Studies on the teratogenicity of anabasine in a rat model


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ABSTRACT

A number of plant toxins have been shown to be teratogenic to livestock. The teratogenic action of some of these alkaloids is mediated by nicotinic acetylcholine receptors (nAChR). However, for many of these alkaloids it is difficult to obtain sufficient quantities of individual alkaloids to perform teratology studies in livestock species. Therefore the objective of this study was to determine if a rat model can be utilized to characterize the teratogenic nature of individual plant toxins that are nAChR agonists. In this study, we evaluated the teratogenicity of anabasine by feeding pregnant rats anabasine-containing rodent chow from gestational day (GD) 6–21. On GD21, the dams were euthanized and the gravid uteri were removed. The gravid uteri and individual pups were weighed. The pups were evaluated for bone malformations including cleft palate and scoliosis. Overall, the results of this study suggest that the rat is not a good model to study the teratogenicity of plant toxins that are nAChR agonists. It is possible that in the rat model, anabasine administered orally via the chow may not result in sufficient reduction in fetal movement to cause the significant malformations observed in livestock species.

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1. Introduction

A number of plant toxins have been shown to be teratogenic to livestock (Panter et al., 2011). The maternal consumption of plants that contain piperidine, pyridine, or quinolizidine alkaloids has the potential to cause developmental defects in animals (Green et al., 2013a). Many of the actions of these alkaloids are mediated by nicotinic acetylcholine receptors (nAChR). In the developing fetus, teratogenic piperidine alkaloid-mediated desensitization of fetal muscle-type nAChR is postulated to inhibit fetal movement, resulting in skeletal flexure defects and cleft palate (Green et al., 2013b; Panter et al., 1999). The inhibition of fetal movement disrupts the normal developmental process to cause multiple congenital contracture-type (MCC-type) deformities (arthrogryposis, kyphosis, lordosis, scoliosis, and torticollis) and cleft palate (Panter and Keeler, 1992; Weinzweig et al., 2008). The inhibition of fetal movement is thought to cause skeletal malformations through sustained alignment and positioning of the embryo/fetus in utero (Panter et al., 1990a, 1999).

The association between the inhibition of fetal movement by plant alkaloids and the formation of MCC-type defects is well-documented in livestock (Green et al., 2013e; Panter et al., 1999). When plants, such as lupine (Lupinus spp.), tobacco (Nicotiana spp.), or poison hemlock (Conium spp.), are consumed by pregnant females at the sensitive stage of development, the teratogenic alkaloids from the plants are believed to cross the placenta into the fetal compartment, to act at fetal muscle-type nAChR, and inhibit fetal movement. This has been documented with ultrasound imaging studies of fetuses in pregnant livestock dosed i.v. with the piperidine alkaloid coniine, or oral

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dosing studies with poison hemlock (*Conium maculatum*) and tree tobacco (*Nicotiana glauca*) (Panter et al., 1999; Panter and Keeler, 1992; Panter et al., 1990b).

There are numerous follow-up studies that need to be conducted to further characterize the teratogenic nature of the individual piperidine, pyridine, and quinolizidine alkaloids. Many of these alkaloids are enantiomers, and characterization of the differences in teratogenicity of the individual enantiomers is also needed. In this regard, studies have documented the stereoselective inhibition of fetal movement and the formation of MCC-type defects and cleft palates (Green et al., 2013b; Panter et al., 1990a; Panter and Keeler, 1992, 1993). However, many of these alkaloids are present in the plants as a mixture, and some of the alkaloids are not very abundant. Thus it is difficult to obtain sufficient quantities of many of these alkaloids to perform teratology studies in livestock species. Therefore, a small rodent model to study the teratogenicity of these alkaloids, including their enantiomers, would be valuable for investigating alkaloids present in only limited quantities. Consequently, the objective of this study was to determine if a rat model can be utilized to study and characterize the teratogenic nature of individual plant toxins that are nAChR agonists.

