

Blood parameters and immune responses during haemonchosis

Dominika Mravčáková^{1*}, Daniel Petrič¹, Katarína Kucková¹, Marián Várady², Zora Váradyová¹

¹Institute of Animal Physiology, Centre of Biosciences of Slovak Academy of Sciences, Košice, Slovakia

²Institute of Parasitology, Slovak Academy of Sciences, Košice, Slovakia

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The aim of this experiment was to determine the effect of medicinal plants (*Artemisia absinthium* and *Malva sylvestris*) as feed supplements on blood parameters, and local immune responses in the abomasum of *Haemonchus contortus* infected lambs. Twenty-four lambs were infected orally with 5000 L3 larvae of *H. contortus* and subsequently randomly divided into four groups: unsupplemented animals (UNS), animals with *A. absinthium* (ART), animals with *M. sylvestris* (MAL), and animals with a mix of two plants (ARTMAL). During the experiment, samples of blood were taken for evaluation of hemoglobin and albumin levels. All animals were humanely killed after 75 days of the experiment, and abomasum tissue samples were taken for histopathology. The hemoglobin concentration in the blood was influenced by time ($p < 0.001$) and in all groups decreased until day 45. The serum albumin levels were influenced by treatment ($p < 0.05$) and time ($p < 0.001$) and in all groups occurred reduction until the end of the experiment compared to day 15. The number of plasma cells was higher in ARTMAL compared to MAL ($p < 0.05$). UNS and ART had significantly higher numbers of mast cells compared to MAL ($p < 0.01$ and 0.05 , respectively). The regeneration of abomasal tissue was most frequent in ARTMAL. The results showed that dietary supplementation by *A. absinthium* and *M. sylvestris* did not positively affect blood parameters but influenced local immune response in the abomasum of *H. contortus* infected sheep.

Keywords: *Haemonchus contortus*, sheep, hemoglobin, abomasum, local immune responses

1 Introduction

Haemonchosis, an infection caused by the gastrointestinal nematode *Haemonchus contortus*, is a prevalent parasitic disease associated with productivity loss, increased morbidity or mortality of small ruminants (Besier et al., 2016). Adult parasites live in the abomasum where they attach to the abomasal mucosa and feed on blood. The control of haemonchosis was mainly limited to repeated use of anthelmintic drugs. Nowadays, nutraceuticals in the form of medicinal plants are rising attention as natural alternatives for controlling this infection, which could eventually replace anthelmintic treatment (Hoste et al., 2016). Medicinal plants contain a wide range of bioactive compounds with different therapeutic effects (Villalba et al., 2017), which can affect parasites directly or indirectly by increasing the resistance of animals. Based on our previous experiments, we concluded that a dry mixture of medicinal plants used as dietary supplements could probably slow the dynamics of infection and increase the resistance of lambs to haemonchosis (Váradyová et al., 2018; Mravčáková et al., 2019). Our study aimed to evaluate the effect of two medicinal plants, *Artemisia absinthium* and *Malva sylvestris*, as dietary supplements for *H. contortus* infected lambs on blood parameters and immune responses in lambs abomasal mucosa.

*Corresponding Author: Dominika Mravčáková, Institute of Animal Physiology, Centre of Biosciences, Slovak Academy of Sciences, Šoltésovej 4-6, 040 01 Košice, Slovak Republic; e-mail: mravcakova@saske.sk

