Final report

Slow-release capsule for reducing winter scours in sheep – Stage: proof of concept

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Abstract

The potential for continuous *Trichostrongylus* antigen administration to sheep as a means to prevent hypersensitivity scours following infection was investigated. The results of the trial demonstrate continuous administration of parasite antigen leads to a suppression of the eosinophil response in the sheep following parasite challenge. This eosinophil suppression correlated with a significantly higher worm count in the antigen treated group. Despite being challenged with 7000 L3s, neither treated nor untreated sheep developed scours, indicating the development of diarrhoea is a multifactorial process. Although scouring did not occur in sheep in this trial following challenge, the suppression of eosinophils, the main mechanism behind the development of immune mediated scouring in sheep, indicates that the continuous administration of low doses of parasite antigen to sheep has the potential to prevent or reduce hypersensitivity diarrhoea in sheep. This concept may serve as a basis development of a commercial intra-ruminal device to prevent scouring in sheep.

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1. Objectives

1) Demonstrate the feasibility of using small, continuous doses of worm antigen to prevent hypersensitivity scouring in sheep.
2) Serve as the basis behind further research into the development of a commercial intra-ruminal device to prevent winter scours in sheep based on this concept.
3) Instigate larger multi-centre research into host-parasite interactions in ruminants to develop novel technologies to reduce the burden of parasitism in red meat production

2. Methodology

MLA is committed to investing in top quality scientific research, performed by suitably qualified, experienced and registered researchers and organisations. In experiments that involve livestock, MLA acknowledges that such research needs to be done under the auspices of a recognised Animal Care and Ethics Committee (AEC). The responsibility for obtaining AEC approval lies with the researcher. MLA has in the past not specifically asked for evidence that such AEC approval had indeed been obtained.

2.1 Animals

Wethers (N=24) were selected for this study from the flock at the Roseworthy Campus of the University of Adelaide. These sheep had grazed the previous season on pasture and were selected based on their propensity for scouring based on the records of the farm manager. Sheep were housed in individual pens within the Livestock Research Centre at the Roseworthy Campus. Upon arrival, at the facility, sheep were treated with Zolvix (2.5 mg/kg P.O) to remove any current nematode infections. Sheep were then allowed to acclimatise to their new conditions for 7 days. Before the experiment was initiated sheep were randomly allocated into 3 groups of 8 animals each as follows.

Group	Treatment
Group 1	Parasite antigen 3 x per week. Challenged on day 94
Group 2	No antigen treatment. Challenged on day 94
Group 3	Unchallenged controls

Faecal egg counts were performed on all sheep to ensure they were free from nematodes prior to the experiment being initiated.

2.2 Parasite antigen

Parasite antigen from this study was obtained from 2 donor sheep infected with *Trichostrongylus* spp. L3s originally used to infect these sheep were kindly provided by Dr Brown Besier, AgWA, Albany Western Australia. Faeces were collected from these sheep as required and larval cultures established to ensure a constant supply of parasite antigen for this study. Infectious L3s were harvested from cultures using the Baermann technique, enumerated, and those to be used in the antigen preparation were frozen immediately in aliquots of 8,000.

The antigen was prepared by thawing an aliquot of L3s at room temperature followed by sonication to disrupt the larvae. Prior to antigen being administered to the sheep, a subsample of the preparation was examined under the microscope to ensure no L3s had survived the antigen preparation process. Antigen was administered to sheep within 30 minutes of preparation.

Beginning on day 1 of the study, sheep in Group 1 received an oral dose of antigen equivalent to 1000 L3s using a syringe. Sheep in this group received this antigen 3 times per week from week 1 to week 13 of the study (37 treatments over 91 days).

Sheep in Group 2 and Group 3 received no treatments during this period of time. During this time, blood samples were collected once per week from all sheep. Faecal egg counts were also performed on sheep every 2 weeks to ensure sheep remained free of nematode infection

On day 94 of the study, sheep Group 1 and Group 2 were challenged with 7000 infectious *Trichostrongylus* spp L3s sourced from the infected donor sheep as described above. The challenge was administered to each sheep using a syringe. Sheep in Group 3 received no challenge.

Blood samples were collected from all sheep on the day prior to challenge and 2, 6, 12, and 24 hours following the administration of the challenge. Additional blood samples were collected 4, 6, 8, 15 and 22 days following the challenge. Complete blood counts (CBCs) were performed on these blood samples. In addition, mast cell tryptase and histamine levels were measured in blood collected 14 days prior to challenge and at 12 hours, 4 days, and 14 days post challenge using commercial ELISA kits according to their manufacturer's instructions. Total IgE in the serum of the sheep was also measured using a commercial ELISA at the same time points.

Faecal samples were collected from all sheep a day prior to challenge and then 3 times per week following challenge. Faecal moisture content was measured from these samples using a precision moisture analyser (MJ33 Infrared Moisture Analyser, Mettler Toledo). Faecal egg counts were also performed on samples collected on days 20, 22 and 25 following challenge to ensure infections were patent.