2. Materials and methods

2.1. Animals

Male and female Wistar rats (8 weeks old) were purchased from Simonsen Laboratories Inc., Gilroy, CA. Rats were acclimated for 3–4 d with free access to a commercially pelleted rodent chow (Harlan Teklad rodent diet (w) 8604) and tap water before beginning experiments. Rats were housed under controlled temperature (20–22 °C) and humidity, in a 12:12 h light:dark cycle. Rats were hand mated (2 females and 1 male per cage) for 12 h each night. Females were evaluated the following morning for the presence of a copulatory (mucus) plug and for the presence of sperm in the vaginal area as evidence of mating. The day sperm in the vaginal area were detected was considered gestational day (GD) 0. The pregnant rats were housed individually and dosed as outlined below beginning on GD6. All procedures were conducted under veterinary supervision and were approved by the Utah State University Institutional Animal Care and Use Committee.

Diets for the treated groups were prepared using the same commercially pelleted rodent chow (Harlan Teklad rodent diet (w) 8604), which was ground and mixed with anabasine and 10% corn starch in hot water to obtain 0, 50, 125, 250, 500, and 1000 μg anabasine/g rat chow. Anabasine was extracted from *N. glauca* following previously published methods (Keeler et al., 1984). The anabasine used for this study has been shown to be greater than 95% pure by gas chromatography–mass ionization detectors (GC-FID) (Welch et al., 2013a). Pellets, approximately 1 × 3 cm in size, were formed and dried overnight in an oven at 37 °C. The rat chow for the control group was prepared in the same manner, but with 90% commercial rodent chow and 10% corn starch.

Chow consumption and animal body weight were measured every two days beginning on GD6 through GD21. On GD21 the rats were euthanized by CO2 asphyxiation and the ovaries and uteri were removed by cesarean section. The gravid uterus was weighed. The fetuses were removed from the uterus, dried of amniotic fluid and individually weighed. The number of implantation sites and resorptions was recorded. After being weighed, the fetuses were incubated in acetone for a minimum of 24 h, and subsequently eviscerated. For examination of the skeleton, the fetuses were submerged in a solution of 0.8% potassium hydroxide with alizarin-red S which was changed daily for 3–5 days (Medeiros et al., 2008; Staples and Schnell, 1964). The fetuses were then stored in a solution of 40% ethyl alcohol, 40% glycerin, and 20% benzyl alcohol. Fetuses were visually evaluated, using a dissecting microscope, for any bone malformations including cleft palate and scoliosis.

2.2. Ultrasound protocol

Rats were restrained using common handling techniques by grasping the rat firmly around the neck and back and held such that their abdomen was facing up. The rats were examined transabdominally using an Aloka SSD-900V scanner fitted with a 7.5 MHz convex electronic thumb transducer (Wallingford, CT). Qualitative assessments of fetal movement were made via ultrasound on GD7, GD14, and immediately prior to euthanasia on GD21.

2.3. Analysis and statistics

All statistical analyses were performed using SigmaPlot (version 12.5, SPSS Inc., Richmond, CA). Statistical comparisons between multiple groups were performed using ANOVA with a Bonferroni posthoc test of significance between individual groups. Statistical comparisons of incidence rates between two groups were performed using a two-sided Fisher’s exact test, using a 2 × 2 contingency table. Correlations between two factors were determined using Pearson Product Moment Correlation. Differences were considered significant at *P* < 0.05.

3. Results

Rats in the treated groups were fed rodent chow that contained anabasine from GD6-21. Rats fed rodent chow that contained 125 μg anabasine/g of chow, or greater,
consumed less chow compared to controls over the 15 day period (Table 1). However, only the rats fed chow containing 500 or 1000 mg/g anabasine had a lower body weight on GD21 compared to controls. Even though there was a dose-dependent decrease in chow consumption ($P < 0.001$), there was still an increase ($P < 0.001$) in the dose of anabasine received in the groups fed chow with higher concentrations of anabasine (Table 1). On GD20 and 21 the rats in the 1000 mg/g group showed clear clinical signs of poisoning with marked muscle weakness and trembling.

On GD21, all of the dams were euthanized and their gravid uteri were removed. Although there were rats in most of the groups that were found to be non-pregnant on GD21, only the 1000 mg/g group had significantly ($P = 0.03$) more non-pregnant rats than the control group (Table 2). There was no indication in the non-pregnant rats that they had conceived, i.e., no sites of implantation or resorption, suggesting that these were rats that had mated but not become pregnant. There was no difference in the number of resorption sites in the dams in any of the treated groups versus the control group (Table 2). The decreased weight of the gravid uteri in the 1000 mg/g group resulted from smaller pups (Table 2).

