POTASSIUM BROMIDE MITIGATES CLINICAL SIGNS OF INTOXICATION CAUSED BY THE INDOLE DITERPENOID TOXIN LOLTREM B.

Martin A. Combs[1, 2], Scott Edwards[1, 2], Allan E. Kessell[3], Adam Hamlin[4], Joshua Scherpenhuizen[1, 2], Edward Narayan[1, 2] and Jane C. Quinn* [1, 2]

Aims

Lolitrem B is an indole diterpenoid toxin found in perennial ryegrass. Although lolitrem B confers beneficial effects to the plant, these are counteracted by adverse effects observed animals that ingest it which can result in behavioural changes, ataxia and tremor and, in the most severe cases, seizures and death (Tor-Agbidye et al 2001). Morbidity and mortality of livestock can be in significant numbers (Cunningham 1959, Combs et al, 2014).
Currently no therapeutic treatment is available that can be delivered at a flock-wide level to mitigate the large-scale effects observed in animals during critical outbreaks. Platforms for drug testing have been difficult to establish due to seasonal variations in lolitrem B in pasture-based feeds and the difficulty in extracting this highly lipophilic toxin (Munday-Finch, 1997). To overcome this, a reproducible and reliable model of PRGT in sheep was established utilizing a feed containing high levels lolitrem B toxin. This model was compared to a rodent model where animals were exposed to pure lolitrem B toxin isolated from perennial ryegrass. Establishment of these model systems allowed a number of therapeutics to be trialled. The most significant improvements were observed in movement, behaviour and reduction of stress by administration of potassium bromide. Pharmaceutical products that could improve clinical outcomes, reduce mortality can now be tested using this experimental model.

Methods

Animals

White Sussex x Merino first cross male lambs of between 10-12 months of age (n=27, live weight (LW) 36.1 ± 3.02 kg) were used. A feed containing toxic levels of lolitrem B, was generated by incorporation into a chaff diet of a novel endophyte-infested perennial rye grass seed (Ga66 AR98, Grasslanz Technology Ltd, New Zealand).All animals receiving toxic feed were exposed to a diet containing a final concentration lolitrem B of 0.16mg / kg LW q 24hrs. After 7 days acclimation, animals were exposed to feeds either + or – for Lolitrem, B. Any animal that had not shown significant movement disorder at 21 days, toxin was increased to 0.27 mg/kg LW for the duration of the trial. Five treatment groups each containing nine animals entered the trial: Group 1 - negative control: lucerne chaff only; Group 2 - positive control: lucerne chaff containing 0.16 mg/kg LW lolitrem B; Group 3 - acute KBr treatment: lucerne chaff containing 0.16mg/kg LW lolitrem B, treated orally with 300mg/Kg KBr (Sigma Aldrich) on first day of dropping during gait analysis, Group 4, prophylactic bromide therapy + lolitrem B0.16mg/kg LW q 24hrs. Group 5 Bromide control

4 groups of adult mice were treat as follows: 1) adult mice were injected with lolitrem B (2mg/kg, i.p.) (n=4) or lolitrem B (2mg/kg, i.p.) + bromide pre-treatment, 2500ppm in drinking water (n=10) or vehicle (90 % DMSO) (n=4) or vehicle (90% DMSO) + bromide pre-treatment, 2500ppm in drinking water.

Gait analysis

Animals underwent physical and observational examination daily. Gait analysis was performed on entry to the trial and then every third day during the trial. To achieve this, animals were circled in the yards for a minimum of three minutes whilst their movement was captured on video and a gait observation score determined using the following scoring criteria on a scale if (0) normal – (5) severe; ataxia, dysdiadochokinesia, rhythmic myoclonus, stumbling or falling and seizures. Entry to the treatment phase of the study was when animals displayed sufficient dysdiadochokinesia or rhythmic myoclonus to induce stumbling or falling. From point animals were assessed daily for gait changes for the subsequent 48 hours.

