Effect of subacute dietary nitrate on production traits and plasma analytes in Suffolk ewes

R. R. Cockrum, K. J. Austin, P. A. Ludden and K. M. Cammack†

Department of Animal Sciences, University of Wyoming, Laramie, WY 82071-3684, USA

(Received 30 January 2009; Accepted 7 November 2009; First published online 16 December 2009)

Elevated dietary nitrate (NO₃⁻) is associated with production losses in ruminant livestock, resulting in substantial economic losses incurred by producers. Severe drought, fertilization practices and poorly maintained pastures increase the risk of elevated NO₃⁻ intake among cattle and sheep. Nitrate is metabolized to nitrite (NO₂⁻) in the rumen and further reduced to ammonia. Ruminants consuming high dietary NO₃⁻ vary in ability to efficiently reduce excess NO₂⁻ to ammonia. This leads to methemoglobin formation and ultimately NO₃⁻ toxicity signs. Variation in individual tolerance to elevated dietary NO₃⁻ can be partially attributed to rate and duration of exposure, rate of elimination, metabolism, species and dose. Our objectives were to confirm and quantify variation in individual tolerance to subacute levels of dietary NO₃⁻, and determine if individuals could be identified as highly or lowly tolerant to elevated dietary NO₃⁻ based on production traits, plasma analytes and(or) signs of subacute NO₃⁻ toxicity. Purebred Suffolk ewes were administered supplement mixed with tap water (control; n = 8) or potassium nitrate (NO₃⁻ treated; 300 mg NO₃⁻/kg BW daily; n = 47) for 8 days. Coefficients of variation (CV) indicated that supplement intake was more variable in NO₃⁻ treated ewes (CV = 59.3%) than in control ewes (CV = 13.6%). Among NO₃⁻ treated ewes, six ewes highly tolerant and six ewes lowly tolerant to elevated dietary NO₃⁻ were identified based on individual performance, NO₃⁻ treated supplement intake, and signs of toxicity. Supplement intake was lower (P < 0.0001) in NO₃⁻ treated ewes than in control ewes, indicating elevated dietary NO₃⁻ influences feed intake. Supplement intake differed (P < 0.0001) between control, highly tolerant and lowly tolerant ewes. Supplement intake of highly and lowly tolerant ewes was 82% and 23%, respectively, of the control ewes’ intake. Weight change and plasma concentrations of NO₃⁻, cortisol, glucose and retinol were not different (P > 0.38) among control, highly tolerant and lowly tolerant ewes. Plasma urea nitrogen (PUN) levels were not different (P = 0.25) between control and lowly tolerant ewes, but were lower (P = 0.02) in highly tolerant ewes than in control ewes. Furthermore, PUN and NO₃⁻ treated supplement intake were highly correlated (0.71; P < 0.0001) in lowly tolerant ewes. These results confirm and quantify variation in response to subacute levels of dietary NO₃⁻ and indicate that individuals can be identified as highly or lowly tolerant to elevated dietary NO₃⁻ based on their performance and NO₃⁻ toxicity signs.

Keywords: nitrate, sheep, tolerance, toxicity

Implications

More than $340 million is conceded annually by Western United States producers due to production losses associated with livestock consuming toxic plants (Nielsen and James, 1992). The economic implications of acute NO₃⁻ toxicity have been recognized; however, the economic impact of subacute NO₃⁻ toxicity is difficult to establish, as the signs are non-specific and often misdiagnosed. Subacute NO₃⁻ toxicity may partially explain the poor performance frequently observed in livestock in drought-stricken regions, including decreased feed intake and nutrient utilization, weight loss, and compromised reproduction and immune function.

