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ORIGINAL PAPER

Outcomes in mice with abdominal angiostrongyliasis treated with enoxaparin

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Abstract Abdominal angiostrongyliasis (AA) is caused by the nematode *Angiostrongylus costaricensis*. Parasite-associated thrombosis of mesenteric vessels may lead to intestinal infarction, which might be prevented with anti-thrombotic agents. This study assessed the effect of enoxaparin on survival and pathological findings in Swiss mice with AA. In this experiment, 24 mice were infected with *A. costaricensis* (10 L3 per animal) followed by treatment with subcutaneous enoxaparin (40 mg/kg/day) or water (sham), starting from 15 days post-infection (dpi) and continued until animal death. Animals were monitored until death or sacrifice at the 50th dpi. Ten mice (42%) were dead after 36±8 dpi. Of these, five (50%) were treated with enoxaparin. Animals treated with enoxaparin and sham did not differ in terms of weight loss (median, 1.3 vs. 4.2 g; P=0.303) and macroscopical findings. Microscopically, no difference was found in regard to vascular granuloma (median grade, 2 vs. 3; P=0.293) and presence of either vasculitis (75% vs. 100%; P=0.217), mesenteric thrombosis (33% vs. 50%; P=0.680), or bowel necrosis (25% vs. 50%; P=0.400). Mice dead before the 50th dpi showed more pneumonia (90% vs. 21%; P=0.002), bowel infarction (40% vs. 0%; P=0.02), and purulent peritonitis (60% vs. 7%; P=0.008) compared to survivors. Prophylactic enoxaparin in mice did not prevent tissue damage and mortality related with AA. The lower prevalence of mesenteric thrombosis and bowel infarction regardless of treatment were notorious. Frequent septic complications suggest the need of studies addressing the effect of antibiotics in AA.

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Introduction

Abdominal angiostrongyliasis (AA) is caused by the nematode *Angiostrongylus costaricensis*, first described by Morera and Céspedes in 1970 (Morera and Cespedes 1970). The parasite lives inside the mesenteric arteries of rodents, having slugs as intermediate hosts (Morera 1970; Morera and Ash 1970). *A. costaricensis* may be found in the stool of rodents as first stage larvae 1 (L1) and in the mucus of slugs as third stage larvae (L3). This latter stage may infect humans after ingestion of contaminated foods, particularly vegetables (Morera and Céspedes 1971).

Infected humans may develop a clinical entity named abdominal angiostrongyliasis (Morera 1973), manifested mainly by an infarct or pseudotumor-like lesions in the ileum–cecum segment of the bowel (Graeff-Teixeira et al. 1991). AA has been detected in many countries of Central

and South America, like southern Brazil (Agostini et al. 1984; Graeff-Teixeira et al. 2005), Costa Rica (Morera 1985), and Ecuador (Morera et al. 1983). Probably imported cases were also detected in North America (Ubelaker and Hall 1979) and Europe (Vazquez et al. 1993). In Brazilian series, asymptomatic patients with positive serology for *A. costaricensis* are believed to be more common than patients with symptomatic disease (Graeff-Teixeira et al. 2005).

The pathogenesis of AA is not well understood. A major hypothesis includes mesenteric vessel damage characterized by eosinophilic infiltration and perivascular granulomas. Such lesions may be associated with thrombotic events, combined with the presence of parasitic eggs or larvae in the vascular lumen (Agostini et al. 1984; Graeff-Teixeira et al. 1991). In addition, recent evidence in mice indicates an inverse correlation between fecal elimination of *A. costaricensis* L1 and survival, suggesting the existence of immunotolerance in animals who did not develop the disease (de Azevedo et al. 2010). Nevertheless, the mechanism of mesenteric thrombosis and its contribution to tissue damage and survival are poorly defined in research models for AA.

Patients with AA have been managed with support therapy in uncomplicated presentations and with surgical treatment in cases of bowel obstruction or infarction (Rodriguez et al. 2008). Several studies have tested pharmacological approaches, including antihelmintics (Mentz and Graeff-Teixeira 2003; Morera and Bontempo 1985), oviposition-blocking agents (Bohrer et al. 2007), and anti-inflammatory drugs (Fante et al. 2008), although with disappointing results. Theoretically, the use of anti-thrombotic agents such as enoxaparin might be beneficial in preventing mesenteric thrombosis as observed in patients with AA. Therefore, the aim of this study was to assess the effect of enoxaparin on survival and pathological features in a validated animal model for AA (de Azevedo et al. 2010). For this purpose, a controlled trial with enoxaparin was performed in mice infected with *A. costaricensis*.