Faecal glucocorticoid metabolite analysis

Faecal cortisol metabolites (FCM) were extracted from sheep faecal samples using the methods as previously described for ruminants. FCM ELISA followed the detailed guidelines provided by Brown et al (2003) for validating the FCM enzyme-immunoassay. Concentrations of FCM were determined using a polyclonal anticortisol antiserum (R4866) diluted 1:15,000, horse-radish peroxidase conjugated cortisol label diluted 1:80,000 and cortisol standards (1.56–400pgwell^{-1}). Samples were assayed (duplicates) on Nunc Maxi-Sorp^{TM} plates (96 wells). Intra- and inter- assay coefficients of variation were determined from internal control samples (30% and 70% bound) included in all assays. Intra-assay coefficients of variation were 1.8% and 5.3% for low- and high- percentage bound controls, and inter-assay coefficients of variation were 5.8% and 1.8%, respectively.

Tremor Analysis on Mice

Tremor analysis was performed on mice using a piezoelectric pressure sensor and ADInstruments Powerlab^{TM} bioamplifier and LabChart^{TM} software to characterise the period and severity of induced tremor. Tremor was analysed by selecting one minute epochs for Fast Fourier Transform (FFT) analysis. The ratio of power output at likely tremor frequencies (9-20Hz) to total power output (0-50Hz), a motion:tremor power ratio (MTPR) was used to assess severity of tremor.

Results

Treatment with a single acute dose of oral potassium bromide decreases severity of tremor, increases time to falling and improves gait in lolitrem B intoxicated animals.

Sheep Gait Analysis

Following admission to the treatment stage of the protocol, animals given a single oral dose of bromide 300mg/KG LW showed reduced incidence of animals falling at 72 hours (Day 3) post treatment compared to untreated positive controls (Group 2) (Figure 1). They also showed improved composite gait scores (Figure 2) indicating that treatment with bromide improved both gait and coordination in this cohort. The majority of KBr treated animals did not exhibit falling during the 3 minute gait testing period on day 2 and 3 of the treatment protocol despite falling on Day 1. By comparison, their untreated counterparts showed greater incidence of ill-coordination and falling over the same time period. This data suggests improved coordination in the acute treatment group (Group 3) and deterioration with increasing toxin load in their untreated counterparts (Figures 1).

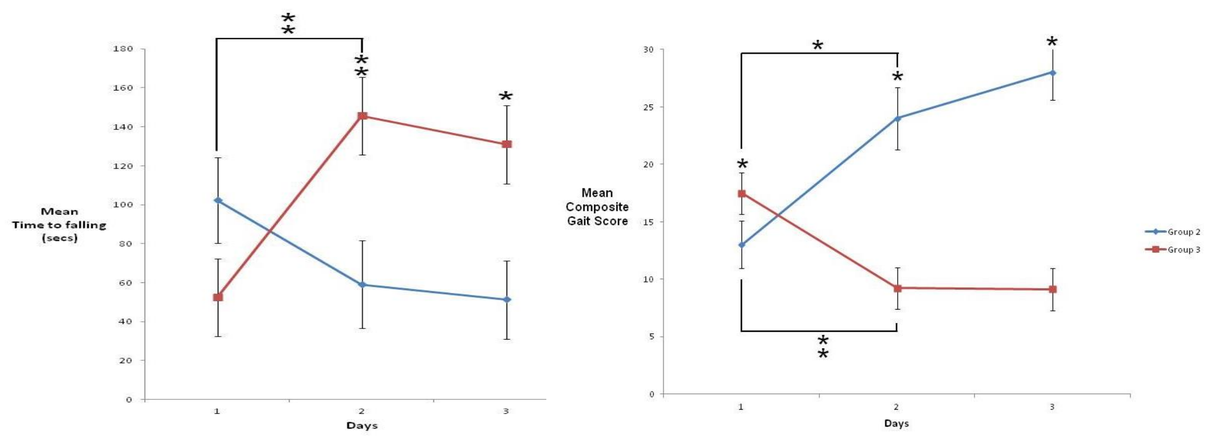

Figure 1. Time to falling (seconds) for animals exposed to lolitrem B toxin only or toxin plus treatment with acute oral potassium bromide. Values shown for Day 1 for Group 3 represent time to falling prior to treatment. Control animals (Group 1) did not exhibit falling. Data not shown.
Mean composite gait score over time for animals intoxicated with lolitrem B toxin and their toxin plus treatment bromide counterparts. (Group 2, n = 9, group 3, n = 8; * p = <0.05; ** p = <0.01). Group 2: lolitrem B toxin only, n = 9; Group 3: lolitrem B toxin plus single acute treatment with potassium bromide, n = 8.
* p = <0.05; ** p = <0.01.