The pups from each group were processed in order to evaluate the stained bones, after which visual observations were made to detect malformed bones including cleft palate and scoliosis (Fig. 1). Out of the 359 pups evaluated, only one pup was found to have a malformed palate (Table 3), suggesting that this may have been a spontaneous occurrence. It is an interesting coincidence, however, that this pup was from the 1000 µg/g group. There was no difference in the number of dams (the true experimental unit for the study) bearing pups with scoliosis from any of the treated groups versus controls (Table 3). However, there were significantly more pups with scoliosis in the 50, 250, 500, and 1000 µg/g groups versus the control group (Table 3), indicating that treatment with anabasine affected the dams such that they had more pups with scoliosis than the control dams.

Several dams from the control and 500 µg/g groups were evaluated by ultrasound imaging on GD7, 14, and 21 to monitor fetal movement. There was no fetal movement detected on GD7 or 14 in the dams from either the control or the treated group (Table 4). Fetal movement was detected on GD21 in both groups (Table 4), although qualitatively the pups from the 500 µg/g group did not appear to be as active as the pups from the control group.

### Table 2

<table>
<thead>
<tr>
<th>Group</th>
<th>Total dams $^a$</th>
<th>Open dams $^b$</th>
<th>Pregnant dams $^c$</th>
<th>Female weight $^d$ (g) Avg SD</th>
<th>Gravid uterine weight (g) Avg SD</th>
<th># Pups/dam Avg SD</th>
<th># Resorptions/dam Avg SD</th>
<th>Pup weight Avg SD n</th>
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</thead>
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<tr>
<td>CNT</td>
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<td>13</td>
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<td>4.4 2.0 155</td>
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<td>4.7 0.4 66</td>
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<td>2</td>
<td>4</td>
<td>319 27 83 15</td>
<td>12.5 2.1 1.3 1.5</td>
<td>4.3 0.5 50</td>
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<tr>
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<td>6</td>
<td>0</td>
<td>6</td>
<td>300 31 73 11</td>
<td>11.8 2.4 1.5 0.8</td>
<td>3.9 0.4 71</td>
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<td></td>
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<tr>
<td>500</td>
<td>10</td>
<td>4</td>
<td>6</td>
<td>219* 14 65 9</td>
<td>14.0 1.9 1.2 1.6</td>
<td>3.1* 0.4 84</td>
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<tr>
<td>1000</td>
<td>6</td>
<td>4*</td>
<td>2</td>
<td>177* 16 38* 4</td>
<td>15.5 0.7 0.5 0.7</td>
<td>1.4* 0.2 31</td>
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</table>

$^a$Denotes difference from control group ($p < 0.05$).
$^b$The total number of dams in each group.
$^c$The number of non-pregnant dams at the time of necropsy on GD21.
$^d$The number of pregnant dams at the time of necropsy on GD21.

The pups from each group were processed in order to evaluate the stained bones, after which visual observations were made to detect malformed bones including cleft palate and scoliosis (Fig. 1). Out of the 359 pups evaluated, only one pup was found to have a malformed palate (Table 3), suggesting that this may have been a spontaneous occurrence. It is an interesting coincidence, however, that this pup was from the 1000 µg/g group. There was no difference in the number of dams (the true experimental unit for the study) bearing pups with scoliosis from any of the treated groups versus controls (Table 3). However, there were significantly more pups with scoliosis in the 50, 250, 500, and 1000 µg/g groups versus the control group (Table 3), indicating that treatment with anabasine affected the dams such that they had more pups with scoliosis than the control dams.

