

Research Paper

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Relationship between histopathological lesions and oxidative stress in mice infected with *Angiostrongylus costaricensis*

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Abstract

This study describes changes in oxidative stress (OS) parameters in mice experimentally infected with *Angiostrongylus costaricensis*, which causes abdominal angiostrongyliasis. For this, 28 Swiss mice were used, divided into two groups (G1 and G2), with 14 animals each. Of these, eight were infected with ten infective larvae each, by gavage, and six were used as a control group. Mice from G1 and G2 were euthanized at 14 days and 24 days post-infection, respectively. Tissue samples were used for histopathological analysis and blood (serum) samples were taken to assess the levels of proteins, non-protein thiols (NPTs) and nitric oxide (NO), from centrifugation and subsequent collection of aliquots of the supernatant. Among OS parameters, infected mice in both groups had higher NO levels than the control group, due to the presence of: eosinophil infiltrate in the liver and intestine; pancreatitis; and intestinal granuloma. However, the infected mice of both groups showed a reduction in the levels of NPTs, in relation to the control group, due to the presence of: eosinophilic infiltrate in the liver and intestine; and intestinal granuloma. Our results suggest that *A. costaricensis* infection has important effects on the intestine, liver and pancreas, and the analyses were performed from the tissue of these organs. The mechanisms for these changes are related to the decrease in the body's main antioxidant defences, as demonstrated by the reduction of NPTs, thus contributing to the development of more severe tissue damage. Thus, the objective of the present study was to evaluate the relationship between histopathological lesions and markers for OS.

Introduction

Angiostrongylus costaricensis is the causative agent of abdominal angiostrongyliasis (AA), a zoonosis that has been reported over an area extending from North America to subtropical regions of South America (Rodriguez *et al.*, 2008). Its life cycle involves terrestrial molluscs as intermediate hosts (Rodriguez *et al.*, 2019) and rodents as definitive hosts (Graeff-Teixeira *et al.*, 1990). Humans are considered to be accidental hosts (Mota & Lenzi, 2005).

The clinical signs of this disease are abdominal pain, anorexia, fever, malaise, vomiting, nausea, diarrhoea or constipation (Graeff-Teixeira *et al.*, 1987). In laboratory tests, leucocytosis, marked eosinophilia and anaemia are observed; and, due to hepatomegaly, there may be changes in liver enzymes (Rodriguez *et al.*, 2008). Therefore, as a result of the biological cycle, not only the intestine is injured, but also other organs end up affected by the action of the parasite (Mota & Lenzi, 2005).

In humans, the diagnosis of AA is based on histopathological findings, through identifying worms, eggs or larvae in arterial or vascular lumina (Rodriguez *et al.*, 2008). Presence of adult nematodes in the mesenteric arteries and the inflammatory reactions that occur as a result of larvae and eggs give rise to pathogenesis of AA. Haemorrhagic or ischaemic infarctions and granulomatous reactions that can progress to intestinal stenosis are detected. These lesions are seen mainly in the ileocaecal region. The macroscopic findings consist of intestinal wall thickening and occurrences of congestive necrotic lesions. Microscopically, there are three histopathological findings: granulomatous reactions; eosinophil vasculitis; and eosinophil infiltration into the intestinal wall layers (Graeff-Teixeira *et al.*, 1991).

An oxidative stress (OS) process can be established as a result of the occurrence of an imbalance between oxidant and oxidant compounds that leads to the excessive generation of free radicals or to a decrease in the speed of their removal (Amer *et al.*, 2015). Free radicals

are linked to a wide variety of diseases, including liver disease, cancer and aging (Lima & Abdalla, 2001). Excess free radicals in the body are countered by antioxidants produced by the body or absorbed from the diet. The human organism suffers constant action of reactive oxygen species (ROS) and reactive nitrogen species generated in inflammatory processes, due to some biological dysfunction or from food. When ROS are produced in excess or for long periods, they can exert toxic effects that damage cells and tissues, resulting in the dysfunction of physiological processes. This process leads to oxidation of biomolecules, with consequent loss of their biological functions and/or homeostatic imbalance. These consequences are manifested as a potential for oxidative damage to cells and tissues, causing OS (Halliwell & Whiteman, 2004). Thus, during its life cycle, *A. costaricensis* may be exposed to different ROS generated by the parasite's metabolism, to the host's immune response, as it occurs with infections by *Trypanosoma cruzi*, in Chagas disease (Docampo, 1990; Turrens, 2004).