On days 121 and 122 of the study (27 days after challenge), sheep were euthanized with an IV injection of sodium pentobarbital. The intestinal contents of each sheep were collected and fixed in buffered formalin and total worm counts were performed according to standard methods. In addition, intestinal samples were collected from the duodenum (3), jejunum (3), ileum (3) and large intestine (2). The intestinal samples were collected from the same locations in each animal, fixed in 10% formalin, processed, sectioned and slides were prepared and stained with H&E for histological examination. The number of eoisinophils within the lamina propria was determined by counting 3 separate high powered fields of view per slide (400X magnification) and eosinophils per 10,000μm^2 was determined. The number of intraepithelial lymphocytes was determined by counting the number along a straight line of epithelium and the total number per 600µm of epithelium determined. Finally, the percentage of lamina propria occupied by lymphocytes and plasma cells was determined. All histological sections were evaluated according to methods employed by Day *et al.*(2008).

Results between each group are expressed as means +/- SEM. Data collected at single time points (ie: total worm counts) were analysed using one way ANOVA. For data collected at multiple time points, two way ANOVA was utilised. Differences of $P < 0.05$ were considered significant.

3. Results

During week 11 of the study, a sheep in the antigen/challenge group was euthanised due to the development of ulcerative posthitis. Following this, the remaining sheep were gradually switched to a lower protein content hay to prevent any further development of cases.

Following anthelmintic treatment upon arrival at the Livestock Research Centre, all faecal egg counts were below detectable levels prior to the nematode challenge, indicating sheep remained free of nematode infection during this period. By 27 days post challenge, strongyle type eggs were detected in 7/7 sheep Group 1 sheep and 7/8 Group 2 sheep indicating infections were established in the sheep. No eggs were detected in the faeces of sheep Group 3.

Parasite challenge did not affect faecal moisture content in sheep during this study. There were no significant differences in faecal moisture content between groups following challenge compared with control sheep (Figure 1). In addition, there was no increase in faecal moisture content in sheep at any time following challenge compared to pre-challenge levels.

Histological analysis of intestinal sections revealed that antigen treatment did have an effect on the number of eosinophils and intraepithelial lymphocytes (IELs) within the small intestine. Figure 2 shows the mean number of eosinophils per 10,000μm^2 of intestine from the duodenum, jejunum and ileum. Following challenge, Group 2 sheep had a significant increase in eosinophils within the jejunum compared to sheep from Group 1, indicating antigen treatment prevented an eosinophil response to challenge in Group 1 sheep. Figure 3 shows the effect of antigen treatment on the number of IELs per 100 enterocytes in the duodenum, jejunum and ileum. In Group 1 sheep, the number of IELs was significantly elevated in the duodenum compared to Group 2 sheep. There was a trend towards increased IELs in the ileum of Group 1 sheep compared to Group 2 sheep, but these were not significant. Antigen treatment had no effect on the percentage of lymphocytes and plasma cells in the lamina propria in this study (Figure 4).

Antigen treatment also had an effect on total worm counts in this study. Figure 5 shows the mean total worm counts for sheep in each treatment group. Group 1 sheep had a significantly higher total worm count of 2777 compared to group 2 sheep which had a mean total worm count of 1600.

Analyses of peripheral blood collected from sheep pre and post challenge did not reveal any significant differences with respect to monocytes, basophils or neutrophils. A trend towards increased eosinophils in Group 2 sheep compared to Group 1 and control sheep was observed 4 days post challenge; however this difference was not significant (Figure 6). A significant decrease in peripheral lymphocytes was observed in Group 1 sheep compared to Group 2 sheep 22 days following challenge (Figure 7).

ELISA results revealed that mast cell tryptase levels (MCT) were significantly elevated in Group 1 sheep compared to Group 2 and Group 3 sheep 14 days prior to challenge (Figure 8). However there was no difference in MCT levels between the 3 groups at 12 hours, 4 days or 14 days post challenge. Total serum IgE was significantly elevated in Group 1 sheep 12 hours following challenge (Figure 9). Differences between the 3 groups were not significant for total IgE for any of the other time points. Serum histamine concentrations were not different between the 3 groups of sheep at any of the time measured (Figure 10).

Figure 1. Faecal moisture content in sheep pre and post challenge with *Trichostrongylus* spp L3s. Data is shown is mean moisture content of faeces ± SEM