Faecal Cortisol Metabolites

There was overall significant difference in mean levels of FCMs between the treatment groups ($F_{4,34}$ = 2.854; p = 0.038; Figure 8). Post-hoc testing showed that mean FCMs levels were significantly different between groups 1 and 2. Also there was significant difference in mean FCM levels between groups 2 (positive control) and group 5 (treatment control). Overall mean levels of FCMs were as follows: Group 1 (7.8 ± 0.65 ng/g dry faecal mass), Group 2 = 13.8 ± 1.46 ng/g dry faecal mass, Group 3 = 10.93 ± 1.28 ng/g dry faecal mass, Group 4 = 11.03 ± 1.77 ng/g dry faecal mass and Group 5 = 9.19 ± 1.56 ng/g dry faecal mass. There were no significant differences in mean FCM levels between group 1 with groups 3, 4, or 5 (p > 0.05 in all comparisons).

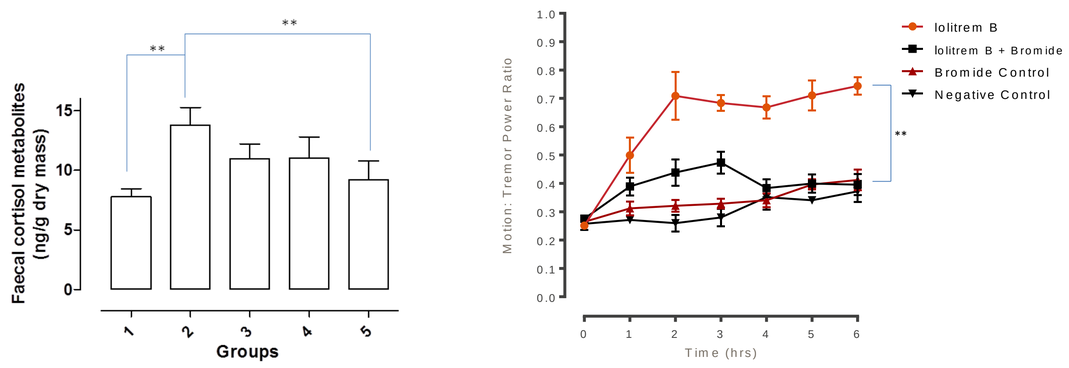

Figure 2: Faecal Cortisol metabolites were significantly increased (p<0.05) in lolitrem B intoxicated animal (group 2) when compared to negative control animal (group 1), however lolitrem B intoxicated animals treated with bromide either as an acute 300mk/kg bolus (group 3) or as a prophylaxis (group 4) did not have significantly increase cortisol when compared to the negative control animals.
Mouse tremor analysis revealed increased MTPR (p<0.01) at all time points after 1 hours in lolitrem B treated animals compared to lolitrem B treat animals given bromide prophylaxis

Tremor Analysis on Mice

Mice administered Lolitrem B at 2mg/kg i.p. exhibited an increased MTPR when compared to lolitrem B + bromide treated mice at all time points except for 0 and 1 hours. At 6 hours intoxicated mice pre-treated with bromide exhibited no significance difference in MTPR and control animals.

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[1]Graham Centre for Agricultural Innovation, (an alliance between Charles Sturt University and NSW Department of Primary Industries), Charles Sturt University, Pugsley Place, Wagga Wagga, NSW 2678, Australia;
[2]School of Animal and Veterinary Sciences, Charles Sturt University, Boorooma Street, Wagga Wagga, NSW, 2678, Australia;
[3]Gribbles Pathology Adelaide, South Australia, 5065, Australia,
[4]School of Science and Technology, University of New England, Armidale, NSW 2351, Australia.
*Corresponding author: Jane Quinn jquinn@csu.edu.au