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Parasitaemia and parasitic load are limited targets of the aetiological treatment to control the progression of cardiac fibrosis and chronic cardiomyopathy in *Trypanosoma cruzi*-infected dogs

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ABSTRACT

It is still unclear whether the progression of acute to chronic Chagas cardiomyopathy is predominantly associated with the limited efficacy of aetiological chemotherapy, or with the pharmacological resistance profiles and pathogenicity of specific *Trypanosoma cruzi* strains. Thus, we tested the hypothesis that parasitic load could be a limited target of aetiological chemotherapy to prevent chronic cardiomyopathy in dogs infected by different *T. cruzi* strains. Animals were infected with benznidazole-susceptible (Berenice-78) and -resistant (VL-10 and AAS) strains of *T. cruzi*. A quantitative real-time PCR strategy was developed to comparatively quantify the parasite load of the three different strains using a single standard curve. For dogs infected with the VL-10 strain, benznidazole treatment reduced cardiac parasitism during the acute phase of infection. However, similar parasite load and collagen deposition were detected in the myocardium of treated and untreated animals in the chronic phase of the infection. In animals infected with the AAS strain, benznidazole reduced parasite load, myocarditis and type III collagen deposition in the acute phase. However, increased type III collagen deposition was verified in the chronic phase. Dogs infected with the Berenice-78 strain showed a parasitological cure and no evidence of myocardial fibrosis. Parasitic load and cardiac fibrosis presented no correlation in acute or chronic phases of *T. cruzi* infection. Our findings in a canine model of Chagas disease suggest that parasite burden is a limited predictor for disease progression after treatment and show that benznidazole, although not inducing parasitological cure, is able to prevent total fibrosis in the early stages of infection, as well as complete prevention of cardiac damage when it eliminates parasites at the onset of infection.

1. Introduction

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi* (Prata, 2001). This disease exhibits multiple clinical chronic manifestations, ranging from asymptomatic to symptomatic forms with cardiac, digestive, neural or mixed events. Despite progressive damage to organs such as the heart, oesophagus and colon, *T. cruzi* parasites are scarce and difficult to detect in chronic infections (Gironès and Fresno, 2003; Teixeira et al., 2006). Several studies have attributed the poor detection of parasites to technical limitations, suggesting active participation of the parasites in the pathogenesis of chronic heart disease (Barbosa, 1986; Higuchi et al., 1993, 2003). However, it remains unclear why some patients develop symptomatic forms while others remain asymptomatic. Apparently, the genetic characteristics of the parasite strains and the immunogenetics of the host contribute equally to the final predictive parameters (Costa et al., 2009).

Aetiological treatment with benznidazole is effective for acute infections, but its true efficacy in chronic stages remains controversial (Viotti et al., 2006; Marin-Neto et al., 2007; Colantonio et al., 2016). A randomized study investigated 2854 patients with chagasic

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cardiomyopathy treated with benznidazole in several regions of South America (Morillo et al., 2015). The authors reported that benznidazole significantly reduced parasite detection but was unable to prevent cardiac lesions after a period of 5 years of follow-up (Morillo et al., 2015). Furthermore, many therapeutic failures have been observed even in the acute phase, which could be attributed to geographic differences where *T. cruzi* strains with different genetic groups circulate, as well as to many other factors influenced by interactions of the parasite with vectors and hosts (Estani et al., 1998; Cançado, 2002; Toledo et al., 2003; Yun et al., 2009). Due to the similarity of the canine disease to the human disease, dogs are a more suitable model for preclinical investigations of chronic Chagas cardiomyopathy (Andrade, 1984; Barr et al., 1991; de Lana et al., 1992; Guedes et al., 2002; Caldas et al., 2017). Nevertheless, dogs infected with strains susceptible to benznidazole exhibit low cure rates when the therapy is administered in chronic infections (Guedes et al., 2002). Previous studies have shown that benznidazole suppresses parasitaemia in dogs when the treatment is given in acute infections, and in the absence of parasitological cure, benznidazole can prevent morphofunctional cardiac injuries (Caldas et al., 2013). In another study with dogs treated in the recent chronic phase of infection, benznidazole was able to reduce parasitic burden, but only transiently (Santos et al., 2016). Follow-up analysis of treated animals showed increased parasitism and diastolic dysfunction, suggesting no impact on disease progression (Santos et al., 2016).

Considering the controversial outcomes of benznidazole treatment, this study used dogs and a quantitative real-time PCR (qPCR) strategy to assess whether parasitic load could be a rational target of aetiological chemotherapy to prevent cardiac fibrosis and chronic cardiomyopathy induced by *T. cruzi* strains with divergent profiles of resistance to specific chemotherapy.

2. Material and methods

2.1. T. cruzi stocks, animals, infection and chemotherapy

Three *T. cruzi* DTU II stocks (Moreno et al., 2010) were used in this study: VL-10, AAS and Berenice-78 strains. The AAS and VL-10 strains are classified as benznidazole-resistant (Caldas et al., 2013), and the Berenice-78 strain as benznidazole-susceptible (Guedes et al., 2002). The VL-10 strain was isolated from a patient with an indeterminate form of Chagas disease (Schlemper et al., 1982), and the AAS strain was isolated from a patient in the acute phase, both in the state of Minas Gerais, Brazil (Filardi and Brener, 1987). Berenice-78, isolated by Lana and Chiari in, 1986 from a human case in Brazil, was used as a benznidazole-susceptible reference strain.