2 Material and methods

2.1 Animals and experimental design

All procedures and animal care followed European Community guidelines (EU Directive 2010/63/EU). The experimental protocol was approved by the Ethical Committee of the Institute of Parasitology of the Slovak Academy of Sciences, following national legislation in Slovakia. Twenty-four parasite-free lambs (Improved Valachian) were housed in common stalls on a commercial sheep farm. After the adaptation period, all lambs were infected orally with 5000 L3 larvae of *H. contortus* susceptible to anthelmintics (MHC01). During the experiment, each animal was fed oats (500 g dry matter (DM)/d), and meadow hay (*ad libitum*). Lambs were randomly divided into four experimental groups ($n = 6$): unsupplemented animals (UNS), animals supplemented with the stem of *Artemisia absinthium* (ART, 1 g DM/d/lamb), animals supplemented with the flower of *Malva sylvestris* (MAL, 15 g DM/d/lamb) and animals supplemented with a mix of *A. absinthium* and *M. sylvestris* (ARTMAL, 16 g DM/d/lamb). The dry plants were obtained from commercial sources (AGROKARPATY, Plavnica, Slovak Republic) and were mixed daily with the oats throughout the experiment. The experiment lasted for 75 days (D) during summer after which all animals were humanely killed, and samples of abomasum were taken from each animal for histopathological examination.

2.2 Blood analysis

Samples of blood were collected from the jugular vein of each animal on D15, D30, D45, and D70. The serum samples were obtained by centrifugation of blood samples at 1,200 g for 10 min at room temperature and stored at -70 °C until analysis. The hemoglobin (Hb) content of blood and albumin concentration in the serum samples was measured spectrophotometrically (Shimadzu UV-2550, Kyoto, Japan) using a commercial kit (RANDOX Laboratories, Ltd., UK) according to the manufacturer's instructions. All samples were analyzed in duplicate.

2.3 Immune cells in the abomasum

After the necropsy, fresh samples of abomasum were removed, washed with water to remove nematodes, and fixed in 10% neutral buffered formalin. Samples were processed in a series of reagents and embedded in paraffin (Paraplast, Sigma-Aldrich, Steinheim, Germany), which were then cut with a rotary microtome into 4 µm thick sections. Paraffin slides were then automatically stained with hematoxylin and eosin (Varistain Gemini Thermo Scientific, U.K.), for eosinophils, plasma cells and mitosis counts. Moreover, a toluidine blue stain was done for the evaluation of mast cell numbers. To determine the number of immune cells and mitotic figures in the abomasal mucosa, the cells were counted in 10 randomly selected fields for each animal at 400× magnification using a BX43 light microscope equipped with an SC30 digital camera (Olympus Optical, Japan). Pictures were analyzed and recorded by computer software (CellSens Entry 2011, Olympus Lifescience).

2.4 Calculations and statistical analysis

Analyses of variance (GraphPad Prism 8, GraphPad Software, Inc., San Diego, CA, USA) were used for analyzing hemoglobin and albumin levels as repeated-measures mixed models representing the four animal groups (UNS, ART, MAL, ARTMAL) and sampling days. The effects included in the model were treatment (T), time, and their interaction (T × time). Data of immune cells in abomasum were evaluated by multiple comparisons of one-way ANOVA. Student's *t*-tests were applied to assess the differences between the immune cells' means of different groups on D75. The significance level was set at $p < 0.05$.

3 Results and discussion

H. contortus causes anemia in infected animals due to its blood-feeding behavior, which can be reflected in reduced blood Hb. The Hb concentration in whole blood (Figure 1) was influenced by time ($p < 0.001$) and all treatments decreased until D45. In lambs treated with ARTMAL Hb content slightly increased and in lambs from the UNS, ART, and MAL remained almost stable from D45 until the end of the experiment. One or two medicinal plants did not have a beneficial effect on the Hb level of the infected lambs.

The serum albumin concentration in our experiment was influenced by treatment ($p < 0.05$) and time ($p < 0.001$), and in all experimental groups showed a reduction until the end of the experiment when compared to D15 (Figure 2). However, albumin concentration was stable from D45 to D75 in lambs treated with MAL, with higher values compared to the other groups. Since the albumin in our experiment reached only threshold values from 25 to 38 g/L, which are

similar to the values previously reported in infected rams (Rouatbi et al., 2016) we cannot state that treatment by medicinal plants affected albumin levels.

