The effects of chronic nitrate supplementation on erythrocytic methaemoglobin reduction in cattle

<table>
<thead>
<tr>
<th>Journal:</th>
<th>Animal Production Science</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>AN13366</td>
</tr>
<tr>
<td>Manuscript Type:</td>
<td>Research paper</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>02-Sep-2013</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Godwin, Ian; University of New England, Animal Science, Li, Li; University of New England, School of Environmental and Rural Science, Luijben, Koen; University of New England, School of Environmental and Rural Science, Oelbrandt, Nicky; University of New England, School of Environmental and Rural Science, Velazco, Jose; University of New England, School of Environmental and Rural Science, Miller, Joseph; University of New England, School of Environmental and Rural Science, Hegarty, Roger; University of New England, School of Environmental and Rural Science</td>
</tr>
<tr>
<td>Keyword:</td>
<td>Animal physiology, Biochemistry, Beef cattle, Cell biology, Toxicology</td>
</tr>
</tbody>
</table>

The effects of chronic nitrate supplementation on erythrocytic methaemoglobin reduction in cattle.

I Godwin, L Li, K Luijben, N Oelbrandt, J Velazco, J Miller and R Hegarty

School of Environmental and Rural Science, University of New England, Armidale, N.S.W. 2351 Australia

Short Title: Chronic nitrate and methaemoglobin reduction
Abstract. Calcium nitrate and urea were fed as a supplement on an isonitrogenous basis to Angus steers and their erythrocytic methaemoglobin and NADH- and NADPH-methaemoglobin reductase levels measured over a 54 day period. Methaemoglobin levels remained elevated despite increases in NADH-methaemoglobin reductase activity. In a second experiment, Brahman cross steers were fed either calcium nitrate or urea supplements for 111 days. Blood cells were then taken washed and exposed to sodium nitrite to convert all haemoglobin to methaemoglobin. The rates of glycolysis and methaemoglobin reduction were measured following incubation of these cells in buffers containing either 1,5 or 10mM inorganic phosphate. Glucose consumption and methaemoglobin reduction was increased by inorganic phosphate and was more rapid in those animals supplemented with nitrate. Lactate production was reduced in those animals fed nitrate. It is concluded that adaptation to chronic nitrite exposure occurs in the erythron resulting in greater methaemoglobin reduction potential and that there is competition between NADH-methaemoglobin reductase and lactate dehydrogenase for NADH.

Additional keywords: inorganic phosphate, methaemoglobin reductase

Introduction

Nitrate supplementation of ruminant animals is being investigated as a means of reducing methane emissions (Nolan et al. 2010; van Zijdderveld et al. 2011; Huishof et al. 2012). Basically, excess hydrogen produced in the rumen fermentation is normally converted to methane by reduction of carbon dioxide. Supplementation of the rumen fermentation with nitrate and its subsequent reduction leads to the production of ammonia. The ammonia can then be incorporated into microbial protein. However, the reduction of nitrate to ammonia results in the generation of the intermediate nitrite. Nitrite absorption at high levels results in toxaemia manifesting as methaemoglobinaemia (Harris and Rhodes 1969).
Methaemoglobin is the oxidised form of haemoglobin and lacks the ability to transport oxygen. High levels of methaemoglobin effectively leads to tissue hypoxia and ultimately death. In vivo, methaemoglobin is reduced back to haemoglobin by an NADH-dependent methaemoglobin reductase (cytochrome b5 reductase) (Agar and Harley 1972). In nitrite poisoning this enzymatic reduction is simply overwhelmed and methaemoglobinaemia results. A second enzyme system, NADPH-dependent methaemoglobin reductase can be activated by the electron donor methylene blue. Methylene blue is reduced to leucomethylene blue and is oxidised back to methylene blue providing electrons for the non-enzymatic reduction of methaemoglobin back to haemoglobin (Janssen et al. 2004). The current study examines the effects of chronic nitrate supplementation on the activity of NADH and NADPH dependent methaemoglobin reductases in cattle. The supply of NADH in erythrocytes is largely provided by the action of the enzyme glyceraldehyde -3-phosphate dehydrogenase which requires inorganic phosphate as a co-factor. In a second study we investigated the effects of incubation of red cells with different levels of inorganic phosphate on the rate of methaemoglobin reduction in vitro.