There is increasing scientific evidence showing that OS has important implications for the mechanisms that culminate in the development of future diseases in humans (Furukawa *et al.*, 2004). OS plays an important role in intestinal, liver and pancreatic damage. It acts as the activating factor for organ barrier dysfunction, thus triggering immune imbalance and inflammation (Que *et al.*, 2010; Han *et al.*, 2016; Yang *et al.*, 2019). In this context, we tested the hypothesis that infection by *A. costaricensis* causes severe intestinal, liver and pancreatic damage, and interferes with the homeostasis of the organism. Thus, the objective of the present study was to evaluate the relationship between histopathological lesions and markers for OS.

Materials and methods

Animals

Twenty-eight male Swiss mice, from the Animal Laboratory of the Institute of Biological Sciences of the University of Passo Fundo, located in the city of Passo Fundo, in the state of Rio Grande do Sul, Brazil, were used in this study. These animals were kept in plastic cages (polypropylene) with stainless steel grids. These do not have 'living corners' inside them, as the animals tend to gnaw at any ledge to try to escape; they are rectangular and closed at the top to allow the accommodation of the pelleted feed (Nuvilab®) and the bottle containing water; they are safe, not allowing the animals to escape; easily allow observation and feeding; have proper ventilation; they are hygienic and easy to clean; comfortable, allowing animals ample freedom in their movements; and allow easy access to food and water. Animals are kept under ideal temperature conditions ($23 \pm 2^\circ\text{C}$), *ad libitum* and lighting conditions (12/12 hour light/dark cycle) (Andrade *et al.*, 2002).

The 'three Rs principle', that is, reduction, replacement and refinement, was followed in this experiment. This principle envisages that, whenever possible, data obtained through previous animal experimental studies should be used, and it encourages reduction of animal use when there is no real need for this (Benvegnú *et al.*, 2021).

Formation of experimental groups

These 28 animals were selected in two groups containing 14 animals each, for this training. The animals were selected based on

their characteristics, such as same weight, size and age. In each of the two groups (G1 and G2) eight animals were infected and six served as uninfected controls. The experimental infection was performed using the gavage method with a dose of 10 infective larvae (L3) per animal (0.1 to 0.3 ml). The G1 animals were euthanized at 14 days post-infection (dpi) and the G2 animals, at 24 dpi. Blood samples were collected at 07:30 am through intracardiac puncture, with previous anaesthesia of the animals with Isoflurane (Isoforine®). The blood was stored in tubes with ethylenediaminetetraacetic acid and the serum was separated by centrifugation at 2500 rotations per minute for five minutes and was frozen at -70°C until use (Benvegnú *et al.*, 2021).

Sample preparation

Serum samples were obtained on the same day that blood was collected and homogenized in phosphate-buffered saline (pH 7.5), using an Ultra-Turrax homogenizer, and centrifuged ($3000 \times g$ for 10 min) (Llesuy *et al.*, 1985). The supernatant was separated and aliquoted to assess the levels of proteins, non-protein thiols (NPTs) and nitric oxide (NO).

Histopathology

The preparations and histopathological analyses were performed according to the methodology previously described by our team (Sandri *et al.*, 2018). Macroscopic evaluation to identify anatomical alterations, such as the presence of ischaemic lesions and formation of pseudotumours, was performed in the animals during necropsies. Specimens (pancreas, liver and intestine) with visible changes were fixed in 10% formalin for 24 h and embedded in paraffin to obtain histological sections ($5 \mu\text{m}$ thick). The slides were prepared for histological analysis from three sections and stained with haematoxylin and eosin, for later analysis under an optical microscope. The following aspects were investigated: (a) presence of infarction; (b) identification of eggs, larvae and adult worms; (c) presence of eosinophilic infiltrate (focal, multifocal and diffuse); (d) granuloma formation (focal, multifocal and diffuse); and (e) characterization of vasculitis, thrombosis and pancreatitis (Hermes *et al.*, 2020; Benvegnú *et al.*, 2021) (figs 1–3).

