Experimental infection by *Haemonchus contortus* in lambs: influence of disease on purine levels in serum

LUCAS T. GRESSLER[1], ALEKSANDRO S. DA SILVA[2]*, CAMILA B. OLIVEIRA[1], ANDRESSA S. SCHAFER[3], ADELINA R. AIRES[3], JOSÉ F. X. ROCHA[3], ALEXANDRE A. TONIN[4], GABRIEL H. SCHIRMBECK[5], EMERSON A. CASALI[5], SONIA T. A. LOPES[4], MARTA L. R. LEAL[3] *and* SILVIA G. MONTEIRO[1]

[1] *Department of Microbiology and Parasitology, Universidade Federal de Santa Maria (UFSM), Brazil*
[2] *Department of Animal Science, Universidade do Estado de Santa Catarina, Brazil*
[3] *Department of Large Animal, UFSM, Brazil*
[4] *Department of Small Animal, UFSM, Brazil*
[5] *Department of Biochemistry and Department of Morphological Sciences, ICBS, Universidade Federal do Rio Grande do Sul, Brazil*

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SUMMARY

The aim of this study was to evaluate the purine levels of lambs experimentally infected with *Haemonchus contortus*. A total of 12 healthy lambs were divided into two groups, composed of 6 animals each: Group A represented the healthy animals (uninfected), while in Group B the animals were infected with 15 000 larvae of *H. contortus*. Blood was drawn on days 15, 45 and 75 post-infection (PI) in order to perform the purine analysis (ATP, ADP, AMP, adenosine, inosine, hypoxanthine, xanthine and uric acid) by high pressure liquid chromatography (HPLC) in serum. On day 15 PI a significant ($P<0{\cdot}05$) increase in the levels of ATP and inosine was observed in the infected animals, unlike the levels of ADP, adenosine, xanthine and uric acid which were reduced. On day 45 PI a significant ($P<0{\cdot}05$) increase in the ATP and xanthine levels in infected animals was observed, contrasting with reduced levels of ADP and uric acid. Finally, on day 75 PI an increase occurred in the levels of ATP, adenosine and hypoxanthine in infected lambs, concomitant with a reduction in the levels of ADP and uric acid ($P<0{\cdot}05$). These changes in purine levels may influence the inflammatory process and the pathological events.

Key words: Sheep, Trichostrongylidae, purinergic system, ATP, ADP, adenosine, uric acid.

INTRODUCTION

Haemonchus contortus is the main helminth of sheep in Brazil (Cavalcante *et al*. 2009), and is also considered endemic in Australia, South Africa and South America, as well as other countries (Waller *et al*. 1995). Animals parasitized may exhibit weight loss, dehydration, diarrhoea and anaemia during the acute phase. In hyperacute infections it is possible to observe pale mucous membranes and even the death of some animals. In the chronic phase the clinical signs usually intensify, often allowing the observation of ventral and submandibular swelling, weakness and apathy. Infected animals can present with mucosa of abomasums swollen, oedematous, anaemic and bright; as well as small ulcers at the site of attachment of *H. contortus* (Cavalcante *et al*. 2009).

Several factors are involved in the pathogenesis of infection by *H. contortus*. For the development of the disease, pathogenicity of the parasite and host response are among the most important factors. The main pathogenic mechanisms of *H. contortus* are the direct injury on the gastric mucosa and the haematophagia. The immune response is complex through cellular and humoral mechanisms (McClure *et al*. 1996; Meeusen, 1999), which may vary depending on the stage of the parasite (Balic *et al*. 2000).

Among the several inflammatory mechanisms, purinergic signalling is well documented (Atkinson *et al*. 2006; Bours *et al*. 2006; Yegutkin, 2008). It represents a common route of cell-to-cell communication involved in many physiological functions, such as immune response, inflammation, pain, platelet aggregation, vasodilation, proliferation and cellular death (Burnstock and Knight, 2004). This system consists of nucleotides (ATP, ADP and AMP) and nucleosides (adenosine and inosine), representing signalling molecules that are involved in activating the immune response (Atkinson *et al*. 2006; Yegutkin, 2008). The ATP has proinflammatory functions, since it controls the stimulation and proliferation of lymphocytes, cells involved in cytokine release (Bours *et al*. 2006). However, the endogenous nucleoside 'adenosine' is formed extracellularly (Ralevic and Burnstock, 1998) and presents itself as an anti-inflammatory molecule (Gessi *et al*. 2007).