Introduction

Death rates from the consumption of high NO₃⁻ forages (45 to 547 mg NO₃⁻/kg body weight (BW) in cattle and 224 to 547 mg NO₃⁻/kg BW in sheep) range from 7% to 44% in cattle and up to 42% in sheep (Harris and Rhodes, 1969). During severe drought conditions, plants do not absorb NO₃⁻; however, eventual moisture leads to rapid absorption of NO₃⁻ by plants. Nitrate itself is not toxic to livestock, but ingested NO₃⁻ is converted to NO₂⁻ by rumen bacteria and further
Effect of nitrate toxicity on ewe production

Reduced to ammonia. Ingestion of high levels of NO$_3^-$ causes accumulation of toxic levels of NO$_2^-$ in the blood, resulting from a conversion of the ferrous ion of hemoglobin to the ferric form, which reduces the ability of red blood cells to carry the oxygen. Signs of NO$_3^-$ toxicity may appear when >20% of hemoglobin is converted to methemoglobin (Yaremcio, 1991). The transfer of rumen NO$_2^-$ into the bloodstream is influenced by NO$_3^-$ intake, rate of feed digestion and subsequent NO$_3^-$ release, rate of NO$_2^-$ reduction to ammonia and absorption of NO$_2^-$ from the rumen. Subacute NO$_3^-$ toxicity can result in lethargy, head pressing, and impaired animal production as evidenced by depressed appetite, reduced or no BW gain, lowered milk production, reproductive inefficiency and increased susceptibility to infection (Yaremcio, 1991). The long- and short-term effects of subacute NO$_3^-$ toxicity have received limited research investigating, resulting in frequent misdiagnosis in livestock operations and incorrect or lack of appropriate treatment. Signs of toxicity exhibited by sheep and cattle are similar in response to high levels of NO$_3^-$ in forage. However, sheep have a higher tolerance to NO$_3^-$ due to their ability to increase the concentration of red cells in the blood (Diven et al., 1964). The observed variation in susceptibility to toxicity can be partially attributed to the rate and duration of exposure as well as individual tolerance and metabolism capacity. The objective of this study was to confirm and quantify individual variation in ewes exposed to subacute levels of dietary NO$_3^-$ and determines if individual animals can be identified as highly tolerant or lowly tolerant based on production traits, plasma analytes, and/or signs of subacute NO$_3^-$ toxicity.

Material and methods

Animal procedures

All animal procedures used were approved by the University of Wyoming Institutional Animal Care and Use Committee. Due to time and labor limitations, purebred Suffolk ewes ($n = 60$; average initial BW = 85.7 ± 46.4 kg) were randomly allotted to one of two project start days (contemporary groups). The variation in BW was attributed to variations in both body condition score (3 to 7) and age of ewes (1 to 8 years). Within each contemporary group, ewes were randomly assigned to a control ($n = 5$) or elevated NO$_3^-$ ($n = 25$) treatment group with age randomly distributed between control (average of 2.4 years) and NO$_3^-$ treated (average of 3.1 years) ewes. The trial period occurred during the summer months of June and July, and animals within the contemporary group were housed in a covered outdoor facility and fed the basal diet and supplement mixture during the trial period. The basal diet consisted of baled bromegrass hay (8.1% CP, 61% NDF; 91% dry matter (DM) basis) fed at 2.5% (2.150 g/day, 60% total digestible nutrient (TDN)) of initial BW. The supplement mixture (11.5% total dietary CP) consisted of (92% DM) 53.9% soybean meal, 28.7% beet pulp, 10.0% molasses, and 7.4% vitamins/minerals and was fed 3× per day (125 g/feeding, as fed basis; 75.6% TDN) for 8 days. The maximum TDN intake per ewe was 1.460 g/d; an 85 kg non-lactating mature ewe requires 720 g/day for maintenance (National Research Council, 2007). Respective treatments included either control supplement (supplement mixture with tap water) or NO$_3^-$ treated supplement (supplement mixture with 100 mg/kg BW of NO$_3^-$ provided as a 1.5 M NO$_3^-$ solution, using potassium nitrate (KNO$_3$) as the NO$_3^-$ source) administered 3× daily. All ewes ($n = 60$) were adjusted to the control supplement for 3 days prior to the initiation of treatment (day 3 to day 1). Starting on day 0, ewes were then administered their respective supplement, control ($n = 10$) or NO$_3^-$ treated ($n = 50$), for the 8 day trial period (day 0 to day 7). Liver biopsies were performed on all ewes using a procedure modified from Ferreira et al. (1996) on day 0 (prior to treatment administration) and again at the end of the study on day 7 to obtain tissues for future gene expression analyses. An 11% death rate typically occurs in sheep when performing liver biopsies as opposed to <0.5% in cattle (Anderson et al., 1962). A total of 120 biopsies were performed in this study, with an 8.3% death loss ($n = 5$) due to surgery complications. With the loss of 2 control ewes and 3 NO$_3^-$ treated ewes, a total of 8 control ewes and 47 NO$_3^-$ treated ewes were available for analyses. After ewe losses, contemporary group 1 was composed of 5 controls and 25 NO$_3^-$ treated ewes and contemporary group 2 was composed of 3 controls and 22 NO$_3^-$ treated ewes.