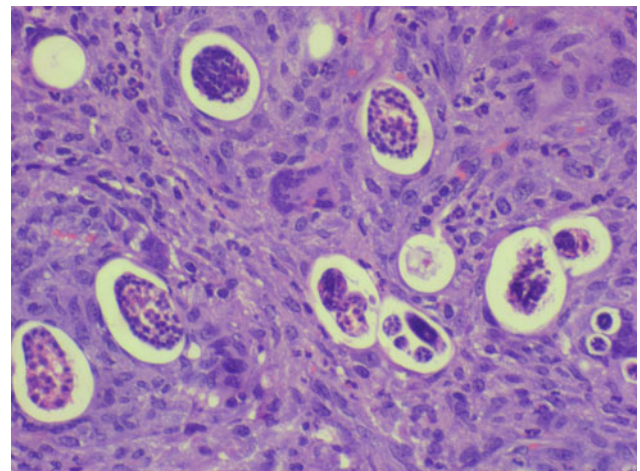


Fig. 1. Submucosa of small intestine with granuloma enveloping the eggs and larvae (haematoxylin and eosin-200 \times).

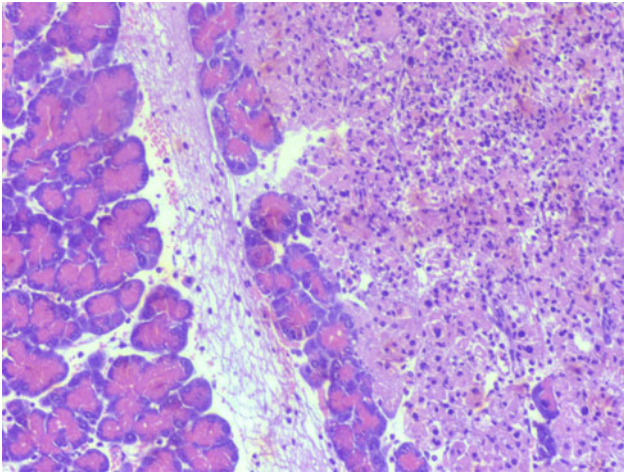


Fig. 2. Acute pancreatitis at 24 days post-infection – necrosis area with eosinophilic infiltrate (haematoxylin and eosin-100 \times).

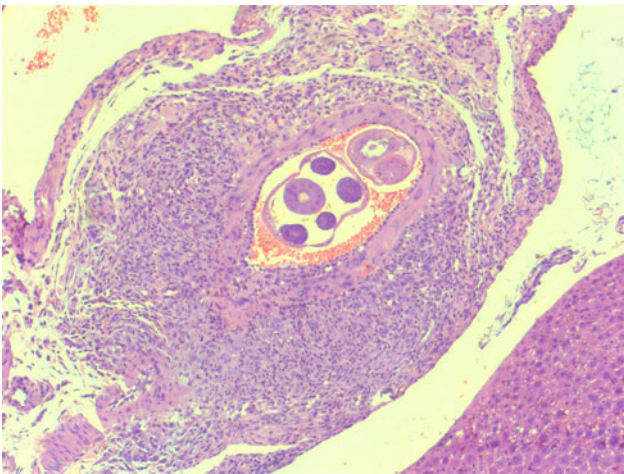


Fig. 3. Artery in the liver with thrombi and worm of *Angiostrongylus costaricensis* (haematoxylin and eosin-50 \times).

Oxidative damage parameters

NO

The NO concentration was indirectly evaluated using the Griess reagent (Bracht & Ishii-Iwamoto, 2003). NO end products (nitrite/nitrate) were colorimetrically quantified by means of spectrophotometry at 540 nm. The results were expressed as $\mu\text{mol/g}$ of protein.

NPTs

The levels of NPTs were measured through indirect intracellular measurement of glutathione levels (Ellman, 1959). The product from this reaction was quantified by means of spectrophotometry at 412 nm. The results were expressed as $\mu\text{mol/g}$ of protein.

Protein quantification

Protein concentrations were determined using Coomassie blue, in the Bradford method (Bradford, 1976) and bovine serum albumin was used as a standard. Absorbance was measured at 595 nm and the results were expressed in g/l.

Statistical analysis

For comparisons between the groups (G1: control vs. infected; G2: control versus infected), the normality and homoscedasticity of the variables of NO and NPTs levels were tested using the Shapiro–Wilk and Levene tests, respectively. The variables that met the above prerequisites, were compared using the *t*-test for independent samples, with the exception of the variable of NO in G2, which showed heteroscedasticity of variances, and thus, required Welch correction in the *t*-test for independent samples.