* Corresponding author: Department of Animal Science, Universidade do Estado de Santa Catarina, Brazil. E-mail: aleksandro_ss@yahoo.com.br

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The activity of ecto-adenosine deaminase (E-ADA) has been recently investigated in lambs infected with *H. contortus*. This enzyme is responsible for the deamination of adenosine, and when measured in erythrocytes, it was found a co-relation between the enzyme activity and anaemia (Da Silva *et al.* 2013*b*). Therefore, this study aimed to assess the purines levels in serum of lambs experimentally infected with *H. contortus*.

MATERIALS AND METHODS

Animals

Our experiment used 12 male lambs crossbred Corriedale × Texel, 5 months old and weighing on average 23 kg each. They were kept in holding pens (one pen/group) during 30 days under a diet [base of 10·7% protein (commercial feed and ryegrass hay)] for adaptation to the experimental environment. In this period, the animals received anthelmintic treatment based on monepantel (Zolvix®). The same diet was provided during the first 20 days of the experiment (post-infection), but after this period it was necessary to change the diet as a consequence of the severe evolution of the disease. From this period onward, the animals were fed with a mixture of hay ground ryegrass (70%), commercial feed with 20% crude protein (CP), and soybean meal with 10% (CP) [totalling a diet with 13% CP]. Each animal consumed 1 kg of dry matter/day. Haematological (erythrogram and leukogram) and biochemical (hepatic and renal function) evaluations were performed three times at 15-day intervals. After 30 days (day 0 of the experiment), the evaluated patterns showed normal values, according to Feldman *et al.* (2000). The animals were apparently healthy, and they had negative fecal exam for eggs, cysts and oocysts of parasites.

Experimental design

The animals were divided into two groups with 6 animals each: Group A was composed of healthy animals (uninfected) and was used as a negative control group; Group B comprised the animals infected by *H. contortus*, representing the positive control. Each animal from group B was infected orally with a total of 15 000 larvae (L3), divided in three episodes of infection of 5000 larvae each time, at intervals of 3 days between them. The larvae were obtained by coproculture technique (Roberts and O'Sullivan, 1950).

Collection of samples

Blood was drawn through a Vacutainer® system on days 15, 45 and 75 PI. To measure the levels of purines, blood samples were stored in tubes without anticoagulant; and to hematocrit the samples was collected and stored in tubes with anticoagulant (EDTA). The determination of microhaematocrit was performed according to the technique described by Feldman *et al.* (2000). To obtain the serum, blood samples were centrifuged (5000 ***g*** for 5 min at 37 °C). The serum was stored at −20 °C until analysis.

Analysis of purine levels in serum by high pressure liquid chromatography (HPLC)

The denaturation of sample proteins was performed using 0·6 mol L^{-1} perchloric acid. All samples were then centrifuged (14 000 ***g*** for 10 min at 4 °C) and the supernatants were neutralized with 4·0 N KOH and clarified with a second centrifugation (14 000 ***g*** for 15 min at 4 °C). Aliquots of 20 μL were applied to a reversed-phase HPLC system (Shimadzu, Japan) using a C_{18} column (Ultra C18, 25 cm × 4·6 mm × 5 μM, Restek – USA). The elution was carried out applying a linear gradient from 100% of solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium phosphate, pH 6·0) to 100% of solvent B (solvent A plus 30% methanol) over a 30 min period (flow rate at 1·4 mL min^{-1}) according to a method previously described (Voelter *et al.* 1980) with minor modifications. The amounts of purines were measured by absorption at 260 nm. The retention time of standards was used as parameter for identification and quantification. Purine concentrations are expressed as nmol of the different compounds per mL of serum.

Stool testing

Fecal samples for quantification of eggs per gram (EPG) were collected on days 15, 45 and 75 PI, and processed according to the technique described by Gordon and Whitlock (1939). Five days after the end of the experiment (day 80 PI), 5 animals from each group were euthanized (10 mg intravenous (IV) of acepromazine; 2 g IV of sodium thiopental; 100 mL IV of potassium chloride), and their parasite loads were determined (Ueno and Gonçalves, 1998). The euthanasia was necessary to confirm that the animals were infected (Group B) or negative (Group A), since the EPG by itself may not be sufficient.