Methods

Animals

We studied 24 male Swiss mice, 10 weeks old, weighing 25 to 38 g, obtained from the Universidade of Passo Fundo Animal House breeding stock (UPF, Passo Fundo, Brazil). The animals were housed under standard caging conditions, with ad libitum consumption of water and pellet chow (Nuvilab®). The experiment was conducted in accordance with Brazilian law 11.794/08 and ethical principles from the Brazilian Society of Laboratory Animal Science. The study was approved by the Ethical Committee–UPF (certificate no 161/2008).

Acquisition of *A. costaricensis* larvae

Larvae L3 were obtained from *Biomphalaria glabrata* snails infected with *A. costaricensis* (Santa Rosa strain, PUCRS). After 20 days, the snails were killed, and tissues were digested using a 0.03% solution of pepsin and 0.7% hydrochloric acid for 3 h at 37°C.

Drugs

Enoxaparin for subcutaneous injection was acquired in bottles of 20 mg (Sanofi–Aventis, São Paulo, Brazil). The drug was diluted in sterile water and prepared in insulin like syringes, in doses ranging 0.16 to 0.25 ml, according to animal weight. Sterile water in identical preparation was used in the sham group. Inhalatory isoflurane (Isoforine®, Cristália, São Paulo, Brazil) was employed to sacrifice the animals.

Study protocol

Experiments with animals were performed by three authors (RR, SMP, and RSF), always in the morning. In the first day, each mouse was infected with ten L3 by oral gavage. Animals were kept in groups of two to five in appropriate cages, with free access to water and food. They were monitored daily to detect signs of pain, including piloerection, tremors, and prostration. Animals were weighed weekly during the entire study. Feces eliminated during animal manipulation were examined to detect gross signs of blood. At the 15-day post-infection (dpi), animals were randomized to receive enoxaparin 40 mg/kg or water subcutaneously. They were treated daily until death or 50th dpi, when survivors were sacrificed with isoflurane. All animals underwent autopsy for characterization of macroscopic findings, followed by fixation of thoracic and abdominal organs in 10% formalin.

Pathological evaluation

Macroscopic findings were described as follows: (1) bowel lesions, including infarction, serosal granulations, peritoneal abscesses, and other signs of peritonitis; (2) spleen infarction; (3) capsular liver abscesses; and (4) congestion and petechia in the lungs.

Microscopic findings were determined after analysis of slices from paraffin-blocked tissue. For this purpose, the entire bowel and mesentery were rolled and fixed in such way to allow a complete view of the intestinal tract in a couple of paraffin blocks. The slices were stained with hematoxylin and eosin and examined by optical microscopy (Axioplan 2 Zeiss, Germany) by two independent pathologists (RR and AMM). A consensus was reached in case of discordant analysis.

Statistical analysis

Data are presented as means (±SD or range) or when otherwise stated. Student's *t* or Wilcoxon–Mann–Whitney tests were used to analyze quantitative data according to variable distribution. Differences in proportions were tested with chi-square test or Fisher exact test. The analyses were carried out using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA, USA). A P value<0.05 was assumed as indicative of statistical significance.

Results

Animals

Ten mice (42%) died from AA after 36±8 dpi on average. Of these, five (50%) were treated with enoxaparin, and five (50%) participated in the sham group. Animals treated with enoxaparin and sham did not differ in terms of weight loss (median, 1.3 vs. 4.2 g; P=0.303). Blood in feces was equally found in both comparisons: between enoxaparin and sham groups (25% vs. 50%; P=0.400) and between survivors and non-survivors (43% vs. 30%; P=0.678). Pain was similarly observed between enoxaparin and sham groups (58% vs. 58%; P=0.104). It was more frequent in non-survivors compared to survivors, however, with borderline significance (80% vs. 43%; P=0.068).