Animals assigned to the NO$_3^-$ supplement treatment were provided a KNO$_3$ treated supplement at a rate of 300 mg/kg BW of NO$_3^-$ (Sigma Aldrich, St Louis, MO, USA) daily (100 mg/kg BW of NO$_3^-$ 3× daily) in a 1.5 M solution consisting of NO$_3^-$ and distilled H$_2$O that was evenly distributed within the 125 g supplement mixture prior to feeding. To avoid acute NO$_3^-$ toxicity, this supplement was delivered over three daily feeding periods at 0700, 1200 and 1700 h. Based on preliminary studies in our laboratory, a molar solution of NO$_3^-$ rather than supplement top-dressed with NO$_3^-$ was effective in eliminating sorting behavior. Furthermore, those preliminary studies indicated that administration of 300 mg/kg BW of NO$_3^-$ resulted in the greatest variation in NO$_3^-$ treated supplement intake among ewes. Control supplement ewes were administered 125 g daily of supplement mixed with tap water only immediately prior to feeding. The control supplement was similarly administered over three daily feeding periods. Tap water used for mixing control supplement was also used as the drinking water source for this study. Concentration of NO$_3^-$ was not tested in the tap water, but assumed to be minimal due to lack of subacute NO$_3^-$ toxicity signs in control animals and other stock animals with access to the tap water. As a precautionary measure against acute NO$_3^-$ toxicity, a mixture of 1% to 4% aqueous solution of methylene blue in saline was prepared for intravenous injection on a 5 to 20 mg/kg live BW basis upon onset of clinical signs of NO$_3^-$ toxicity. Blood was drawn for analyses on day 0, 12 h after initial NO$_3^-$ exposure, every 24 h for the remaining 8 days of the trial, and 3 days after the cessation of treatment. Blood was drawn via the
jugular vein into ethylenediaminetetraacetic acid-lined tubes (Tyco Healthcare Group LP, Mansfield, MA, USA) to prevent clotting. All samples were immediately mixed and put on ice for 1 h. Samples were then centrifuged for 20 min at 1520 × g at 2°C after which plasma was obtained and stored at −20°C.

**Supplement weight back**

Once respective supplements (control or NO₃⁻ treated) were administered to ewes, a maximum period of 30 min was allotted for feeding so that accurate measures of supplement intake could be obtained. In a preliminary trial, it was determined that a feeding period of 30 min was sufficient for maximum supplement intake, or *ad libitum* supplement intake. Ewes tended to waste supplement when feeding periods exceeded 30 min, making it difficult to obtain accurate supplement intake measures. Any remaining supplement was placed into individual bags, labeled with date and ewe tag number, and stored at −20°C until weighed.