Materials and methods

Experiment 1

Eighteen Lowline Angus steers (BW 353 ± 17.7kg) were fed a standard feedlot ration containing either supplemental urea and calcium carbonate (8 animals) or calcium/ammonium nitrate (10 animals). The levels of urea and nitrate fed were initially 0.25 and 1.0% and increased gradually to 0.865 and 2.57% of the dry matter intake respectively, over the first 25 days to provide an isonitrogenous (0.4%N of DM) and isocalcic diet. Blood samples were taken at approximately weekly intervals via the tail vein into heparinised vacutainers and immediately stored on ice. Methaemoglobin levels were determined by the method of Hegesh et al (1970) on these samples within an hour of collection. Whole blood samples were then stored at 4°C, for up to 2 weeks and the activity of both NADH and NADPH-methaemoglobin reductases determined.
using the methods of Beutler (1986) and Agar & Harley (1972) respectively. All spectrophotometric measurements were undertaken using a Helios Gamma Spectrophotometer (Thermo Electron Corporation, UK).

Experiment 2

Ten Brahman X Senepol X Charolais cross steers maintained in a feedlot setting were fed commercial diets containing either 1.255% nitrate (calcium nitrate) or 0.502% urea for 20 days and then 2.51% nitrate (calcium nitrate) or 1.00% urea on an isonitrogenous and isocalcic basis for a further 91 days. The composition of the diets and food intakes are given in Table 1.

(Table 1 near here)

On day 66, 10 mL of blood was obtained from the tail vein of each steer and methaemoglobin levels were determined. On day 111, 50 mL of blood was sampled from each steer via the tail vein into heparinised vacutainers. The blood samples were immediately placed on ice and transported to the laboratory. Red cell NADH-methaemoglobin reductase was determined by the method of Beutler (1986), NADPH methaemoglobin reductase by the method outlined by Agar and Harley (1972) and haemoglobin using the method of van Kampen and Zijlsta (1961).

Red blood cells were prepared by centrifuging the samples at 3000g followed by washing three times in cold isotonic saline with the plasma and buffy coat removed by aspiration with each washing. The cells were then incubated in an equal volume of 150mM sodium nitrite in saline for 15 minutes at 38°C. This procedure allowed the production of 100% methaemoglobin in the cells. The cells were then washed 5 times in isotonic saline to remove all nitrite.

The cells were then resuspended in one of three buffers P1, P5 and P10 (Table 2) to achieve haematocrits of approximately 50%.

(Table 2 near here)
The resuspended cells were then incubated at 38°C for 6 hours. Samples were taken at 0, 2, 4 and 6 hours for the measurement of methaemoglobin, glucose, lactate and pyruvate.

Methaemoglobin was measured using the method of Hegesh et al., (1970). For glucose, lactate and pyruvate measurement, an aliquot was mixed with an equal volume of cold 8% trichloroacetic acid, vortexed and frozen at -20°C until analysis.

Glucose and lactate concentrations were determined on the supernatant using a clinical analyser (Dade Behring Dimension RXL Clinical Chemistry System, Newmark, USA).

Data from experiment 1 were analysed using a repeated measures analysis of variance while data from experiment 2 were analysed using a 2 way analysis of variance. Tukey post hoc tests were used following both analyses. The program StatistiXL was used for all analyses (Roberts and Withers 2007).