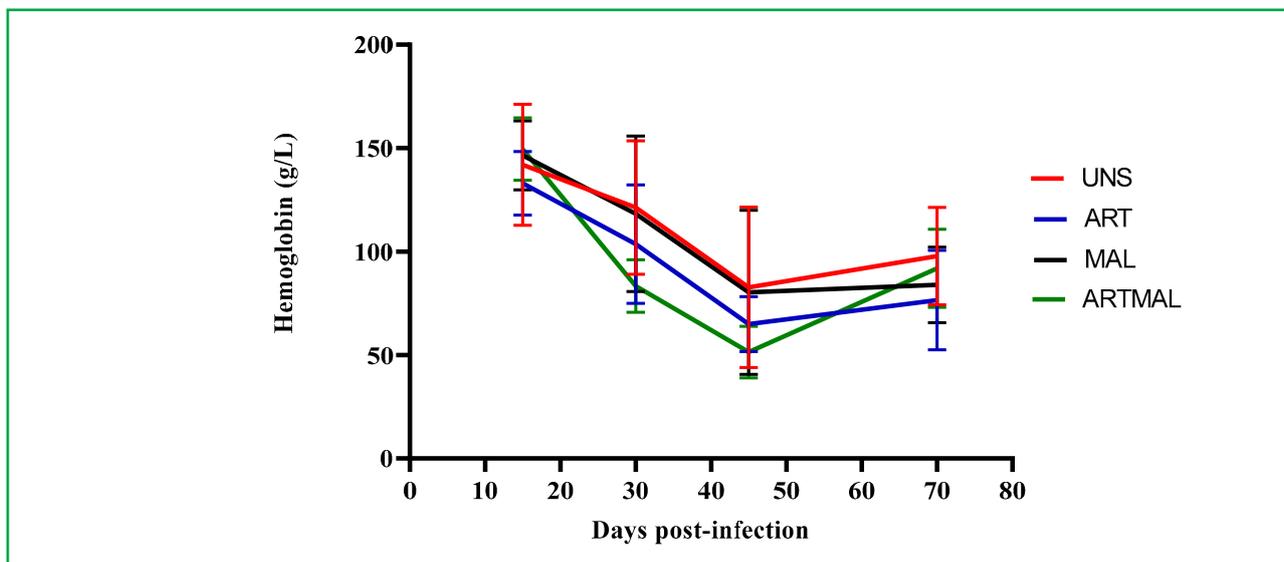


Figure 1 Hemoglobin concentration in the blood of infected lambs throughout the study (Treatment: $p > 0.05$; Time: $p < 0.001$; Treatment \times time: $p > 0.05$)