For comparison between histopathology lesions of G1 and G2, a cross table was used for analysing the observed frequencies, and differences in proportions were analysed by Yates' correction Chi-square.

To describe the effects of lesions on the levels of NO and NPTs, multiple linear regression models were used for the different periods of time analysed (G1 and G2). Given the large number of predictor variables in each model, they were selected through their *t*-values (values that presented $P < 0.05$), and in terms of whether the model with any given variable was significantly better than the model without it. To select the best models, they were subjected to simplification using the methodology of the Akaike information criterion, with correction for small sample size (corrected Akaike information criterion (AICc)) (Hurvich & Tsai, 1989; Burnham *et al.*, 2011; Symonds & Moussalli, 2011). The adjusted coefficient of determination (R^2) was calculated to determine the variability of the best model. Data were considered significantly different with a probability (*P*) of less than 5%. The R statistical package was used for the statistical analysis and the GraphPad Prism software, version 7.01, was used to generate graphs.

Results

Histopathology findings

Liver nodules were identified in both infected groups (28% at 14 dpi and 62.5% at 24 dpi) and the infected group at 24 dpi had splenic infarction (37.5%). Changes in the pancreas, such as pancreatitis, were observed in both infected groups (37.5% at 14 dpi and 75% at 24 dpi). At 24 dpi, 62.5% of the mice had eosinophilic infiltrate, in addition to vasculitis, granuloma necrosis and thrombi. The animals in G2 (24 dpi) presented, in their entirety, worms, eggs and larvae in different organs (table 1 and figs 1–3) indicating patent infection.

Changes to OS parameters in mice experimentally infected with *A. costaricensis*

Among the OS parameters, the infected animals of both groups presented NO release, in comparison with the control group, as shown in fig. 4.

In relation to NPTs, G1 showed a significant decrease, as can be seen in fig. 5.

Effects of lesions on levels of NO and NPTs

The multiple linear regression model that was proposed to describe the variability of NO in G1 (AICc = 178.1; difference in corrected Akaike information criterion values of the best model (dAICc) = 0.00; model weight = 0.96; df = 5) included the degree of eosinophil infiltration in the liver and intestine, and any

Table 1. Microscopic findings of mice infected with *Angiostrongylus costaricensis* at days 14 (G1) and 24 (G2) post infection.

Variables	G1		G2		P
	n	%	n	%	
granuloma (bowel)					
absent	8	100.0	0	0.0	<0.001*
mild	0	0.0	3	37.5	
moderate	0	0.0	3	37.5	
severe	0	0.0	2	25.0	
eosinophils (bowel)					
absent	1	12.5	0	0.0	0.022*
mild	0	0.0	0	0.0	
moderate	7	87.5	3	37.5	
severe	0	0.0	5	62.5	
vasculitis (bowel)					
no	5	62.5	0	0.0	0.027*
yes	3	37.5	8	100.0	
thrombus (bowel)					
no	4	50.0	5	62.5	1.00
yes	4	50.0	3	37.5	
necrosis (bowel)					
no	8	100.0	5	62.5	0.200
yes	0	0.0	3	37.5	
bronchopneumonia					
no	7	87.5	5	62.5	0.564
yes	1	12.5	3	37.5	
splenitis					
no	7	87.5	0	0.0	0.002*
yes	1	12.5	8	100.0	
eosinophils (spleen)					
absent	7	87.5	0	0.0	0.001*
mild	1	12.5	2	25.0	
moderate	0	0.0	6	75.0	
severe	0	0.0	0	0.0	
granuloma (spleen)					
no	8	100.0	4	50.0	0.083
yes	0	0.0	4	50.0	
infarct (spleen)					
no	8	100.0	5	62.5	0.200
yes	0	0.0	3	37.5	
granuloma (liver)					
absent	8	100.0	2	25.0	0.008*
mild	0	0.0	3	37.5	
moderate	0	0.0	3	37.5	
severe	0	0.0	0	0.0	

(Continued)

Table 1. (Continued.)