Results

Experiment 1

There was no significant effect of diet on blood haemoglobin levels (Fig. 1). However, methaemoglobin levels showed a substantial rise by day 15 after the introduction of supplementary nitrate feeding in the nitrate fed animals (Fig. 2). There was no change in erythrocyte NADH-MetHb reductase activity until about 6 weeks after the introduction of nitrate into the diet. The nitrate fed animals then exhibited a much higher activity of the enzyme (Fig. 3). There was no change in NADPH-MetHb reductase activity over time or with diet (Fig. 4).

(Figure 1 near here)

(Figure 2 near here)

(Figure 3 near here)

(Figure 4 near here)
Experiment 2

The nitrate fed animals had significantly higher methaemoglobin levels than the urea fed animals (P<0.05) (Fig. 5).

(Figure 5 near here)

NADH-MetHb reductase activities of the cattle at day 111 were significantly higher (P<0.01) in the nitrate fed animals 34.8 ± 1.88 vs 23.3 ± 0.88 IU/gHb. There was no difference in the NADPH-methaemoglobin reductase levels between the two groups (1.8 ± 0.17 vs 1.7 ± 0.16 IU/gHb).

Glucose consumption and lactate and pyruvate production rates of erythrocytes averaged over the 6 hours of incubation are shown in Fig. 6. Glucose consumption by the erythrocytes from both groups of cattle were similar. As Pi concentration was increased from 1 to 10mM the rate of glucose consumption increased (P<0.001) with no difference between dietary treatments. The blood cells of the animals fed the urea diet produced lactate in a pattern similar to that of glucose consumption, with a significant increase (P<0.05) with increased Pi concentration. Pyruvate production followed a similar trend, but the changes were not statistically significant. The lactate production of the cells from the nitrate fed animals was substantially reduced compared to the cells from the urea fed animals incubated with the corresponding Pi level (P<0.05). There was a trend for pyruvate production to increase with increasing Pi concentration but this was not statistically significant (P=0.06)

(Figure 6 near here)

Methaemoglobin levels steadily reduced from 100% to around 40% after 6 hours. Following 6 hours of incubation methaemoglobin had reduced further in the nitrate treated animals (P<0.01) and was slowest at the lowest Pi concentration (P<0.05) (Table 3).

(Table 3 near here)
Discussion

Methane emissions from ruminant livestock can be reduced substantially by feeding nitrate salts (Nolan et al. 2010; van Zijderveld et al. 2011; Hulshof et al. 2012). Unfortunately a major limitation of this methodology is the toxicity of nitrite, the intermediate of nitrate reduction to ammonia in the rumen. Nitrite poisoning of livestock is also a naturally occurring phenomenon as many plant species accumulate nitrate under certain conditions (McKenzie et al. 2004).

Absorbed nitrite rapidly leads to oxidation of the haem component of haemoglobin leading to the formation of methaemoglobin, which is unable to carry oxygen. The in vivo methaemoglobin levels are normally low amounting to less than 1% of total haemoglobin (Power et al. 2007) because red cell methaemoglobin reducing enzymes, particularly NADH-methaemoglobin reductase continually convert oxidized haemoglobin back to haemoglobin (Vetrella et al. 1971). In the presence of methylene blue, an artificial electron acceptor, a secondary “rescue pathway” is activated involving the enzyme NADPH methaemoglobin reductase (Janssen et al. 2004). In the present study NADPH-methaemoglobin reductase was not altered by dietary treatment, confirming its normally inactive role (Hultquist et al. 1993). However, NADH-methaemoglobin reductase did increase over time with the chronic feeding of nitrate. Interestingly, there was no change in levels of this enzyme until about 44 days after the introduction of nitrate to the diet. This may simply indicate that as bovine red cells have a lifespan of about 130 days in the circulation (Vacha and Znojil 1981) the exposure to nitrite and/or the elevation of methaemoglobin levels leading to an increase in NADH-methaemoglobin reductase, did not become evident until sufficient new cells carrying increased levels of the enzyme entered the circulation. As red cells age, the activity of NADH-methaemoglobin reductase declines exponentially (Takeshita et al. 1983). It is not unusual for methaemoglobin laden cells containing Heinz bodies to be prematurely removed from the circulation by the spleen (Sugawara et al.
For Review Only

2010), leading to an increase in younger cells with higher enzyme activity. Despite the increased
levels of NADH-methaemoglobin reductase, methaemoglobin levels in the nitrate fed steers
remained relatively elevated throughout experiment 1. Studies in rats have shown that following
chronic nitrite intake the levels of methaemoglobin return to normal within 25 weeks (Csallany
and Ayaz 1978) and that this adaptation is probably due to increases in NADH-methaemoglobin
reductase activity (Pankow et al. 1975).