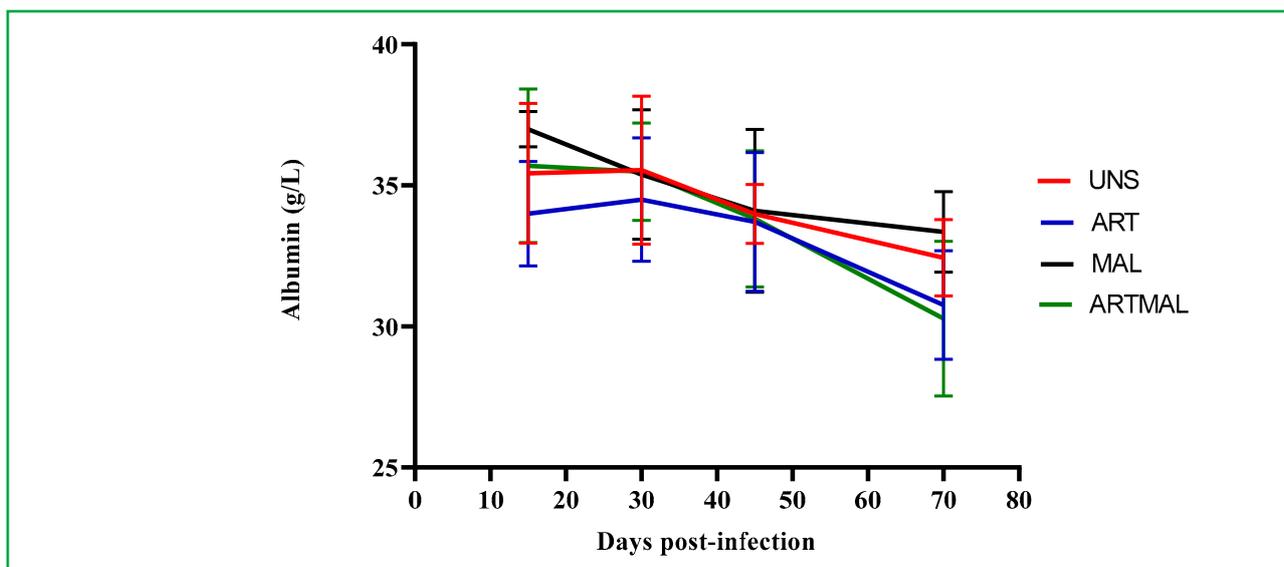


Figure 2 Albumin concentration in sera of infected lambs throughout the study (Treatment: $p < 0.05$; Time: $p < 0.001$; Treatment \times time: $p > 0.05$)

Natural immune responses against *H. contortus* in sheep are connected with the infiltration of immune cells into the infected tissue (McRae et al., 2015). Table 1 shows the mean numbers of eosinophils, plasma cells, mast cells, and mitotic figures in the abomasum of each experimental group on D75. Eosinophil numbers did not significantly differ between the groups, which could mean that two medicinal plants did not affect this blood parameter or the necropsy was inappropriately timed. The number of plasma cells in MAL was significantly lower compared to ARTMAL ($p < 0.05$), which could be affected by *M. sylvestris* bioactive compounds with anti-inflammatory properties. Mean numbers of mast cells were lowest again in MAL, and groups UNS and ART had significantly higher numbers of mast cells compared to this group ($p < 0.01$ and 0.05 , respectively). Abomasum tissue is usually damaged during haemonchosis due to the feeding activity of parasites. Within improved health conditions, regeneration of tissue typically occurs, which is presented by an increase of mitotic figures in tissue. Mitotic figures in our experiment were more frequent in

ARTMAL compared to MAL ($p < 0.05$), but also UNS and ART ($p < 0.001$). *A. absinthium*, in combination with *M. sylvestris*, could improve the regeneration of damaged tissue caused by parasitic infection.

Table 1 Immune cells and mitotic figures in the abomasum of infected lambs ($n = 6$)

Groups	Eosinophils	Plasma cells	Mast cells	Mitotic figures
UNS	2.70 ± 2.72	1.15 ± 0.58 ^{ab}	0.78 ± 0.21 ^b	1.02 ± 0.56 ^a
ART	3.14 ± 2.80	1.30 ± 0.61 ^{ab}	0.98 ± 0.44 ^b	1.19 ± 0.32 ^a
MAL	2.40 ± 1.34	1.00 ± 0.42 ^a	0.42 ± 0.11 ^a	2.00 ± 0.69 ^b
ARTMAL	2.39 ± 2.41	1.64 ± 0.41 ^b	0.84 ± 0.53 ^{ab}	2.80 ± 0.54 ^c

Values are mean numbers ± SEM; UNS – unsupplemented animals; ART – animal supplemented with *A. absinthium*; MAL – animals supplemented with *M. sylvestris*; ARTMAL – ART plus MAL; a–c different letters within a column indicate significant differences

4 Conclusions

Treatment with two medicinal plants (*A. absinthium* and *M. sylvestris*) did not show any positive effect on blood parameters, but on the other hand, affected local immune response in the abomasum of *H. contortus* infected lambs. Supplementation by medicinal plants, therefore, could improve sheep immunity against parasites, but further research is needed.

Acknowledgments

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