**Selection**

Nitrate treated ewes (*n* = 47) were ranked on average NO₃⁻ treated supplement intake during the experimental feeding period. During the adjustment period (day 3 to day 1) all ewes consumed 100% of the control supplement offered. The 20% (*n* = 9) of ewes consuming the greatest amount of NO₃⁻ treated supplement during the 8 day trial period were considered for selection as highly tolerant to NO₃⁻. The 20% (*n* = 9) consuming the least amount of NO₃⁻ treated supplement were considered for selection as lowly tolerant to NO₃⁻. Lowly tolerant ewes selected for further analyses consumed <25% of the NO₃⁻ treated supplement. Highly tolerant ewes selected for further analyses consumed >70% of the NO₃⁻ treated supplement. From the initial 20% considered, six ewes were selected as lowly tolerant and six ewes as highly tolerant to NO₃⁻ based on behaviors and toxicity signs, or lack thereof, such as lethargy, decreased supplement intake, head pressing and upper respiratory congestion attributed to suppressed immune function. For each toxicity sign, ewes were assigned a score of 1 if the sign was expressed and a score of 0 if not. Lethargic ewes would hold their heads and ears low and position themselves away from the surrounding ewes. Ewes that demonstrated head pressing would hold their heads down and press them against the pen for extended periods of time. Though ewes that demonstrated upper respiratory congestion were not examined by a licensed veterinarian, ewes that consistently coughed and had noticeable nasal mucous discharge where classified as exhibiting upper respiratory congestion. Lowly tolerant ewes demonstrated ≥2 of those signs whereas highly tolerant ewes did not demonstrate any signs associated with subacute NO₃⁻ toxicity. Of the eight control ewes, six were selected for further analyses based on health status and collection of a liver sample of adequate quality and quantity. Of the two control ewes not selected for further analyses, one had a tumor on the neck prior to treatment and the other exhibited blood clots in the liver sample obtained via biopsy.

**Feed analyses**

Feed samples were analyzed for DM (Association of Official Analytical Chemists, 1990), nitrogen (LECO Model FP-528 Nitrogen analyzer; LECO Corporation, St Joseph, MI, USA), and NDF contents (ANKOM 200 fiber analyzer; ANKOM Technology, Fairport, NY, USA). Nitrate levels in the bromegrass hay and supplement were analyzed using a Standard Range Lab Nitrate Test Kit (L-NTK; NECi, Lake Linden, MI, USA) and measured on a Nanodrop spectrophotometer (Nanodrop Technologies, Inc., Wilmington, DE, USA). Nitrate measurements in bromegrass hay and supplement were obtained to determine the basal level of NO₃⁻ that all animals were consuming regardless of treatment assignment. In this assay, NO₃⁻ was measured by the reduction of NO₃⁻ to NO₂⁻ using a NO₃⁻ reductase and an electron donor, NADH (Campbell *et al.*, 2006). Nitrate was evenly mixed with the supplement immediately prior to administration and ewes were unable to sort the NO₃⁻ solution from supplement; therefore, NO₃⁻ intake of NO₃⁻ treated ewes was estimated by NO₃⁻ treated supplement consumption.

**Plasma analyses**

Plasma samples from selected ewes were tested for NO₂⁻ levels as described for analysis of the supplement and bromegrass hay with the omission of the NO₃⁻ reductase and NADH reagents from the assay. Plasma NO₂⁻ levels were not determined as toxicosis is caused by circulating NO₂⁻ rather than NO₃⁻. Daily plasma urea nitrogen (PUN) levels were confirmed in control, highly tolerant and lowly tolerant ewes using a QuantiChrom™ Urea Assay Kit (DIUR-500; BioAssay Systems, Hayward, CA, USA) and measured on a Beckman Coulter DU 640 spectrophotometer (Beckman Coulter, Fullerton, CA, USA). The urea assay kit utilizes a chromogenic reagent that forms a colored complex specifically with urea (Jung *et al.*, 1975). Daily plasma glucose levels in control, highly tolerant and lowly tolerant ewes were determined using a rapid multi-assay analyzer protocol (reagent GMRD-002A and standard GMRD-011; 8 mmol/l per 144.1 mg/dl) and instrument (Analox Instruments Ltd, London, UK). Samples were analyzed using 10 ml of plasma until duplicate results of ±4.0 mg/dl were reached. Daily plasma cortisol levels in control, highly tolerant and lowly tolerant ewes were analyzed via radioimmunoassay using a TCK05 Cortisol RIA Coat-a-Count kit (Diagnostic Products Corporation, Los Angeles, CA, USA) on a Cobra II autogamma counter (Packard, Downers Grove, IL, USA). All results were compared against internal controls of non-specific bound and standard samples. Retinol levels in control, highly tolerant and lowly tolerant ewes on day 1, 4 and 7 were analyzed on an HPLC (Waters Separation Module 2690) as an indicator of vitamin A in plasma by the Michigan State University’s Diagnostic Center for Population and Animal Health (Lansing, MI, USA). Plasma rather than liver tissue...
was analyzed for retinol levels due to the limited amount of hepatic tissue available.