The reduction of methaemoglobin by NADH-methaemoglobin reductase is dependent on a ready
supply of NADH (Kennett et al. 2005). This NADH is generated in the erythrocyte by the action of
the enzyme, glyceraldehyde-3-phosphate dehydrogenase, in the glycolytic pathway. This enzyme
phosphorylates glyceraldehyde-3-phosphate to yield 1,3-diphosphoglycerate and hence requires
inorganic phosphate as one of its substrates. Ogawa et al. (1987, 1989) found elevated levels of
methaemoglobin in phosphate deficient post-parturient cows, that was corrected with
intravenous phosphate.

The consumption of glucose was elevated with the increase in inorganic phosphate level of the
incubation media and this was paralleled by an increase in lactate production, but only in the cells
from the urea fed animals. An increase in inorganic phosphate concentration is known to increase
the glycolytic rate of red blood cells (Agar et al. 1986).

The glycolytic pathway in erythrocytes ends with either pyruvate or lactate, depending on the
availability of NADH (Kennett et al. 2005). Normally NADH is not limiting and lactate exits the cell
as the end product of glycolysis. However, if NADH is oxidised by the action of NADH-
methaemoglobin reductase it would be unavailable as a cofactor for the conversion of pyruvate
to lactate by lactate dehydrogenase. In the cells from the cattle fed urea, the levels of lactate
produced were much higher than those in the animals fed nitrate, despite similar rates of glucose
consumption. This suggests that as the nitrate treated group had the additional drain on NADH to
reduce methaemoglobin, the production of lactate was curtailed. Indeed the percentage of pyruvate produced as an end-product was much higher in the cells from nitrate fed cattle.

The rates of methaemoglobin reduction were faster in the cells from the nitrate fed animals, which was presumably due to the higher activity of NADH-methaemoglobin reductase. The level of inorganic phosphate in the incubation suspension did influence the rate of methaemoglobin reduction, with the higher levels of Pi resulting in a greater reduction of methaemoglobin. The effect of phosphate could be due to both an increase in the overall glycolytic rate (Wegener and Krause 2002) making more NADH available or to the direct action of Pi to stimulate the NADH generating step catalysed by glyceraldehyde-3-phosphate dehydrogenase (Reis et al. 2013). It is somewhat surprising that the effect of inorganic phosphate on methaemoglobin reduction was greater in the cells from the urea fed cattle as it would be expected that a higher enzyme activity, fuelled with greater amounts of substrate would yield a much faster rate of methaemoglobin reduction. As many northern Australian cattle graze phosphate deficient pastures, it would be prudent to ensure adequate phosphate supplementation, if nitrate is to be used as a nitrogen supplement.

Acknowledgements

The authors gratefully acknowledge the skilled technical help of Graeme Bremner and Andrew Blakely. The project was funded by Cargill Inc. and the Australian Government Department of Agriculture, Forestry and Fisheries Action on the Ground projects.

References

Agar, NS, Godwin, IR, Suzuki, M, Hablethwaite, J, Roberts, J, Hume, ID (1986) Comparative red blood cell metabolism in three wallaby species, Macropus eugenii, Macropus parma and
Thylogale thetis (Macropodidae: Marsupialia). Comparative Biochemistry and Physiology 85A, 297-299.


Roberts, A, Withers, P (2007) 'StatistiXL 1.8.' Nedlands, WA


Table 1. Composition of the diets and dry matter intake.