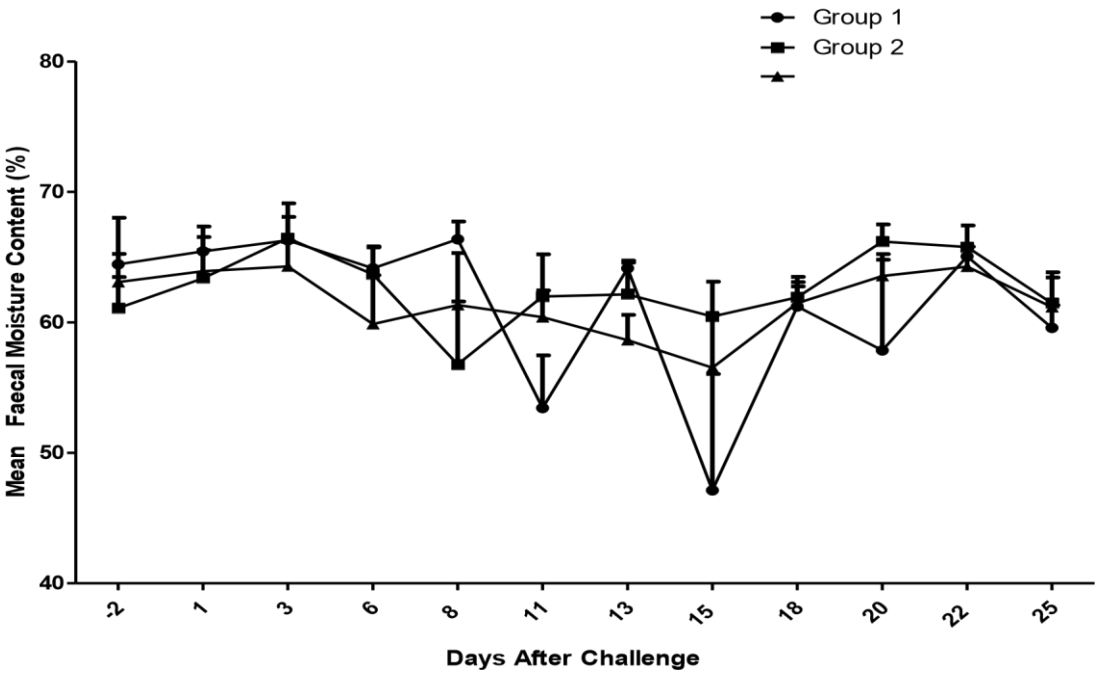


Figure 2. Mean number of eosinophils per 10,000µm^2 of intestine in the duodenum, jejunum and ileum of sheep following challenge with 7000 *Trichostrongylus* spp L3s. *indicates P< 0.5