Variables	G1		G2		P
	n	%	n	%	
eosinophils (liver)					
absent	3	37.5	0	0.0	0.221
mild	1	12.5	1	12.5	
moderate	4	50.0	6	75.0	
severe	0	0.0	1	12.5	
hepatitis (liver)					
no	4	50.0	0	0.0	0.083
yes	4	50.0	8	100.0	
infarct (liver)					
no	4	50.0	1	12.5	0.281
yes	4	50.0	7	87.5	
thrombus (liver)					
no	7	87.5	6	75.0	1.000
yes	1	12.5	2	25.0	
pancreatitis					
no	5	62.5	2	25.0	0.313
yes	3	37.5	6	75.0	
vasculitis (pancreas)					
no	8	100.0	4	50.0	0.083
yes	0	0.0	4	50.0	
granuloma (pancreas)					
absent	8	100.0	2	25.0	0.008
mild	0	0.0	2	25.0	
moderate	0	0.0	4	50.0	
severe	0	0.0	0	0.0	
eosinophils (pancreas)					
absent	7	87.5	3	37.5	0.121
mild	0	0.0	0	0.0	
moderate	1	12.5	5	62.5	
severe	0	0.0	0	0.0	
thrombus (pancreas)					
no	8	100.0	7	87.5	1.000
yes	0	0.0	1	12.5	
necrosis (pancreas)					
no	8	100.0	6	75.0	0.450
yes	0	0.0	2	25.0	

*Probability less than 5% ($P < 0.05$) indicates a significant difference (Chi-square test with Yates correction).

presence of pancreatitis. The model was able to explain 97.72% (R^2) of this variability. Thus, the presence of a mild or moderate infiltrate of eosinophils in the liver promoted on average increases in serum NO of 1520.19 and 40.99 $\mu\text{mol/g}$, respectively. Moreover, the presence of moderate eosinophil infiltrate in the

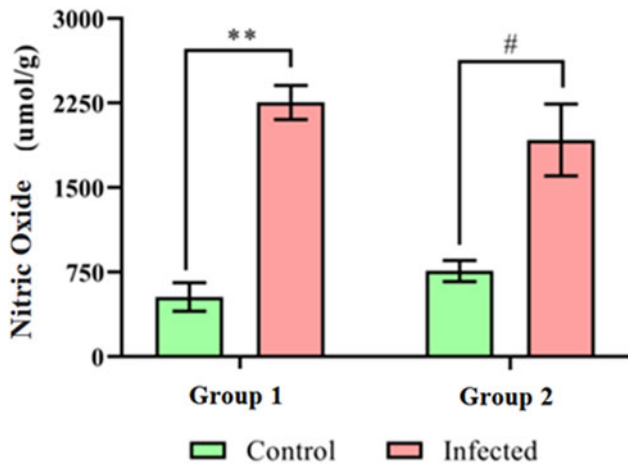


Fig. 4. Serum nitric oxide in mice experimentally infected with *Angiostrongylus costaricensis* (** $P < 0.001$, $n = 13$, t -test for independent samples; # $P < 0.001$, $n = 12$, t -test for independent samples with Welch correction).

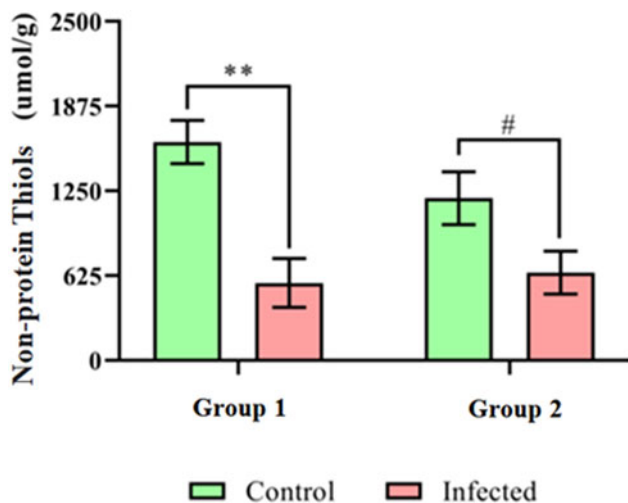


Fig. 5. Serum non-protein thiols in mice experimentally infected with *Angiostrongylus costaricensis* (** $P < 0.001$, $n = 13$, t -test for independent samples; # $P < 0.001$, $n = 12$, t -test for independent samples).

bowel and pancreatitis added, on average, 1666.46 and 132.29 $\mu\text{mol/g}$ of serum NO (table 2).