Macroscopic findings

These include lesions in the bowel, spleen, liver, and lungs. Bowel lesions were: serosal granulations in nine mice, adherences of intestines with the anterior abdominal wall in eight (Fig. 1a), a fibropurulent deposit in the peritoneal surface in seven, pseudotumor-like ileocecal abscesses in six mice (Fig. 1b), and others, including intestinal infarction in four animals (Fig. 1c). Spleen infarction was identified in only one animal. A fibropurulent deposit in the hepatic capsule was found in three mice, while signs of pneumonia were present in six animals. The comparison of mice treated with enoxaparin and sham revealed no statistical difference in regard to gross findings. However, the comparison of survivors (n=14) and non-survivors (n=10) revealed that

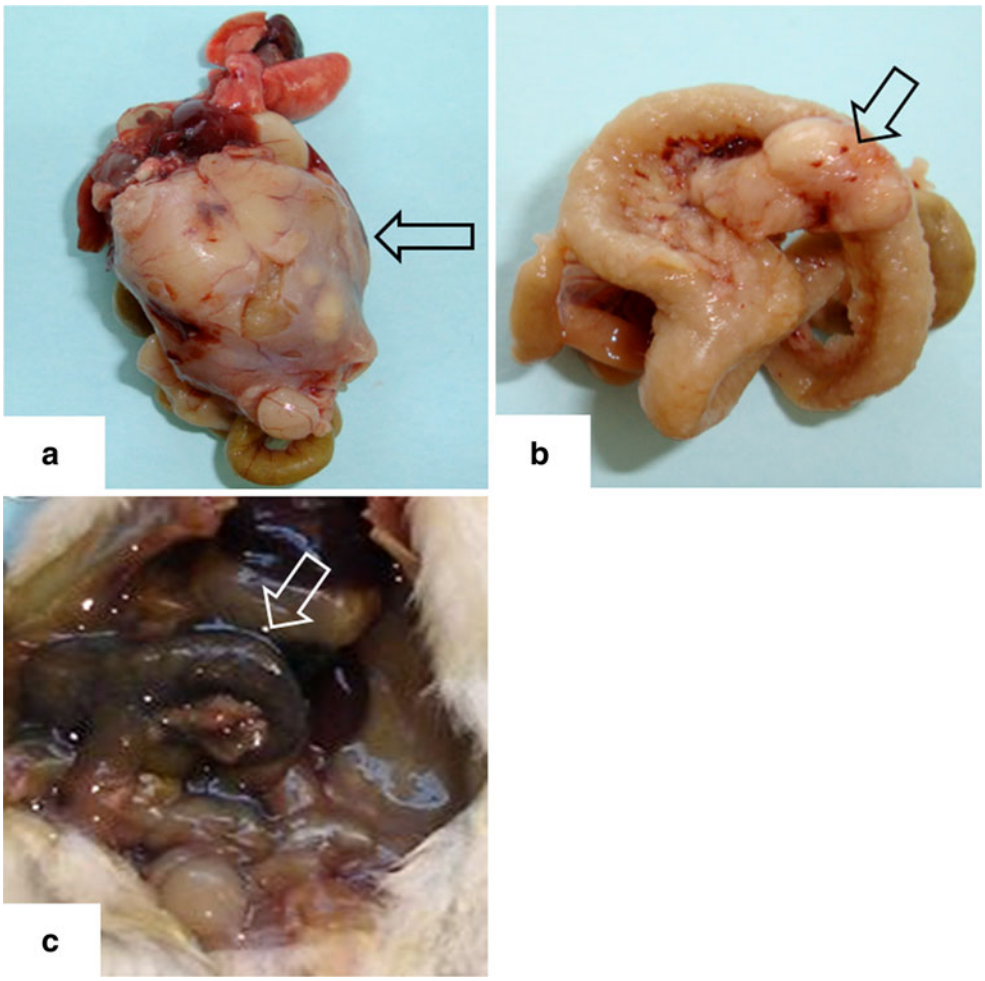

Fig. 1 Macroscopic findings. **a** Adherences of intestines with the anterior abdominal wall (*arrow*), **b** pseudotumor-like ileocecal abscesses (*arrow*), and **c** bowel infarction (*arrow*)

the first showed significantly more pseudotumor-like ileocecal abscesses (43% vs. 0%; P=0.023), more serosal granulations (57% vs. 10%; P=0.033), and less fibropurulent deposits (7% vs. 60%; P=0.008) in the peritoneal surface, as well as less bowel infarction (0% vs. 40%; P=0.019). Survivors also tended to have less pneumonia than non-survivors (7% vs. 50%; P=0.050).