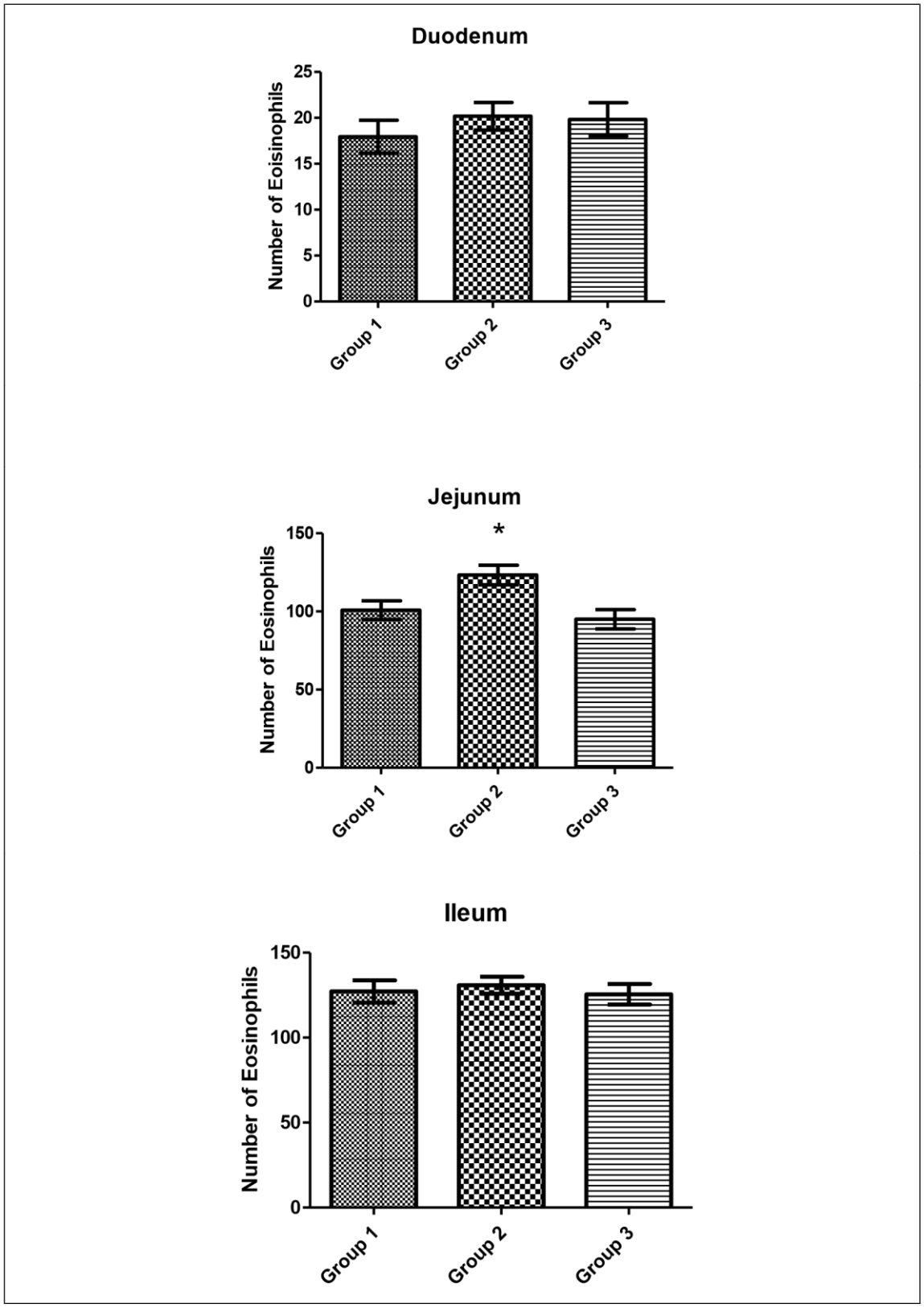


Figure 3. Mean number of intraepithelial lymphocytes per 600µm^2 of epithelim in the duodenum, jejunum and ileum of sheep following challenge with 7000 *Trichostrongylus* spp L3s. *indicates P<0.5

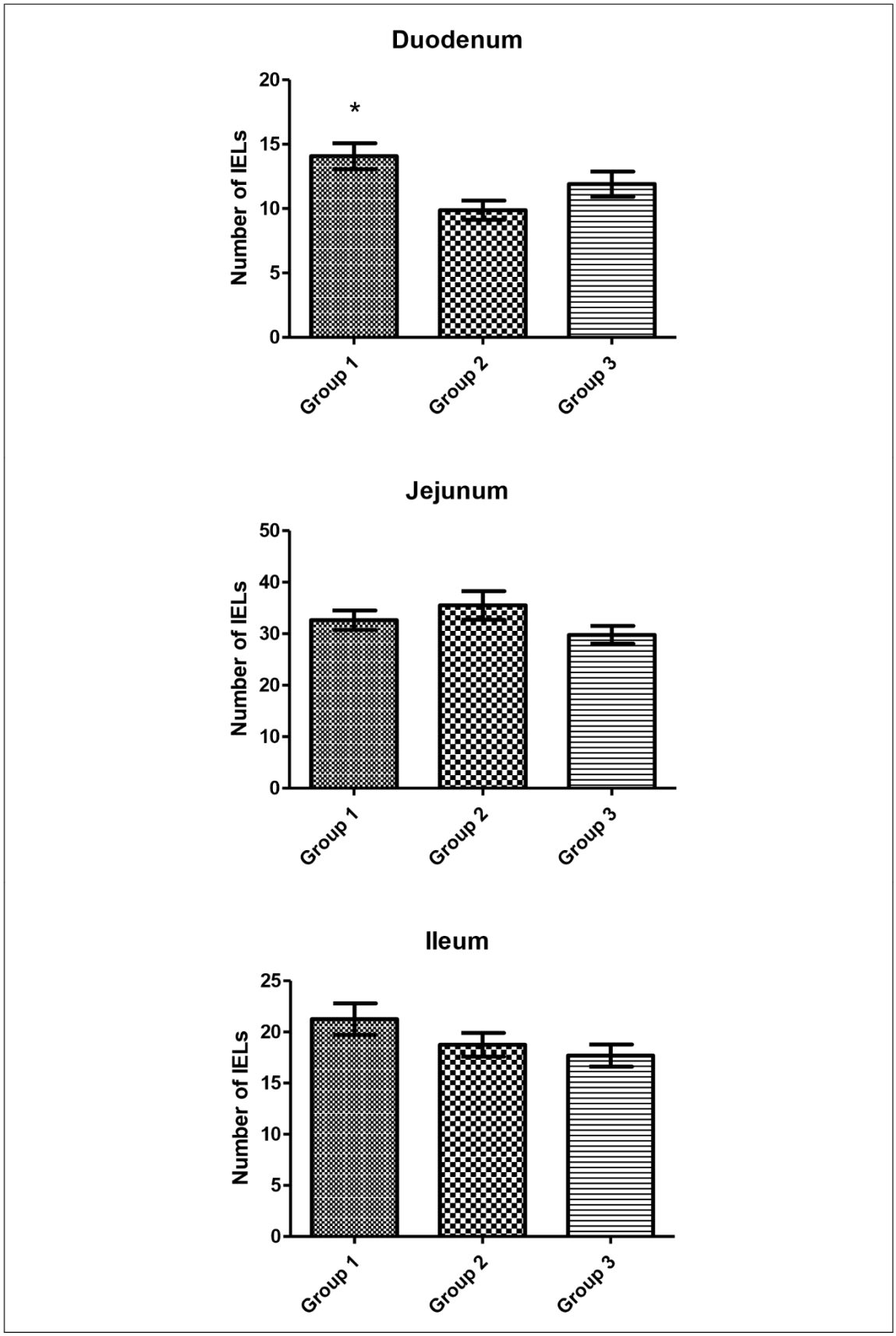


Figure 4. Percentage of lamina propria comprised of plasma cells and lymphocytes in the duodenum, jejunum and ileum of sheep following challenge with 7000 *Trichostrongylus* spp L3s.