The multiple linear regression model proposed for the levels of NPTs in G1 (AICc = 182.3; dAICc = 0.00; model weight = 0.49; df = 5) included the degree of infiltration of eosinophils in the liver and intestine. This model was able to explain 92.16% (R^2) of the variability of this parameter. Thus, the presence of mild or moderate eosinophil infiltrate in the liver promoted reductions in serum NPTs of, on average, 747.94 and 8.83 $\mu\text{mol/g}$, respectively. Lastly, the presence of moderate eosinophil infiltrate in the intestine promoted a reduction in serum NPTs of, on average, 1081.92 $\mu\text{mol/g}$ (table 3).

For G2, it was included in the descriptive model for NO (AICc = 169.5; dAICc = 0.00; model weight = 0.33; df = 5). There was presence of intestinal granuloma that, as the degree of this lesion increased (mild to moderate to severe), promoted increases in serum NO levels of, on average, 1194.64, 1320.36 and 621.20 $\mu\text{mol/g}$, respectively (table 2). This model explained 94.60% (R^2) of the variability of this molecule. The model

proposed for the levels of NPTs (AICc = 173.1; dAICc = 0.00; model weight = 0.50; df = 5) also included the presence of intestinal granuloma, which promoted reductions in the serum levels of NPTs of, on average, 462.41, 529.70 and 779.86 $\mu\text{mol/g}$, as the degree of granuloma increased from mild to moderate, and from moderate to severe, respectively (table 3).

Discussion

Studies that help to better elucidate AA are important for understanding the parasite–host relationship and, especially, its pathophysiology. Hermes *et al.* (2018) found that higher infectious doses (with 30 L3) of *A. costaricensis* L3 increase the amount of faecal shedding of L1 in Swiss mice with no effect on disease development (Hermes *et al.*, 2020). Higher doses resulted in abundant presence of eggs and larvae, hepatic infarction, splenitis and intestinal thrombosis, but did not alter the survival of these rodents; this is due to the co-evolutionary relationship between the parasite and the host, in which the parasite benefits from the survival of the parasite. host. enough time to allow the parasite to reproduce and propagate (Gandon *et al.*, 2008).

The mechanisms of immune response in helminth infections are multiple, due to the metabolic diversity of the parasites, which are antigenically complex. An additional problem is that the parasites can survive for many years in the host, by being able to evade the host immune system (Neva & Brown, 1994). Another characteristic of parasitic infections, in situations in which the parasite has not yet adapted well to its definitive host, is that its persistence can induce an inadequate immune response, in addition to inducing morbidity and severe pathogenesis, as has already been observed in infections with *A. costaricensis*, in BALB/c mice (Geiger *et al.*, 2001).

At 14 dpi, we observed the presence of moderate eosinophil infiltration in the liver and intestine, along with pancreatitis, which contributed to the increase in NO levels. During this same period, there was the presence of a mild or moderate eosinophil infiltrate in the liver and intestine, and this may have led to the reduction in the serum levels of NPTs. In an experiment carried out by Benvegnú *et al.* (2021), 28% of the infected animals in their G1 (14 dpi) presented liver nodules and 37.5% of the infected animals showed pancreatitis.

However, at 24 dpi, the presence of an intestinal granuloma was observed and, as the size of the granuloma increased, it led to an increase in the serum levels of NO. During this same period, there was a reduction in the levels of NPTs as the severity of the granuloma increased. Benvegnú *et al.* (2021) observed the following among their animals at 24 dpi: splenic infarction (37.5%); liver nodules (62.5%); pancreatic changes (75%); and eosinophil infiltration (62.5%), besides granulomas, thrombi, vasculitis and necrosis.

In our study, the groups infected by *A. costaricensis* showed greater NO levels of systemic release. This may have been an attempt by the animals to wrestle the infection caused by the parasite, since NO is an important neurotransmitter and acts as a muscle relaxant, thereby increasing blood flow and showing a possible protective effect (Boeckxstaens *et al.*, 1991; Whittle *et al.*, 1992; Di Lorenzo *et al.*, 1995). Moreover, NO expression can be the result of an inflammatory response to the infection that is regulated by pro-inflammatory cytokines such as interferon gamma and tumour necrosis factor α (Body *et al.*, 1995; Creagh & O'Neill, 2006; Vieira, 2007; Benvegnú *et al.*, 2021).

Table 2. Multiple linear regression model (MLR) proposed to describe the variability of nitric oxide (NO) in mice infected by *Angiostrongylus costaricensis*, over different periods of infection (G1 and G2).