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### 4. Discussion

Numerous plants that are teratogenic to livestock species are known to contain toxins that alter the activity of nAChRs, which results in decreased muscle function (Green et al., 2013a,b,c,d,e; Welch et al., 2013a,b). These
teratogenic plants contain a number of different piperidine, pyridine, and quinolizidine alkaloids, many of which have not been fully characterized (Panter et al., 1999). For example, nine piperidine alkaloids have been identified with known or suspected teratogenic activity. Whereas, only one quinolizidine alkaloid, out of the more than 150 quinolizidine alkaloids that have been structurally identified, has been determined to be teratogenic (Schmeller et al., 1994). Additionally, many of these alkaloids are enantiomers, which can have different biological activities (Lee et al., 2013, 2008). Many of these enantiomeric alkaloids are present in the plants in small quantities and as mixtures of the racemates, making it difficult to obtain sufficient quantities of many of the alkaloids to perform teratology studies in livestock. Consequently, the objective of this study was to determine if a rat model can be utilized to study and characterize the teratogenic nature of individual plant toxins that are nAChR agonists.

A rat model has been used to study birth defects caused by the plant *Mimosa tenuiflora* (Gardner et al., 2014; Medeiros et al., 2008). *M. tenuiflora* has been implicated as the cause of several birth defects in sheep and cattle in Brazil (Silva et al., 2011). The main defects in livestock species include permanent flexure of the forelimbs, craniofacial anomalies including cleft palate, brachygnathia, agnathia, cleft lip and eye malformations including microphthalmia, ocular dermoids, and corneal opacity. In the rat model study of *M. tenuiflora* teratogenicity, 40 of the 101 pups from the experimental group had bone malformations including scoliosis, lordosis, and a shortened head, whereas only three of the 58 fetuses born in the control group had malformations (Medeiros et al., 2008). The susceptibility of pregnant Wistar rats to the teratogenic effect of *M. tenuiflora* suggests that the rat model can be used as an experimental model to study the teratogenicity of the type of plant toxin(s) (Gardner et al., 2014) in *M. tenuiflora*.

It has been suggested that coniine does not cause teratogenicity in rats (Forsyth and Frank, 1993). This may indicate the rat is not the optimum research model for compounds that cause teratogenicity via inhibition of fetal movement. However, a recent study demonstrated that sulfoxaflor, a nAChR agonist, caused skeletal deformities in rats (Rasoulpour et al., 2012). *In utero* exposure to high doses of sulfoxaflor induced forelimb flexure, hindlimb rotation, bent clavicle, and neonatal death.

In this study, we dosed pregnant rats with anabasine by feeding them rat chow that contained 50, 125, 250, 500, and 1000 μg anabasine/g rat chow from GD6-21. Based upon the amount of chow consumed and the final body weights, the rats received a dose of approximately 43, 110, 168, 247, and 485 mg/kg anabasine over the 15 d period (Table 1). The inclusion of anabasine at the higher doses did appear to result in feed refusal as the rats fed chow with 125 μg/g anabasine, or greater, consumed significantly less chow than the controls, which also resulted in significantly smaller rats in the 500 and 1000 μg/g groups. Consequently, the rats in these two groups could have been malnourished, which could have had a significant impact on fetal growth and development.

Although there were more non-pregnant rats on GD21 in the 1000 μg/g group, there was no indication at necropsy in any of the non-pregnant rats that they had conceived, i.e., there were no implantation or resorption sites observed in the uteri of the non-pregnant rats, which suggests that these rats had mated but never became pregnant. Additionally, there was no difference in the number of resorption sites in any of the groups versus the control group (Table 2), suggesting that anabasine did not cause embryonic losses or abortions. Implantation in rats occurs on GD6–7 (DeSesso, 2006), consequently it is possible that anabasine treatment impaired implantation in the 1000 μg/g group. However, this is not likely as the rats in the 1000 μg/g group only consumed, on average, 1 g

### Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Dam&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cleft palate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Scoliosis&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Pup&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cleft palate&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Scoliosis&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>n</td>
<td># Incidence&lt;sup&gt;c&lt;/sup&gt; (%)</td>
<td># Incidence&lt;sup&gt;c&lt;/sup&gt; (%)</td>
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<td># Incidence&lt;sup&gt;c&lt;/sup&gt; (%)</td>
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<sup>a</sup> Denotes difference from control group (<i>p</i> < 0.05).

<sup>b</sup> Data reported with dams as the experimental unit, i.e., the number of dams with pups with malformations.