**Statistical analyses**
Change in BW was analyzed for effects of treatment (control or NO$_3^-$ treated; control, highly tolerant or lowly tolerant), contemporary group and their interaction using PROC GLM of SAS (SAS Institute Inc., Cary, NC, USA). The NO$_3^-$ treated supplement intake and NO$_3^-$ intake were analyzed for the fixed effect of treatment (control or NO$_3^-$ treated; control, highly tolerant or lowly tolerant) and random effects of contemporary group, day, and the interaction of treatment $\times$ day as repeated measures using PROC MIXED of SAS. Plasma parameters, including NO$_2$, nitrogen, cortisol, glucose, and retinol, were analyzed for the random effect of treatment (control, highly tolerant or lowly tolerant) and random effects of contemporary group, day, and the interaction of treatment $\times$ day as repeated measures using PROC MIXED of SAS. Mean separation of least-squares means was performed using a least-squares difference with a Tukey’s adjustment with s.e.m. of least-squares means was performed using a least-squares means using PROC MIXED of SAS. Mean separation of least-squares means was performed using a least-squares difference with a Tukey’s adjustment with s.e.m. was assumed. Relationships between plasma analytes (when significance was detected) and supplement intake were determined using PROC CORR of SAS.

**Results and discussion**
Nitrate levels in bromegrass hay and basal supplement (without added NO$_3^-$) were within the range considered safe for ruminant livestock (Table 1) at <4 ppm NO$_3^-$. Ewe age did not affect supplement intake ($P = 0.54$) or weight change ($P = 0.35$). There were no differences in ewe age or BW between control and NO$_3^-$ treated ewe groups as age and BW were evenly distributed upon assignment of treatment.

**Table 1** % NO$_3^-$ levels (dry matter basis) and potential effects on ruminants

<table>
<thead>
<tr>
<th>% NO$_3^-$</th>
<th>NO$_3^-$ (ppm)</th>
<th>Effect on ruminants</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;0.15%</td>
<td>&lt;1500</td>
<td>Safe</td>
</tr>
<tr>
<td>0.15% to 0.5%</td>
<td>1500 to 5000</td>
<td>Moderately safe. Limit use for stressed or pregnant animals to 50% of the total ration.</td>
</tr>
<tr>
<td>0.5% to 1.0%</td>
<td>5000 to 10 000</td>
<td>Potentially toxic. Limit feed from 25% to 50% of total ration for non-pregnant livestock. Do not feed.</td>
</tr>
<tr>
<td>&gt;1.0%</td>
<td>&gt;10 000</td>
<td>Dangerous. Will cause death and acute toxicosis. Do not feed.</td>
</tr>
</tbody>
</table>

Ewes fed the NO$_3^-$ treated supplement consumed less ($P < 0.0001$) supplement than controls, but change in BW did not differ ($P = 0.97$; Table 2). The pH of KNO$_3$ was approximately 8, and its addition did not change the overall pH of the supplement; however, a potential effect of the NO$_3^-$ on supplement taste or on appetite cannot be ruled out as a possible contributor to the decreased supplement intake in NO$_3^-$ treated ewes. Highly tolerant ewes did not demonstrate a taste aversion to the NO$_3^-$ treated supplement as the majority of supplement offered (81.8%) was consumed with no subacute NO$_3^-$ toxicity signs. Lowly tolerant ewes consumed 23.2% of the NO$_3^-$ treated supplement offered and demonstrated ≥2 subacute NO$_3^-$ toxicity signs. Therefore, the decrease in treated supplement intake of NO$_3^-$ treated ewes, especially in lowly tolerant ewes, coincides with elevated dietary NO$_3^-$. Coefficients of variation (CV) indicated that supplement intake was more variable in NO$_3^-$ treated ewes (CV = 59.3%) than in control ewes (CV = 13.6%). Furthermore, approximately 74% of NO$_3^-$ treated ewes ($n = 35$; Figure 1) ingested 10.1 to 30.0 g/day of NO$_3^-$, whereas 17% ($n = 8$) ingested <10.0 g/day of NO$_3^-$ and 9% ($n = 4$) ingested >30.0 g/day of NO$_3^-$. 