Microscopic findings

In the comparison of groups treated with enoxaparin and sham, no difference was found in regard to pathological findings in the intestines, as follows: vascular granuloma [median grade (IQR 25–75%), 2 (1–3) vs. 3 (2–3); P=0.267] (Fig. 2a), eosinophilic vasculitis (75% vs. 100%; P=0.217) (Fig. 2b), thrombosis (42% vs. 58%; P=0.414) (Fig. 2c), bowel necrosis (42% vs. 58%; P=0.414), and eosinophilic infiltrate [median grade (IQR 25–75%), 2 (1.5–2) vs. 2 (2–2); P=0.342]. A characteristic pattern of granulomatous reaction was observed in the bowel of most animals (92%), in which ova or larvae of *A. costaricensis* are retained into small vessels, particularly in submucosa and muscular layers (Fig. 2d).

Lung damage was similar between mice treated with enoxaparin and sham, including pneumonia and granulomatous reaction. Ova and/or larvae of *A. costaricensis* were found in 42% of animals in both groups, as illustrated in Fig. 2a. Splenitis was found in most mice treated either with enoxaparin (75%) or sham (83%). Ova and/or larvae of *A. costaricensis* in the spleen were found in two animals treated with enoxaparin. Hepatic features in the enoxaparin and sham groups were as follows: granulomatous reaction (58% vs. 75%; P=0.666), eosinophilic infiltrate (92% vs. 92%; P=0.999), and reactive hepatitis (83% vs. 100%; P=0.478). Ova and/or larvae of *A. costaricensis* in the liver were found in nine animals (75%) in each group. Infrequent findings included abscesses (7% in each group) and liver infarct (25% vs. 17%; P=0.999).

The comparison of non-survivors and survivors revealed that the first showed more pneumonia (90% vs. 21%; P= 0.002), more bowel infarction (40% vs. 0%; P=0.02), and more purulent peritonitis (60% vs. 7%; P=0.008) than survivors.

Discussion

AA has no specific treatment. Since mesenteric thrombosis associated with *A. costaricensis* likely participates on disease pathogenesis, the use of anti-thrombotic agents became a rational approach. In the light of this, the aim of the present study was to assess the effect of enoxaparin on survival and pathological features in mice with AA.

The main findings of our study were: (1) survival rates did not differ between mice treated with prophylactic doses of subcutaneous enoxaparin and sham; (2) macroscopic and microscopic features were both similar in the comparison of these treatment groups; and (3) in a separate analysis of