<sup>c</sup> Data reported with pups as the experimental unit, i.e., the number of pups with malformations.

<sup>d</sup> The number of animals with the indicated malformation.

<sup>e</sup> Denotes difference from control group (<i>p</i> < 0.05).

<sup>f</sup> Incidence of the indicated malformation for the designated experimental unit.

<sup>g</sup> Refers to cleft of the hard palate.
of chow/d for the first 3–4 days. In comparison the 500 µg/g group consumed 2 g of chow/d during the first 4 d of treatment. Thus they received the same dose of anabasine during the first 4 d of treatment. However, there was no statistical difference in the number of non-pregnant rats in 500 µg/g group compared to controls. Thus there is no indication that anabasine affected implantation.

The dams in the 1000 µg/g group had a significantly lower gravid uterine weight compared to controls (Table 2), because the pups in this group were significantly smaller. Again this could be due to anabasine treatment, or perhaps due to poor nutrition, as the dams in this group were very small for GD21 (Fig. 1). In this regard, there was a positive correlation between pup weight and chow consumption \((r = 0.86; P = 0.014)\) as well as between pup weight and dam weight \((r = 0.93; P = 0.003)\), which suggests that the decreased chow consumption resulted in a decreased body weight of the dams and the pups.

The mechanism of action of piperidine alkaloid-induced malformations is thought to be a result of toxin-induced reduction in fetal movement (Panter et al., 1990a). The resultant malformations can include skeletal contracture malformations and cleft palate depending upon the timing of the exposure (Panter et al., 1990a; Panter and Keeler, 1992). Even though the rat has a much shorter gestation than livestock species, there are still similarities in the timing of development. For example, in the rat, fetal movement begins on GD16 (Robinson et al., 2008; Smotherman and Robinson, 1986) with palate closure occurring on GD17-18 (DeSesso, 2006). Whereas in a goat, dam weight \(\left(< \right)\) correlation between pup weight and chow consumption with palate closure could be a potential explanation for the lack of anabasine-what occurs in the goat model. However, we only observed GD16-18 could result in cleft palate formations similar to malformations and cleft palate depending upon the timing of the exposure (Panter et al., 1990a; Panter and Keeler, 1992). Consequently, in theory, exposing a rat to anabasine during GD16-18 could result in cleft palate formations similar to what occurs in the goat model. However, we only observed 1 malformed palate out of 299 pups from anabasine-treated dams. It is interesting that the one pup with a cleft palate was from a dam in the 1000 µg/g group, however, this was likely a spontaneous occurrence. Overall this study indicates that anabasine will not cause cleft palate formation in rats.

On GD21 the pups from dams in the 500 µg/g group were observed by ultrasound imaging to be moving, which could be a potential explanation for the lack of anabasine-induced cleft palates in the rat model. It has been shown that in order for malformations to occur, fetal movement has to be completely inhibited (Panter et al., 1990a). Thus, essentially any fetal movement of the head and neck area, which would result in movement of the tongue in the mouth, should prevent cleft palate formations. Other less severe malformations such as slight to moderate carpal flexure can occur in sheep, goats, and cattle, but these spontaneously resolve soon after birth (Panter et al., 1990a, 1990b). Consequently, it is possible that in the rat model anabasine administered orally via the chow does not result in sufficient reduction in fetal movement to cause cleft palate formation, but it may inhibit movement enough to cause minor malformations.

An argument could be made that administering a higher dose of anabasine to the dams could result in complete inhibition of fetal movement. However, the doses used in this study resulted in feed refusal, with potential nutritional deficiencies as well as clear clinical signs of poisoning in the dams. Therefore the utilization of doses above those employed in this study would likely cause severe nutritional deficiencies in the dams and their pups as well as possible death of the dam from overt toxicity and would therefore not be relevant. In conclusion, the data presented in this study suggest that the rat model is not a good model to study the teratogenicity of plant toxins that cause birth defects in livestock by inhibition of nicotinic acetylcholine receptors.

**Ethical statement**

The authors all certify that all ethical considerations were made for the study and preparation of the manuscript, such that there are no ethical issues with the manuscript.

**Acknowledgments**

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**Conflict of interest**

There is no conflict of interest for this work.

**References**