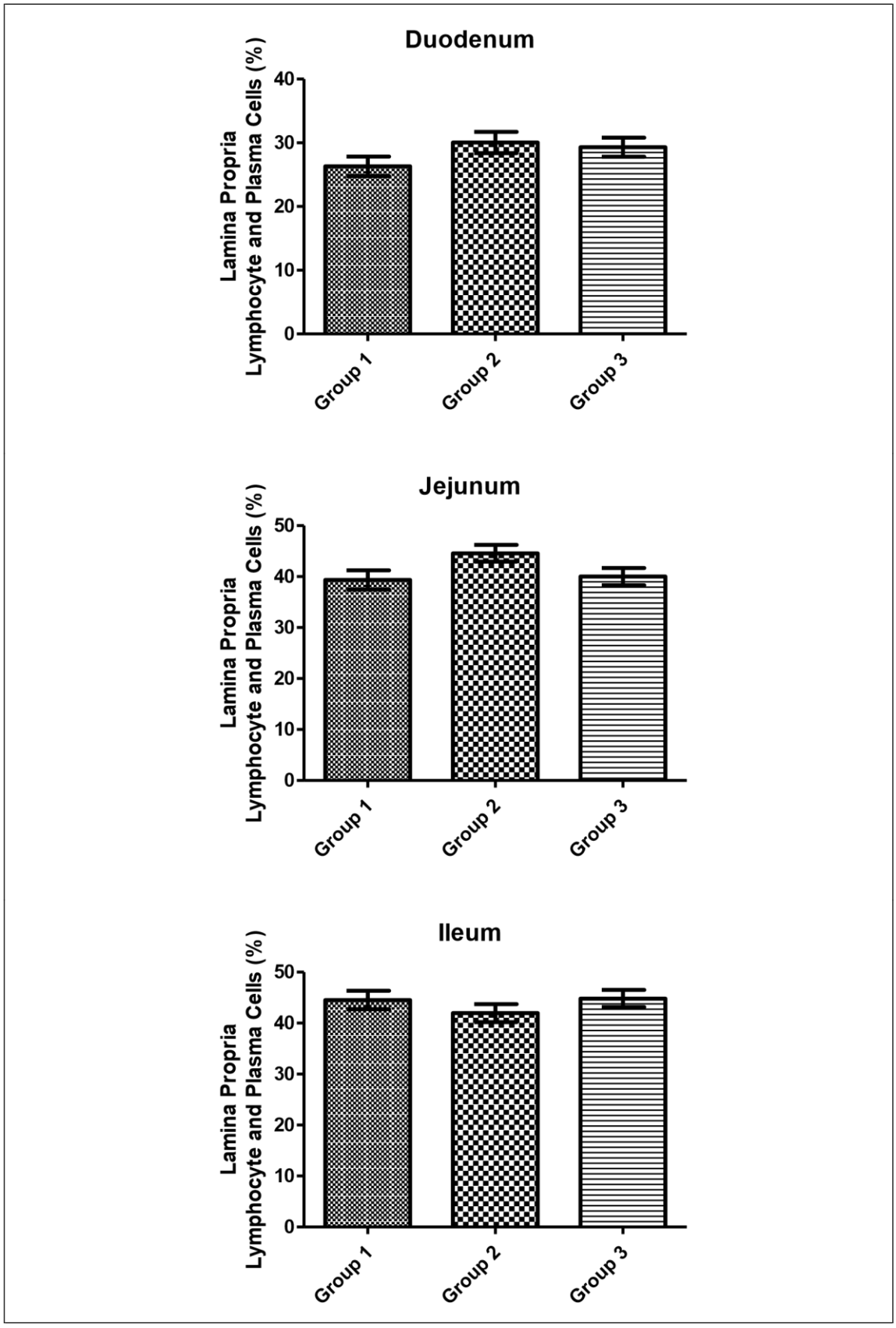


Figure 5. Total worm counts from the small intestine of sheep in each treatment group. Data is expressed as mean worm count ±SEM. * indicates a difference of P<0.05.