**Figure 1** Average daily NO$_3^-$ intake distribution among all NO$_3^-$ treated ewes.

**Table 2** Mean intake and BW change of control and NO$_3^-$ treated ewes

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>NO$_3^-$ treated$^1$</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of ewes</td>
<td>$n = 8$</td>
<td>$n = 47$</td>
<td>&lt;$0.0001$</td>
</tr>
<tr>
<td>Daily supplement intake, %</td>
<td>96.39 ± 7.80</td>
<td>45.10 ± 2.83</td>
<td>&lt;$0.0001$</td>
</tr>
<tr>
<td>NO$_3^-$ intake g/day$^2$</td>
<td>0.00 ± 3.21</td>
<td>19.10 ± 1.17</td>
<td>&lt;$0.0001$</td>
</tr>
<tr>
<td>% of dry matter$^2$</td>
<td>0.00 ± 0.14</td>
<td>0.89 ± 0.05</td>
<td>&lt;$0.0001$</td>
</tr>
</tbody>
</table>

$^1$Treated ewes received 300 mg/kg of BW of NO$_3^-$ daily  
$^2$Total NO$_3^-$ intake from supplement in addition to <4 ppm measured in bromegrass hay.

*Effect of nitrate toxicity on ewe production*
This variation in intake may translate to other feedstuffs containing subacute levels of NO$_3^-$ and may assist livestock producers in recognizing elevated NO$_3^-$ as a potential culprit in populations experiencing variation in intake and performance, especially in regions prone to high NO$_3^-$ feedstuffs. Nitrate treated ewes ingested 0.89 ± 0.05% of NO$_3^-$ or 19.07 ± 1.17% NO$_3^-$ g/day on a DM basis (Table 2). Supplement intake (control and NO$_3^-$ treated) tended (P = 0.057) to differ between the two contemporary groups, but there was no treatment × contemporary group interaction effect on supplement (P = 0.28) or NO$_3^-$ intake (P = 0.10). No ewes exhibited signs of acute NO$_3^-$ toxicity that required a methylene blue injection. Supplement intake differences between the two contemporary groups may potentially be explained by the large CV and/or small replicate numbers in the treatment groups, especially the control group.

Supplement intake differed (P = 0.0002; Table 3) between control and highly tolerant ewes. Additionally, lowly tolerant ewes consumed less (< 0.0001) supplement than control and highly tolerant ewes. Control, highly tolerant and lowly tolerant ewes consumed 100% of bromegrass hay offered daily at 2.5% of BW. Nitrate intake differed (P = 0.008; Table 3) between lowly tolerant and highly tolerant ewes, with lowly tolerant and highly tolerant ewes ingesting 0.49% (10.37 ± 2.12 g/day) and 1.53% (30.33 ± 2.05 g/day) NO$_3^-$ daily on a DM basis, respectively. Generally, forages containing acutely toxic levels of NO$_3^-$ on a DM basis range from 1% to 3%, or 10 000 to 30 000 ppm, NO$_3^-$ (Adams et al., 1992). Wright and Davison (1964) suggested that 0.34% to 0.45% NO$_3^-$ should be considered toxic, while Case (1957) found that forages containing 0.70% NO$_3^-$ resulted in death. Interestingly, ewes identified as highly tolerant to NO$_3^-$ in this study were able to tolerate consuming a higher percentage of NO$_3^-$ than lowly tolerant ewes and yet were noticeably lethargic and had significantly decreased NO$_3^-$ treated supplement intake. Both highly and lowly tolerant ewes consumed 100% of bromegrass hay offered; therefore, demonstration of health problems were attributed to NO$_3^-$ toxicity rather than metabolic needs as the bromegrass hay alone met all nutrient requirements. The difference in percentage NO$_3^-$ intake between highly tolerant and lowly tolerant ewes in this study, in combination with the reports of Case (1957), Wright and Davison (1964) and Adams et al. (1992), provides further evidence of variation in tolerance to elevated dietary NO$_3^-$ in ruminant animals.