<table>
<thead>
<tr>
<th>Ingredient (% as fed)</th>
<th>Urea Diet</th>
<th>Nitrate Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>70.25</td>
<td>70.25</td>
</tr>
<tr>
<td>Corn Silage</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Cotton Seed</td>
<td>8.5</td>
<td>8.5</td>
</tr>
<tr>
<td>Straw</td>
<td>3.25</td>
<td>3.25</td>
</tr>
<tr>
<td>Liquid Supplement 1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.5</td>
<td>0</td>
</tr>
<tr>
<td>Liquid Supplement 2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0</td>
<td>9.5</td>
</tr>
</tbody>
</table>

Dry Matter Intake (kg/d) 10.86  9.91

<sup>a</sup> Liquid Supplement 1 contained 9.1% urea, 15.7% limestone, 49.8% molasses and micronutrients

<sup>b</sup> Liquid Supplement 2 contained 0.3% urea, 2.8% limestone, 26.1% Bolifor CNF, 49.1% molasses and micronutrients
Table 2. Composition of incubation buffers adjusted to pH 7.4

<table>
<thead>
<tr>
<th>Substance (mmol/L)</th>
<th>P1</th>
<th>P5</th>
<th>P10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Tris</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>KCl</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>1.2</td>
<td>1.2</td>
<td>1.2</td>
</tr>
<tr>
<td>NaCl</td>
<td>164</td>
<td>158</td>
<td>150</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1</td>
<td>5</td>
<td>10</td>
</tr>
</tbody>
</table>
Table 3. Erythrocyte methaemoglobin reduction rate (% of total after 6 hours) of red blood cells obtained from steers supplemented with either nitrate or urea. The cells were treated with sodium nitrite and incubated at 38°C with different concentrations of inorganic phosphate for 6 hours (means ± s.e.).

<table>
<thead>
<tr>
<th>Diet</th>
<th>Pi 1mM (%)</th>
<th>Pi 5mM (%)</th>
<th>Pi 10mM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urea</td>
<td>45.4 ± 3.67</td>
<td>41.4 ± 2.96</td>
<td>39.4 ± 1.03</td>
</tr>
<tr>
<td>Nitrate</td>
<td>38.8 ± 2.32</td>
<td>39.1 ± 0.93</td>
<td>36.9 ± 0.37</td>
</tr>
</tbody>
</table>

*Diet effect was significant (P<0.01), Pi effect was significant (P<0.05)*
Figure 1. The effect of dietary nitrate supplementation on blood haemoglobin levels in Angus steers (mean ± s.e.)

Figure 2. The effect of dietary nitrate supplementation on blood methaemoglobin levels in Angus steers (mean ± s.e.)

Figure 3. The effect of dietary nitrate supplementation on erythrocyte NADH-methaemoglobin reductase activity in Angus steers (mean ± s.e.).

Figure 4. The effect of dietary nitrate supplementation on erythrocyte NADPH-methaemoglobin reductase activity in Angus steers (means ± s.e.)

Figure 5. The effect of nitrate supplementation on blood methaemoglobin levels in Brahman cross steers (means ± s.e.)

Figure 6. The consumption of glucose and the production of lactate and pyruvate by red blood cells obtained from steers supplemented with either nitrate or urea. The cells were treated with sodium nitrite and incubated at 38°C with different concentrations of inorganic phosphate (means ± s.e.).
Figure 1

Haemoglobin (g/dL) vs Days

- Urea
- Nitrate

Figure 3

![Graph showing NADH-MetHb reductase activity over days]

- Red line: Urea
- Blue line: Nitrate

Days: 1, 8, 15, 23, 37, 44, 54

NADH-MetHb reductase (IU/gHb)

Days

Figure 4

NADPH-MetHb reductase (IU/gHb)

Days

NADPH-MetHb reductase (IU/gHb)

Days

Urea

Nitrate
Figure 6