As an experimental model, 70 four-month-old dogs were obtained, and maintained in the kennel of the Federal University of Ouro Preto, Minas Gerais, Brazil. The animals were fed with commercial ration and water ad libitum. Before the study, dogs were dewormed and vaccinated against several infectious diseases. For each *T. cruzi* strain, 20 animals were inoculated intraperitoneally with 2000 blood trypomastigotes/kg of body weight and then divided into two experimental groups: (i) half of the dogs were treated with benznidazole (N-benzyl-2-nitro-1-imidazolacetamide; Lafepe, Pernambuco, Brazil) at 7.0 mg/kg bid (Q12) in a suspension made with 4% gum Arabic, for 60 consecutive days (Guedes et al., 2002); and (ii) the other half remained untreated as a control group. Benznidazole treatment started on the first day following confirmation of infection by microscopic examination of fresh blood. An additional 10 animals were maintained as an uninfected control group. The experimental protocols were performed according to guidelines issued by the Brazilian College of Animal Experimentation (COBEA) and approved by the UFOP Ethics Committee on Animal Research (number 2009/15).

Parasitaemia curves were generated by optical microscopy analysis of 5 µL of blood taken daily from the marginal ear vein of dogs, according to the technique described by Brener (1962).

2.2. Evaluation of parasitological cure after treatment with benznidazole

To assess the parasitological cure of treated animals, we performed blood culture, conventional PCR and ELISA (enzyme-linked immunosorbent assay) using blood or serum from treated dogs. Thus, animals with positive serological results and negative blood culture were submitted to molecular blood evaluation by conventional PCR with targets for *T. cruzi* kDNA. All animals showing negative results for blood culture and PCR were considered parasitologically cured.

2.2.1. Blood culture

The blood culture assay was performed as described by Guedes et al. (2002). The test was performed immediately prior to euthanasia, at 90 days of infection, in animals from the acute phase. In dogs euthanized in the chronic phase, at 270 days of infection, one test was performed at 30 days and another at 6 months after treatment. Parasite detection was performed by culturing 10 mL of the collected blood, as well as by ELISA and conventional PCR. Cultures were maintained at 28 °C and examined monthly for up to 120 days for parasite detection.

2.2.2. Conventional PCR

DNA extraction and PCR were performed according to Gomes et al. (1998), with some modifications. PCR amplification was carried out in a 20 µL reaction mixture containing 0.1% Triton X-100, 10 mM Tris-HCl (pH 9.0), 75 mM KCl, 5 mM $MgCl_2$, 0.2 mM (each) dATP, dTTP, dGTP and dCTP (Sigma-Aldrich), 1 µL of Taq DNA polymerase (Invitrogen, USA), 20 pmol of S35 (5′- AAATAATGTACGGG(T/G)GAGATGCA TGA-3′) and S36 (5′- GGGTTCGATTGGGGTTGGTGT-3′) primers and 2 µL of DNA for each sample (Avila et al., 1991). Cycling conditions were as follows: denaturation at 95 °C for 1 min (with a longer initial time of 5 min at 95 °C), 35 cycles of 65 °C for 1 min for primer annealing and 72 °C for 1 min for extension, with a final incubation at 72 °C for 10 min to extend the annealed primers. PCR products were analysed by electrophoresis on a 6% polyacrylamide gel and visualized by silver staining (Santos et al., 1993).

2.2.3. ELISA

ELISA was performed according to Voller et al. (1976). ELISA plates were coated with *T. cruzi* antigen prepared from alkaline extraction of *T. cruzi* Y strain at exponential growth in LIT medium. Anti-dog IgG peroxidase-conjugated antibody (Sigma Chemical Co.) was used. The mean absorbance for 10 negative control samples plus two standard deviations was defined as the cut-off value to discriminate positive and negative results.

2.3. Necropsy and microscopic evaluation of cardiac tissue

Half of the dogs were sacrificed during the acute phase (3 months of infection), and the remaining animals at 6 months after treatment (9 months of infection). Fragments of the right atrial wall were fixed in formalin and embedded in paraffin. Then, tissue fragments were cut into 4 µm-thick sections and stained with Sirius Red dye (Sirius Red F3B; Mobay Chemical, Union, NJ, USA) for assessing the fibrotic process. Cardiac fibrosis was assessed by analysing the distribution of collagen using a segmentation function based on the birefringence properties of the collagen fibres under polarized light. The volume density (Vv[col]) of the myocardium occupied by collagen fibres was determined by point counting according to the formula Vv = Pp/Pt, where Pp is the number of points hitting the collagen fibres, and Pt is the total number of points used in the test system (n = 100). In this analysis, 60 microscopic fields were investigated (magnification: ×200) by random sampling, and a total myocardium area of $4.06 \times 10^3\ \mu m^2$ was analysed for each group. All morphological analyses were performed using the image analysis software Image Pro-Plus 4.5 (Media Cybernetics, Silver Spring, MD, USA).