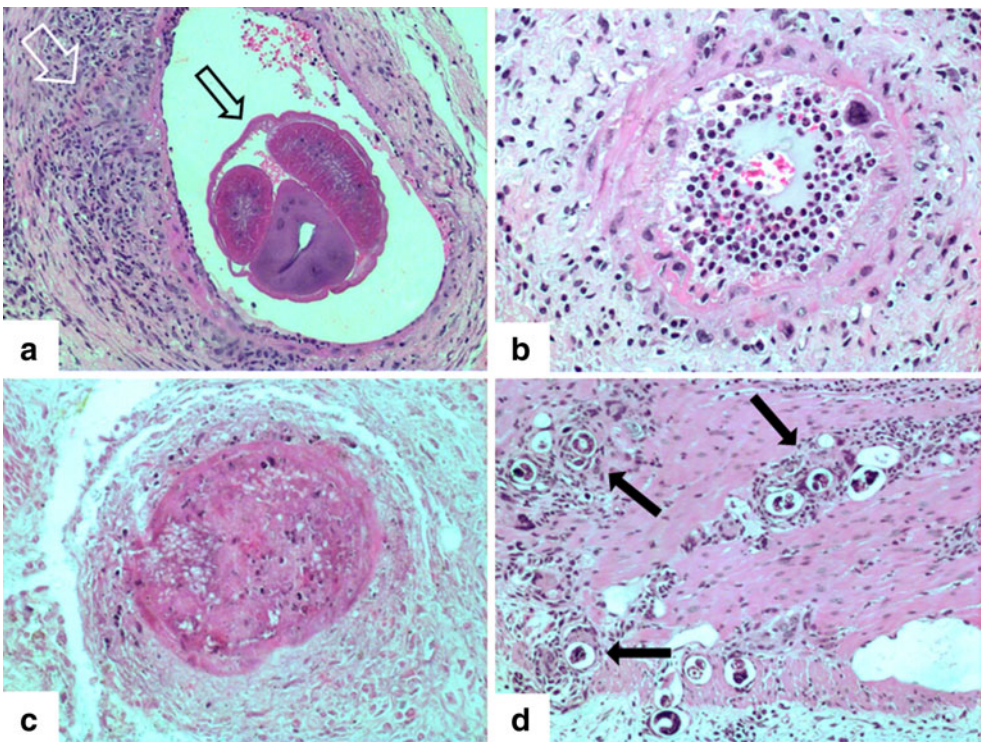

Fig. 2 Microscopic findings. **a** A granulomatous reaction surrounding a large-size mesenteric artery (*major arrow*), which contains an adult worm of *A. costaricensis* (*minor arrow*). **b** Vasculitis showing eosinophils infiltrating both muscle layer and vascular endothelium. **c** Thrombosis in a mesenteric artery of medium-size. **d** Granulomatous reaction engulfing embryonated eggs of *A. costaricensis* (*arrows*), following small vessels in the muscular layer

survivors and non-survivors, the latter group showed more bowel infarction and septic complications, including pneumonia and purulent peritonitis.

To the best of our knowledge, this is the first study addressing the effect of an anti-thrombotic agent in AA. The rationale for its use is the involvement of thromboembolic events in the pathogenesis of AA, which may result in intestinal infarction, a major cause of AA-related morbidity (Graeff-Teixeira et al. 1991; Waisberg et al. 1999). However, the use of enoxaparin in prophylactic doses in the present study did not show a benefit, neither in mortality nor in tissue damage. The lack of benefit might be related to insufficient doses of enoxaparin or a decreased sample size (type II error).

Macroscopic features were similar in mice treated with enoxaparin and sham. Abdominal lesions included serosal granulations, adherences of intestines with the anterior abdominal wall, a peritoneal fibropurulent deposit, and pseudotumor-like ileocecal abscesses. It was noteworthy that intestinal infarction was found in a minority of animals. Based on literature and clinical experience, gross findings in humans with AA are usually limited to pseudotumor and/or ischemic–congestive lesions (Graeff-Teixeira et al. 1991; Rodriguez et al. 2008). Peculiar findings of AA in mice, such as severe peritoneal involvement resulting in adherences and fibropurulent deposit, deserve further studies.

Microscopic findings also did not differ between treatment groups. Animals treated with enoxaparin and sham showed a similar scenario on microscopy, including vascular granuloma, eosinophilic vasculitis, thrombosis, bowel necrosis, and eosinophilic infiltrate. Interestingly, most animals presented vascular lesions characterized by eosinophilic vasculitis and granuloma, particularly in places of AA ova and larvae retention. Such features were found mainly in the submucosa and muscular layer of the bowel wall, following the pathway of arterioles and capillaries (Rodriguez 1997). We are tempted to speculate that vasculitis and granuloma formation may culminate in vascular occlusion and tissue ischemia rather than thrombotic events. Further studies are needed to address the importance of these inflammatory reactions in the pathogenesis of AA.

We performed a separate analysis of mice categorized according to survival, regardless of treatment. Thus, non-survivors showed more bowel infarction and septic complications compared to those considered as survivors, i.e., mice suffering from AA who were sacrificed at the end of the study. A possible explanation for these outcomes may be related with findings of a recent study showing a correlation between survival and L1 elimination in feces from mice infected with *A. costaricensis* (de Azevedo et al. 2010). Such correlation suggests a phenomenon of tolerance between host and parasite, indicating that the less vascular inflammation, the higher the amount of L1 elimination and the longer the survival.

In conclusion, we assessed the effect of enoxaparin on survival and pathological features in mice with AA. We found that neither survival nor pathological features differed between mice treated with prophylactic doses of enoxaparin and sham. Caution should be assumed in the interpretation of these data, considering the study in an experimental scenario and with prophylactic doses of enoxaparin. An interesting finding indicating septic complications as an important cause of death in our animal model points to the need of studies addressing a new approach with early diagnosis and treatment of sepsis as a complication of AA.

Conflict of interest The authors declare no conflict of interest.

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