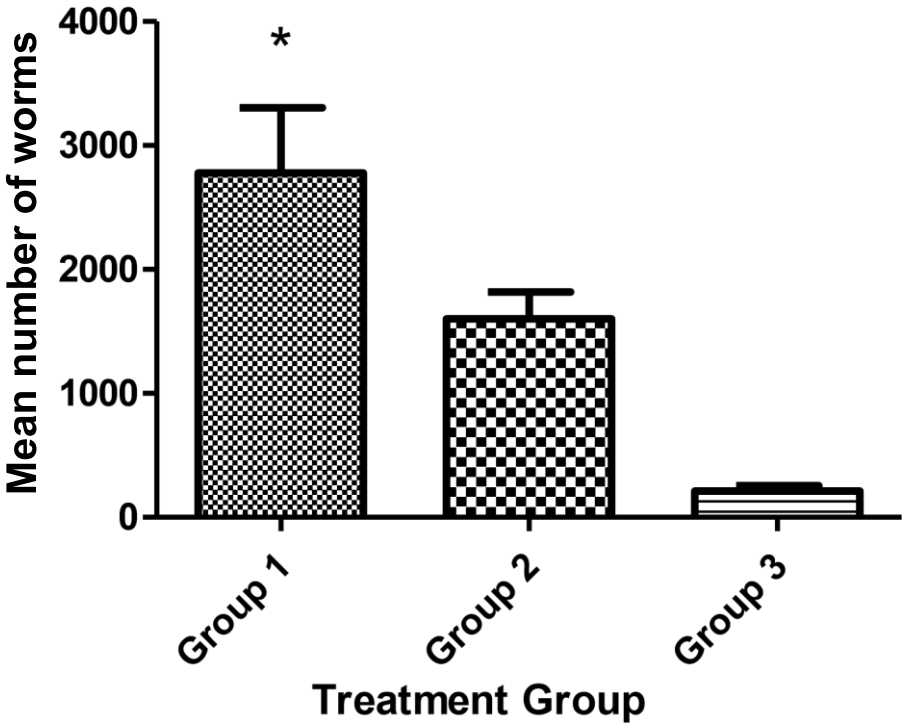


Figure 6. Eosinophils in peripheral blood pre and post challenge in treatment groups. Data is expressed as mean number of eosinophils per litre of blood ±SEM.

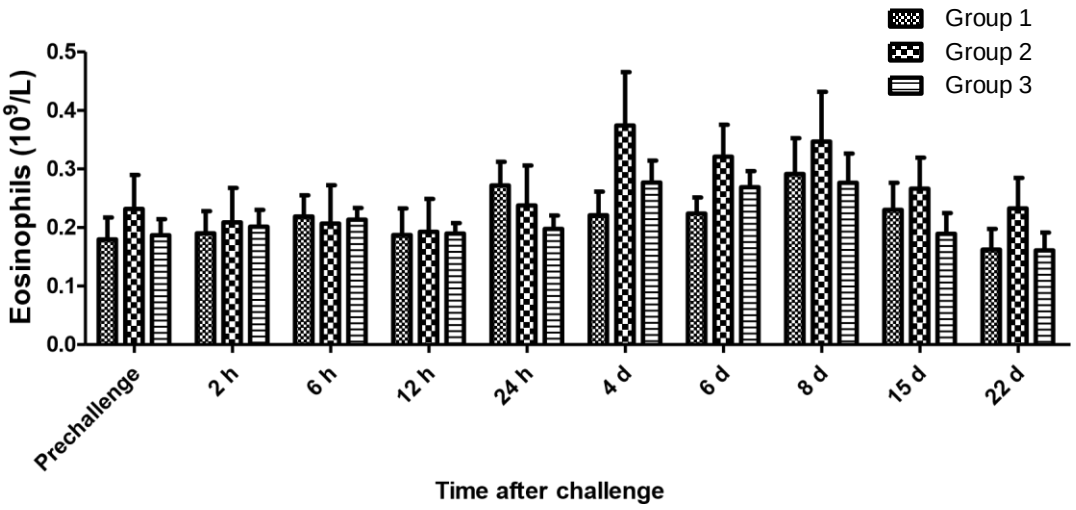


Figure 7. Lymphocytes in peripheral blood pre and post challenge in treatment groups. Data is expressed as mean number of Lymphocytes per litre of blood ±SEM. * indicates difference P < 0.05.