While within day plasma NO$_2^-$ levels did not differ (P = 0.59) between controls, highly tolerant and lowly tolerant ewes, plasma NO$_2^-$ was consistently numerically lower in control ewes (19.25 ± 2.33 ppm) and higher in lowly tolerant ewes (23.30 ± 2.53 ppm) during the 8 day treatment period (Figure 2). Lowly tolerant ewes were noticeably lethargic compared to control and highly tolerant ewes and demonstrated signs consistent with NO$_3^-$ toxicity such as head pressing, listlessness and diarrhea. Thus, behavior displayed by the lowly tolerant ewes may reflect the level of NO$_3^-$ consumed rather than nutrient or energy deficiency.

PUN levels were not different between lowly tolerant and control (P = 0.25) or highly tolerant (P = 0.38) ewes (Figure 3); however, PUN levels were lower (P = 0.02) in highly tolerant ewes than control ewes. Circulating plasma NO$_2^-$ and PUN levels can be indicators of individual NO$_3^-$ metabolism. PUN levels increase over 50% when glomerular filtration rate is impaired, indicating impaired kidney function (Schnellmann, 2008). Interestingly, highly tolerant ewes ingested a significantly higher percentage NO$_3^-$ than lowly tolerant ewes but had numerically lower PUN and circulating NO$_2^-$ levels. Additionally, there was a positive and moderate correlation (0.35; P = 0.0005) between PUN levels and percentage NO$_3^-$ treated supplement intake among selected NO$_3^-$ ewes, indicating that as

![Figure 2](image-url) Average daily circulating plasma NO$_2^-$ levels in control, lowly tolerant and highly tolerant ewes did not differ (P = 0.59) from day 1 to 7.

<table>
<thead>
<tr>
<th>Item</th>
<th>Control</th>
<th>Lowly tolerant$^1$</th>
<th>Highly tolerant$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of ewes</td>
<td>n = 6</td>
<td>n = 6</td>
<td>n = 6</td>
</tr>
<tr>
<td>Daily supplement intake, %$^2$</td>
<td>99.17 ± 2.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.17 ± 2.66&lt;sup&gt;c&lt;/sup&gt;</td>
<td>81.79 ± 2.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>NO$_3^-$ intake g/day$^3$</td>
<td>−0.34 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.37 ± 2.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.33 ± 2.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>% of dry matter&lt;sup&gt;C&lt;/sup&gt;</td>
<td>−0.02 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.49 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.53 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

$^a$<sup>A</sup>$^b$<sup>B</sup>$^c$<sup>C</sup>Means with unlike subscripts within row are different (P < 0.05).

$^1$Highly tolerant and lowly tolerant ewes were offered 300 mg/kg of BW of NO$_3^-$ daily.

$^2$Percentage of 300 mg/kg of BW of supplement offered daily.

$^3$Total NO$_3^-$ intake from supplement in addition to <4 ppm measured in bromegrass hay.
**NO₃⁻ intake increased PUN values also increased.** Lowly tolerant ewes displayed a high positive correlation (0.71; *P*, 0.0001; Figure 4) between PUN levels and treated supplement intake; however, there was no correlation between PUN levels and percentage supplement intake in highly tolerant ewes (0.24; *P* > 0.10). The regression of PUN values on percentage of NO₃⁻ treated supplement intake in highly tolerant and lowly tolerant ewes, respectively, further demonstrates the relationship of PUN levels with NO₃⁻ treated supplement intake in lowly tolerant ewes.