Statistical analysis

EPG data were initially tested for normality, however they did not present a normal distribution and they were converted to logarithm before statistical analysis. Then, the purines and hematocrit results were subjected to the Student's *t*-test. Values with probability (*P*) less than 5% were considered statistically different.

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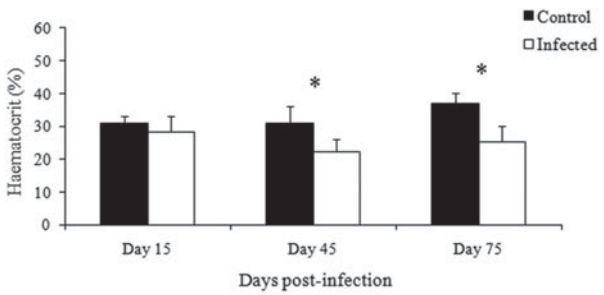

Fig. 1. Haematocrit of lambs experimentally infected with *Haemonchus contortus* assessed on days 15, 45 and 75 post-infection (*$P<0{\cdot}01$).

RESULTS

Infection course

The animals of Group A (negative control) showed negative EPG throughout the experiment; necropsy confirmed that the lambs were negative for helminths. The animals in group B (positive control) showed negative EPG on day 15 PI, but in the other two analyses EPG was positive for eggs of *H. contortus*, with a mean (S.D.) of 9828 EPG (±5426) at day 45 PI and 4100 EPG (±2277) at day 75 PI. Other helminths were not found during the necropsy of these animals.

The hematocrit was assessed to check the course of the disease in animals, and the results are shown in Fig. 1. On day 15 PI there were no significant ($P>0{\cdot}05$) differences between groups in hematocrit. However, a significant ($P<0{\cdot}01$) reduction in hematocrit were observed in the infected lambs on days 45 and 75 PI.

Purine levels in serum

Results of the purine levels are shown in Table 1. On day 15 PI a significant ($P<0{\cdot}05$) increase in the levels of ATP and inosine was observed, concomitant with reduced levels of ADP, adenosine, xanthine and uric acid levels in the infected animals when compared with the negative control group. However, on day 45 PI group B showed a significant ($P<0{\cdot}05$) increase in the levels of ATP and xanthine, together with reduced levels of ADP and uric acid. Finally, on day 75 PI there was an increase in the levels of ATP, adenosine and hypoxanthine, as well as reduction in levels of ADP and uric acid ($P<0{\cdot}05$) in experimentally infected lambs. AMP levels did not differ between groups throughout the experiment.

DISCUSSION

According to the results found in our study, at 15 days PI the animals tested negative for the presence of eggs in their feces. This can be attributed to the pre-patent period of *H. contortus*, which is on average 14–21 days (Cavalcante *et al*. 2009). The literature explains that the penetration of the fourth stage larvae (L4) in the abomasum of the host occurs during the first week of infection, followed by subsequent changes to the fifth stage larvae (adults) (Monteiro, 2010). During this period a response of the host against the infection can be observed, since the L4 perform haematophagy, allowing rapid growth and metabolic activity. Consequently, L4 excrete immunogenic products, as well as proteases and digestive enzymes, besides causing direct damage to the abomasum (Gamble and Mansfield, 1996), leading to an immune response. A Th2-type response to infection by helminths such as *H. contortus* is generally stimulated (Miller and Horohov, 2006).

In our study, during the three evaluated periods there was an increase in the serum levels of ATP. It is well known that ATP is an important molecule for the functioning of the cells, mainly related to energy storage for basic life activity of the cells. In the event of cellular injury or cell stimulation by pathogens, the concentration of extracellular ATP increases, initiating an inflammatory response characterized by the stimulation of leucocytes and secretion of various inflammatory mediators such as cytokines (Langston *et al*. 2003; La Sala *et al*. 2003). Therefore, the immune response against infection with *H. contortus* in sheep is mediated by ATP, a molecule that whose function is well documented (Langston *et al*. 2003; La Sala *et al*. 2003; Bours *et al*. 2006). The higher ATP levels on day 15 PI compared with the days 45 and 75 PI in infected animals can be related to an overreaction of the host against the infection, which occurs during the parasitism by larvae in the abomasal mucosa of the host, reflecting an acute inflammatory response (ATP molecule acute phase; Bours *et al*. 2006).