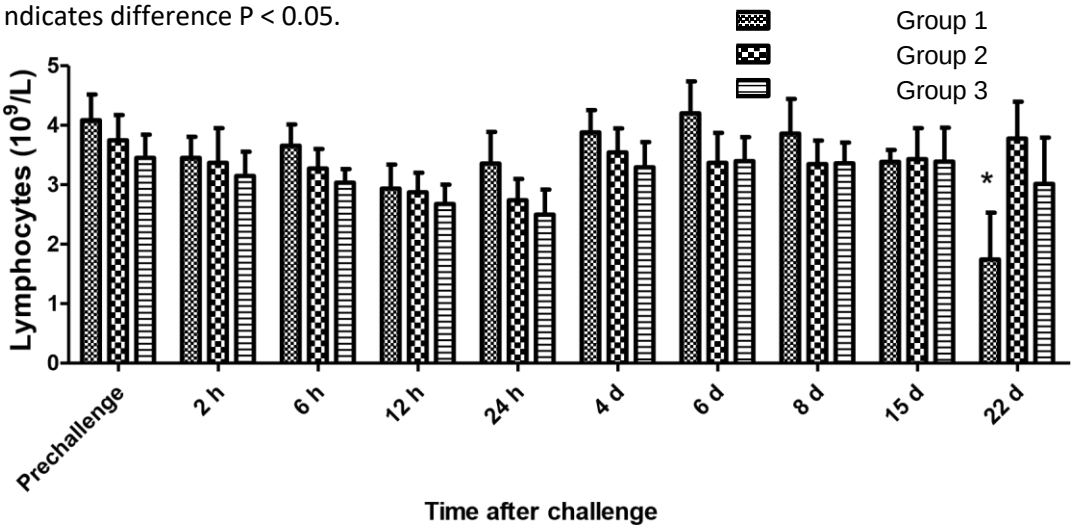
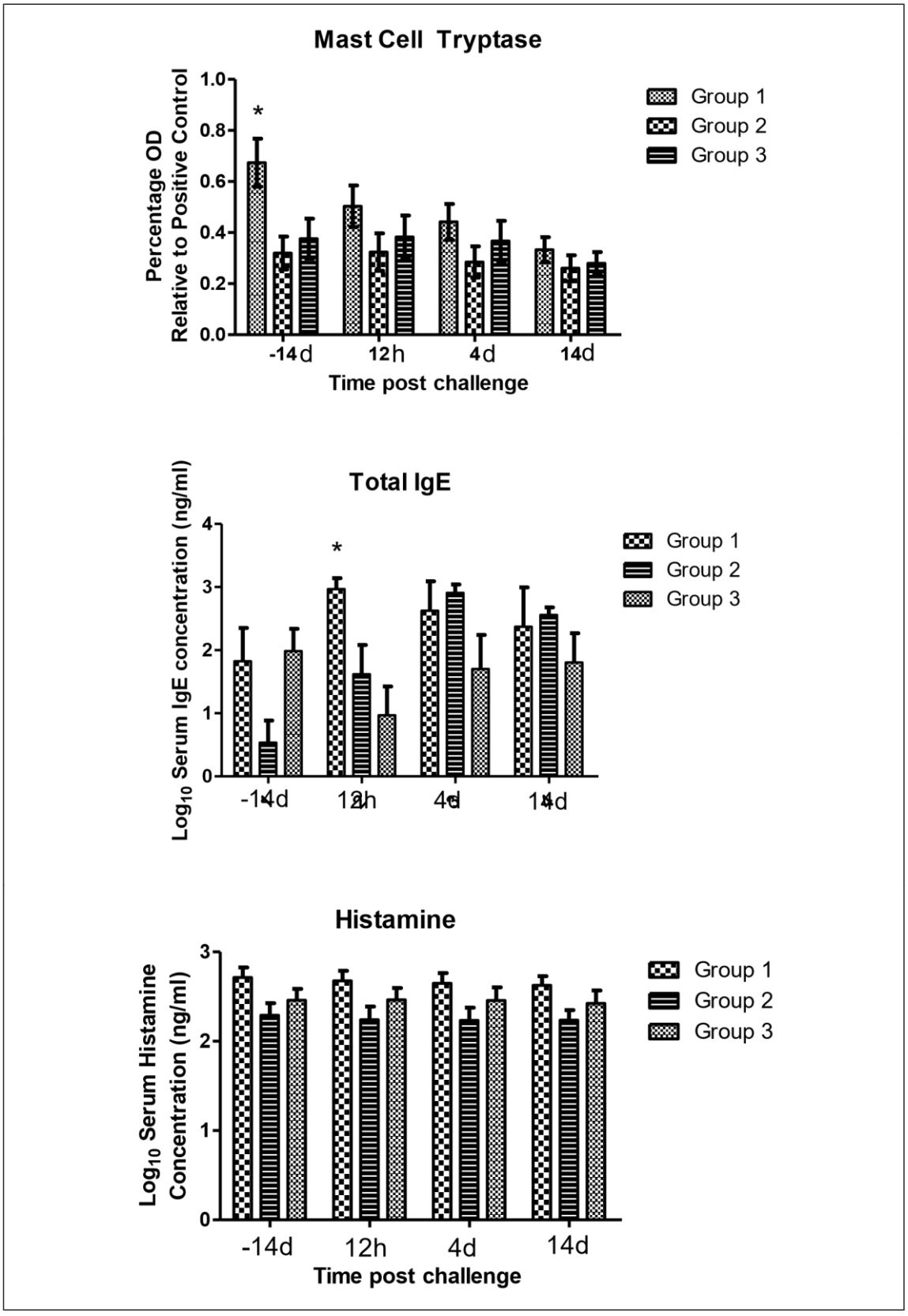


Figure 8. Serum concentrations of mast cell tryptase, IgE and histamine as measured by ELISA. Data is expressed as percentage relative to positive control (mast cell tryptase) and Log_{10} concentration of IgE and histamine in ng/ml. * indicates a difference P< 0.05.



