasound imaging of each fetus for a 5 min period at 0, 1, 8, and 16 h after dosing on GD 37, 44, 51, and 58 as well as 0 h on GD 65. The dams were examined transabdominally using an Aloka SSD-900V scanner fitted with a 5 MHz convex electronic transducer (Wallingford, CT). The dams were restrained on their backs to facilitate access to the hairless areas of the abdominal wall just in front of the udder.

2.5. Analysis and statistics

Statistical comparisons of serum alkaloid profiles were performed using ANOVA with a Bonferroni posthoc test of significance between individual groups using SigmaPlot for Windows (version 12.5, SPSS Inc., Richmond, CA). Correlations were determined with a Pearson Product Moment Correlation analysis using SigmaPlot for Windows (version 12.5, SPSS Inc., Richmond, CA). Differences were considered statistically significant at $P < 0.05$.