Similar to the ATP pattern, on day 15 PI there was an increase in the levels of inosine, unlike what happened with the levels of ADP and adenosine which were reduced in the serum of infected lambs. The findings may be related to reduction of NTPDase activity, which consequently causes a reduction in ATP hydrolysis to ADP, and an increase in the activity of the E-ADA, leading to an increase in the adenosine deamination to inosine (Yegutkin, 2008). However, in a study with a similar design, a reduction in E-ADA in serum of lambs infected with *H. contortus* was observed (Da Silva *et al*. 2013*a*). In another study, a negative correlation was verified between E-ADA activity in erythrocytes of lambs infected by *H. contortus*, and therefore, the researchers showed that E-ADA had participated in the pathogenesis of anaemia (Da Silva *et al*. 2013*b*). In our study, adenosine levels fluctuated during infection, reducing on day 15 PI, increasing on day 45 PI (but did not differ from the control group), and on day 75 PI there was a large increase of adenosine in serum. The high concentration of adenosine probably acted as an anti-inflammatory mechanism,

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Table 1. Mean and S.D. of purine levels in serum of lambs uninfected and infected with gastrointestinal nematode (*H. contortus*) on days 15, 45 and 75 post-infection

Purine	Day	Group A: control	Group B: infected	Probability (P)[#]
ATP (nmoles mL^{-1})	15	8·71 ± 1·23	18·5 ± 7·18	$P<0·05$[#]
	45	8·92 ± 2·12	12·77 ± 2·55	$P<0·05$[#]
	75	7·70 ± 2·35	13·65 ± 2·31	$P<0·01$[#]
ADP (nmoles mL^{-1})	15	19·10 ± 2·44	14·0 ± 3·75	$P<0·05$[#]
	45	20·60 ± 4·02	8·56 ± 1·85	$P<0·01$[#]
	75	18·06 ± 3·14	10·32 ± 1·81	$P<0·01$[#]
AMP (nmoles mL^{-1})	15	6·17 ± 0·97	7·46 ± 0·87	$P>0·05$
	45	7·52 ± 3·10	6·86 ± 0·41	$P>0·05$
	75	6·34 ± 0·92	5·90 ± 0·88	$P>0·05$
Adenosine (nmoles mL^{-1})	15	7·32 ± 4·13	3·91 ± 1·42	$P<0·05$[#]
	45	7·72 ± 3·94	9·26 ± 2·51	$P>0·05$
	75	7·02 ± 3·90	12·7 ± 2·30	$P<0·01$[#]
Inosine (nmoles mL^{-1})	15	2·50 ± 0·39	10·5 ± 1·60	$P<0·01$[#]
	45	2·52 ± 0·34	3·36 ± 0·90	$P>0·05$
	75	2·22 ± 0·71	2·21 ± 0·97	$P>0·05$
Hypoxanthine (nmoles mL^{-1})	15	5·33 ± 0·93	4·96 ± 1·04	$P>0·05$
	45	4·85 ± 1·22	5·36 ± 2·19	$P>0·05$
	75	4·82 ± 1·26	8·60 ± 1·71	$P<0·01$[#]
Xanthine (nmoles mL^{-1})	15	13·6 ± 0·40	8·50 ± 2·70	$P<0·05$[#]
	45	10·9 ± 3·71	16·3 ± 3·2	$P<0·05$[#]
	75	10·9 ± 3·75	11·7 ± 2·62	$P>0·05$
Uric acid (nmoles mL^{-1})	15	85·3 ± 15·9	50·9 ± 27·2	$P<0·05$[#]
	45	78·3 ± 20·8	47·3 ± 7·10	$P<0·01$[#]
	75	83·0 ± 14·6	44·6 ± 17·3	$P<0·01$[#]

[#]In the same line, indicates a significant difference between groups in the Student's *t*-test, when presenting $P<0·05$ or $P<0·01$.