Independent variables	β	SE	<i>t</i>	<i>P</i>
MLR for NO in G1				
constant	529.05	55.78	9.485	<0.001
moderate eosinophil infiltrate in intestine	1666.46	193.23	8.624	<0.001
mild eosinophil infiltrate in liver	1520.19	147.58	10.301	<0.001
moderate eosinophil infiltrate in liver	40.99	167.34	0.245	0.813
pancreatitis	132.29	157.77	0.838	0.426
MLR for NO in G2				
constant	760.51	61.33	12.401	<0.001
mild granuloma in intestine	1194.64	122.65	9.470	<0.001
moderate granuloma in intestine	1320.36	106.22	12.431	<0.001
severe granuloma in intestine	621.20	162.25	3.829	<0.001

β , coefficient; SE, standard error.

Table 3. Multiple linear regression (MLR) model proposed to describe the variability of non-protein thiols (NPTs) in mice infected with *Angiostrongylus costaricensis*, at different periods of infection (G1 and G2).

Independent variables	β	SE	<i>t</i>	<i>p</i>
MLR for NPTs in G1				
constant	1609.74	64.428	24.985	< 0.001
moderate eosinophilic infiltrate in intestine	-1081.92	128.856	-8.396	<0.001
mild eosinophilic infiltrate in liver	-747.94	170.46	-4.388	<0.001
moderate eosinophilic infiltrate in liver	-8.83	136.672	-0.065	0.949
MLR for NPTs in G2				
constant	1195.95	71.33	16.767	<0.001
mild granuloma in the intestine	-462.41	142.65	-3.242	0.012
moderate grade granuloma in the intestine	-529.70	123.54	-4.288	<0.001
severe grade granuloma in intestine	-779.86	188.71	-4.133	<0.001

β , coefficient; SE, standard error.

In addition, we observed that oxidative damage occurred in the G1 animals, as shown by the decrease in the levels of NPTs. Glutathione (GSH) is an antioxidant that has been recognized as the most important NPT in living systems. It exhibits a state of balance with oxidized glutathione (GSSG). The ratio between GSSG and GSH is a reliable indicator of OS, as it reflects the balance between an antioxidant state and the pro-oxidant reactions in cells (Viña, 1990; Jones, 2006). Changes to GSH metabolism have been linked to the pathogenicity of several diseases, such as acute pancreatitis, in which reduction of GSH in pancreatic tissue is a hallmark during the early stage of this disease (Neuschwander-Tetri *et al.*, 1992; Schoenberg *et al.*, 1995). In this way, a change from cellular GSH to GSSG can lead to changes in the redox balance that favour continual presence of ROS, as occurred in our study.

The results obtained in the present study complement what was done by our team, by Hermes *et al.* (2020) and Benvegnú *et al.* (2021). This indicated that the degree of inflammatory lesions is a result of parasite migration during the development of the biological cycle and is not related to the dose of infection.

OS plays an important role in intestinal, liver and pancreatic damage, acting as an activating factor for organic barrier dysfunction, thus triggering immune and inflammatory imbalance (Que *et al.*, 2010; Han *et al.*, 2016; Yang *et al.*, 2019). The lesions caused by AA damage one of the main antioxidant defences, in addition to interfering with the body's homeostasis; therefore, infected animals become susceptible to greater tissue damage.

Conclusion

From the histopathological lesions found in the intestine, liver and pancreas, in mice experimentally infected with *A. costaricensis*, we concluded that they contributed to the increase in NO levels and decrease in NPTs, thus favouring the emergence of OS, causing damage to one of the body's main antioxidant defences.

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Conflicts of interest. None.

Ethical standards. This experiment was approved by the Ethics Committee on Animal Use of the University of Passo Fundo (CEUA-UPF - protocol no. 034/2016).

Authors' contributions. All authors contributed to the conception and design of the study. Natalie Renata Zorzi and Maria Isabel Botelho Vieira conceived the ideas. Natalie Renata Zorzi, Elise Benvegnú, Caroline Hermes, Rubens Rodriguez, Natália Freddo, Bruno Webber, Francieli Ubirajara do Amaral, Cláudia Almeida Scariot, Márcio Costa, Luciana Grazziotin Rossato-Grando and Maria Isabel Botelho Vieira designed the methodology and collected the data. Márcio Costa, Luciana Grazziotin Rossato-Grando, Rubens Rodriguez and Maria Isabel Botelho Vieira analysed the data. Natalie Renata Zorzi wrote the manuscript. All authors read and approved the final manuscript.

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