Neither plasma cortisol (*P*= 0.93; Figure 5) nor glucose levels (*P*= 0.97; Figure 6) were different among highly tolerant, lowly tolerant and control ewes. Cortisol levels increase when animals are exposed to high levels of NO₃⁻ (Yaremci, 1991; Zraly et al., 1997); however, measuring cortisol as an indicator of stress does not identify the nature of the actual stressor. It should be noted that cortisol levels numerically increased (*P* > 0.10) on day 0 and day 3. During those 2 days, ewes were being weighed, prepared for a liver biopsy, blood sampled, or exposed to extra handling such as shearing of the necks, which may have caused the observed increases in cortisol. Gluconeogenesis is a continual process in ruminants as almost all dietary carbohydrates are fermented to volatile fatty acids in the rumen (Young, 1976). Therefore, it is difficult to establish how glucocorticoids affect glucose production in ruminant animals. Blood glucose levels in ruminants range from 40 to 60 mg/100 ml and tend to fall after fasting and remain unchanged after feeding (Van Soest, 1994). Because 100% of the bromegrass hay provided daily was consumed by all ewes, differences in cortisol and glucose levels were not expected between ewe groups.

Circulating plasma retinol levels on day 1, 4 and 7 did not differ (*P* = 0.23).
levels (O’Donovan and Conway, 1967). Circulating retinol levels may not have been affected in this study due to the subacute nature of the NO$_3^-$ exposure. Bruning-Fann and Kaneene (1993) observed a reduction in vitamin A levels upon an acute dose of NO$_3^-$ in steers fed a high concentrate diet and concluded that a chronic NO$_3^-$ exposure or an acute NO$_3^-$ dose is needed to produce noticeable effect on vitamin A or retinol metabolism in cattle and sheep. However, O’Donovan and Conway (1967) found that ewes grazing a pasture with NO$_3^-$ content on a DM basis ranging from 0.05% to 1.72% for 5 months did not have significantly reduced vitamin A levels. Furthermore, it can take up to 1 to 2 years for the liver to be fully depleted of vitamin A, which may explain why little change was observed in circulating plasma concentrations of vitamin A in the short term (NRC, 2007). Finally, differences in basal diet in terms of presence or absence of green forages, or providing β-carotene might contribute to differences between studies.

Overall, there were no differences (P > 0.25) in plasma analytes between highly tolerant, lowly tolerant and control ewes in this study except for PUN levels between control and highly tolerant ewes (P = 0.02). This may indicate that ewes did not adapt to the higher dietary NO$_3^-$, or perhaps were not provided an adequate time to do so. Alternatively, NO$_3^-$ treated ewes may have been reducing the NO$_3^-$ at different rates, resulting in plasma analytes that were similar among the selected groups (highly tolerant and lowly tolerant). Therefore, we hypothesize that individual variation in metabolizing NO$_3^-$ may be genetically based which will be investigated in future gene expression analyses.

Conclusions

This study confirms and quantifies the variation observed among ruminant animals exposed to a short term, subacute NO$_3^-$ exposure. Variation in NO$_3^-$ treated supplement intake, NO$_3^-$ intake and expression of signs associated with NO$_3^-$ toxicity, combined with no differences in BW change or blood analytes with limited differences in PUN values, indicate that animals do not reach a biological threshold when administered subacute dietary NO$_3^-$ . These results imply that animals cannot be identified as highly tolerant or lowly tolerant to elevated dietary NO$_3^-$ based on blood analytes alone. The variation in response to subacute levels of dietary NO$_3^-$ may help producers recognize NO$_3^-$ as a potential cause for reduced intake and performance in a herd or flock that is exhibiting disparities in production and behavior traits among individuals. Identification of lowly tolerant ewes according to production and behavior traits may allow producers to employ alternative management strategies for those individuals, eliminate them from their breeding stock, or cull them from the herd or flock. Furthermore, future trait selection based on genomic marker research for tolerance to elevated dietary NO$_3^-$ in forages would enhance the role for sheep in biological weed and insect pest control and improve profitability in sheep production systems.

Acknowledgements

This research was supported in part by a University of Wyoming Agricultural Experiment Station Competitive Grant. The authors would like to thank Brent Larson and Ed Van Kirk for their assistance with animal care and sample collection.

References