4. Discussion

The results of this study clearly demonstrate continuous exposure to a small dose of parasite antigen has an effect on the immune response in sheep challenged with *Trichostrongylus* spp. The lack of an eosinophil response to challenge in sheep that received antigen, along with a corresponding elevated worm count, indicates it is possible to exhaust the immune response in sheep to parasite challenge. The administration of a small continuous dose of parasite antigen could prevent the development of immune mediated diarrhoea in sheep. These results also indicate that the development of an intra-ruminal device to prevent winter scours in sheep based on this concept would be feasible. In addition, these results also provide further evidence to support the role of the eosinophil as the major effector cell against nematode parasites in sheep and indicate that IELs may play an important regulatory role in the immune response in sheep. Further research is warranted to better understand these mechanisms.

We were unable to induce diarrhoea, or any change to faecal moisture content, in this experiment. Diarrhoea is often multifactorial and it would appear that factors other than the presence of the parasite are required to induce diarrhoea in these sheep. Williams et al (Williams et al., 2010a) observed and increase in faecal moisture content in sheep housed indoors following challenge, however they used rams known to be highly susceptible to scouring and administered a continuous dose of L3s rather than a single dose. Even so, these authors were not able to induce scouring in sheep and increases in faecal moisture content were less than 10% in that study.

Although challenge did not have an effect on faecal moisture content, trickle administration of antigen prevented an eosinophil response within the small intestine upon subsequent challenge infestation with live worm larvae. This lack of an eosinophil response also correlated with an increased worm count in these sheep. It is well known that eosinophils increase following parasite infection in sheep and that the increased eosinophils are associated with diarrhoea and the development of dag, in addition to lower worm counts (Balic et al., 2003; Balic et al., 2002; Larsen et al., 1994; Williams et al., 2010a; Williams et al., 2010b). Parasitic infection initiates the degranulation and release of potent inflammatory mediators by eosinophils. It is these mediators (eosinophil perioxidase and lectin-binding protein galectin-14) in combination with histamine, bradykinin and arachidonic acid metabolites, that leads to the 'flushing' of the gut through increased peristalsis and mucus secretion (Doligalska et al., 1999; Prussin and Metcalfe, 2006). Therefore, the eosinophil likely plays a key role in controlling parasite levels in sheep and in immune mediated diarrhoea. It is clear from the results of this study that antigen treatment reduced the eosinophil response in sheep and therefore has the potential to prevent immune mediated diarrhoea. Although worm counts were higher in antigen treated sheep, in well managed flocks, on a reasonable plain of nutrition, an immune response that reduces the number of intestinal parasites but causes diarrhoea may be counterproductive. In addition, immune mediated diarrhoea in sheep following challenge with trichostrongylid nematodes is not influenced by the level of infection as sheep can develop immune mediated scours irrespective of infectious dose (Larsen et al., 1995). Therefore, the ability to modify the immune response in sheep, as demonstrated in this study, to prevent the development of scours would be of great benefit in many situations.

In addition to preventing the eosinophil response following challenge, the antigen treatment also resulted in an increase in intraepithelial lymphocytes in the duodenum. Further research is required to understand the role of these IELs, however current research demonstrates IELs can play a regulatory role and are associated with tolerance (Cheroutre et al., 2011). It is possible that IELs were regulating the immune response in these sheep and may have played a direct role in

preventing the eosinophil response to the challenge. Obviously, further research would be required to understand the role of IELs in this experimental model.

Peripheral blood analyses did not reveal any major differences between treatment groups following challenge. Results were quite variable and it is therefore not surprising that differences were not observed. Mast cell tryptase levels were significantly elevated 2 in sheep that received the antigen treatment 2 weeks prior to challenge. It is unknown why this was elevated at this time. Total IgE levels in sheep that received antigen treatment were significantly elevated 12 hours post infection. This may have been an anamnestic response to challenge with L3s. An IgE response following parasite challenge has been reported to occur previously in mice (Urban et al., 1991), however the importance of this response does not appear to have been protective in this experiment. Histamine levels were not influenced by the treatment or the challenge in this study. Collectively, these results indicate that future work should focus on the localised immune response rather than the peripheral immune response.

5. Conclusion

All facets of the experimental aspects of this project were completed within budget. The results of the study indicate that it is potentially feasible to use a small continuous dose of worm antigen to prevent immune scouring in sheep as we clearly suppressed the eosinophil response using the antigen treatment. The possible deleterious effects of the resultant higher worm burden needs further investigation. As a proof of concept trial, this study has successfully served as a basis from which further research into the development of a commercial intra-ruminal device can be developed based on this concept to reduce scouring in sheep. These findings will also serve as the basis from which a larger multi-centred study can be initiated examining host-parasite interactions and to develop novel technologies to reduce parasitism in ruminants.

6. Future research and recommendations

It is recommended that additional research be undertaken in the field, and with sheep more inclined towards scouring, to investigate to what extent the antigen treatment can prevent or reduce diarrhoea. If successful in field conditions, work towards an intra-ruminal device based on this concept would be feasible.

In addition, further research is recommended on the role of eosinophils and intraepithelial lymphocytes in parasite immunology and tolerance. This study demonstrated the immune response associated with parasite infection in sheep can be influenced. By understanding the mechanisms by which the immune response can be modified, improved parasite control measures could be developed.

7. References

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