providing an immunomodulatory effect against the chronicity of the disease (Sala-Newby *et al*. 1999; Sawynok and Liu, 2003). Deamination of adenosine to inosine is favourable for the maintenance and survival of invading organisms, since adenosine promotes chemotaxis, activation and degranulation of mast cells (Jin *et al*. 1997; Gounaris and Selkirk, 2005). Studies have shown that mast cells are mucosal effectors against various intestinal nematodes such as *Trichinella spiralis*, and thus assist in eliminating the parasite (Knight *et al*. 2000). It is important to emphasize that adenosine inhibits platelet aggregation and it has vasodilator activity, and these events are favourable to the survival of blood-sucking parasites. Researchers have already shown that adenosine is involved in a response to the stimulation of cellular and tissue damage, protecting against organ damage (Newby, 1984). A recent study reported that adenosine assists in the maintenance of tissue integrity by modulating immune system function (Haskó and Cronstein, 2004). Different types of cells are able to produce extracellular adenosine, some examples being endothelial cells and neutrophils which are constantly co-related to high levels of adenosine in areas of inflammation (Cronstein, 1994).

Haemonchus contortus is a blood-sucking parasite, with mechanisms to neutralize the haemostatic system of the host, such as anti-platelet and anticoagulant proteins (Seymour *et al*. 1990; Stanssens *et al*. 1996; Depraetere *et al*. 1999; Francischetti *et al*. 2000; Crab *et al*. 2002). In this study, we observed a reduction in the level of ADP in all analysed periods in the infected group; however this reduction was more pronounced on days 45 and 75 PI, coinciding with a large number of parasite eggs in the feces. The reduction in ATP hydrolysis (involved in the inflammatory response) can be the main cause of reduction in serum ADP in lambs infected with *H. contortus*. This reduction in ADP probably favours the parasite, because it hinders platelet aggregation and formation of a homeostatic plug, and thus facilitates the feeding of the parasite.

Hypoxanthine, xanthine and uric acid are products of catabolism of purines (Chen and Gomes, 1992). In ruminants most of the uric acid comes from the diet and in extension, from the breakdown of endogenous nucleic acid (González and Silva, 2006). Thus, the a lower intake and/or malabsorption of nutrients (proteins, vitamins and minerals), a situation previously reported due to infection by *H. contortus*, can result in lower blood levels of uric acid. Our results showed reduction of uric acid in all evaluated periods. Researchers have emphasized that infected animals require higher protein intake compared with healthy animals, mainly due to the loss of endogenous nitrogen into the intestine and the low degree of protein synthesis in muscle (Veloso *et al*. 2004). Thus, the ingested protein promotes the restoration of tissue loss, repairing and replacing damaged tissues.

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The chronicity of infection by *H. contortus* in the animals of group B led to an increase in the levels of xanthine and hypoxanthine. These results may be related to a possible reduction in the activity of enzymes such as xanthine oxidase and xanthine oxidoreductase, responsible for the catabolism of hypoxanthine to xanthine and xanthine to uric acid, the latter a potent antioxidant (Haskó *et al*. 2004). It is important to highlight that increased levels of hypoxanthine may be due to cell damage during ischaemia (Shahbazian *et al*. 2006). It is worth reporting that anaemia is the main pathological symptom observed in infections by *H. contortus*, and when the parasite is ingesting blood, it can cause ischaemia or bleeding after its detachment from the abomasal mucosa. Consequently, it may be related to the increase of hypoxanthine and xanthine observed in this study.

We conclude that infection by *H. contortus* causes changes in purine levels in lambs. Due to all the functions of the purines aforementioned, we suggest that these changes in concentrations of ATP, ADP, adenosine, inosine, hypoxanthine, xanthine and uric acid may influence the pathophysiology of disease and the host immune response against the parasite. It is believed that the increase in serum levels of ATP and adenosine may be related to the inflammatory response, influencing the release of pro-inflammatory and anti-inflammatory molecules, respectively. Changes in the levels of certain nucleotides such as ADP appear to be linked to homeostatic factors, resulting in massive blood loss, leading to anaemia, a major clinical sign of disease.

ETHICAL PROCEDURES

The procedure was approved by the Comissão de Ética no Uso de Animais (CEUA) from the Universidade Federal de Santa Maria (UFSM), under the number 012/2011.

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