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Chagas cardiomyopathy: The potential effect of benznidazole treatment on diastolic dysfunction and cardiac damage in dogs chronically infected with *Trypanosoma cruzi*

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ABSTRACT

Cardiac involvement represents the main cause of mortality among patients with Chagas disease, and the relevance of trypanocidal treatment to improving diastolic dysfunction is still doubtful. In the present study, we used a canine model infected with the benznidazole-sensitive Berenice-78 *Trypanosoma cruzi* strain to verify the efficacy of an etiologic treatment in reducing the parasite load and ameliorating cardiac muscle tissue damage and left ventricular diastolic dysfunction in the chronic phase of the infection. The effect of the treatment on reducing the parasite load was monitored by blood PCR and blood culture assays, and the effect of the treatment on the outcome of heart tissue damage and on diastolic function was evaluated by histopathology and echo Doppler cardiogram. The benefit of the benznidazole-treatment in reducing the parasite burden was demonstrated by a marked decrease in positive blood culture and PCR assay results until 30 days post-treatment. At this time, the PCR and blood culture assays yielded negative results for 82% of the treated animals, compared with only 36% of the untreated dogs. However, a progressive increase in the parasite load could be detected in the peripheral blood for one year post-treatment, as evidenced by a progressive increase in positive results for both the PCR and the blood culture assays at follow-up. The parasite load reduction induced by treatment was compatible with the lower degree of tissue damage among animals euthanized in the first month after treatment and with the increased cardiac damage after this period, reaching levels similar to those in untreated animals at the one-year follow-up. The two infected groups also presented similar, significantly smaller values for early tissue septal velocity (E' SIV) than the non-infected dogs did at this later time. Moreover, in the treated animals, an increase in the E/E' septal tissue filling pressure ratio was observed when compared with basal values as well as with values in non-infected dogs. These findings strongly suggest that the temporary reduction in the parasite load that was induced by benznidazole treatment was not able to prevent myocardial lesions and diastolic dysfunction for long after treatment.

1. Introduction

Chagas disease is a vector-borne parasitic infection caused by the kinetoplastid protozoan *Trypanosoma cruzi* and is an important cause of end-stage cardiomyopathy and loss of disability-adjusted life years (DALYs) among young, economically active adults in endemic countries (Coura, 2007; Martins-Melo et al., 2012). An estimated 14,000 people die from Chagas disease each year, mainly due to a chronic cardiac condition that may be associated with other chronic diseases, further increasing mortality (Schmunis and Yadon, 2010; Guariento et al., 2011).

The role of parasite persistence in the pathogenesis of Chagas heart disease has been demonstrated by the presence of *T.*

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cruzi in the heart, along with a low-grade but relentless inflammatory process and myocardial autoimmune injury (Gutierrez et al., 2009; Marin-Neto et al., 2009; Caldas et al., 2013). However, the immunopathological mechanisms involved in the pathogenesis of chagasic cardiomyopathy and the effect of trypanocidal therapy on the clinical course of patients with chronic Chagas heart disease have not been completely elucidated.

The myocardial abnormalities observed in the chronic phase of the *T. cruzi* infection are extremely variable, ranging from mild forms, such as digitiform apical aneurysms and abnormalities of the left ventricular diastolic function only, to significant cardiac chamber dilatation coupled with severe systolic dysfunction (Ianni et al., 2001; Migliore et al., 2004). Echocardiography is a well-established test in clinical practice that provides parameters by which chagasic patients can be analyzed and stratified (Rassi et al., 2014). Early diagnosis of diastolic dysfunction is a good tool for the management of patients with Chagas cardiomyopathy and can improve prognosis (Cianciulli et al., 2006; Garcia-Alvarez et al., 2010).

A number of clinical trials have demonstrated the beneficial effect of etiologic treatment with benznidazole in the acute and recent chronic phases of Chagas disease (Andrade et al., 1996; Andrade et al., 2004; Sosa Estani et al., 1998). However, no definitive consensus has been reached concerning whether benznidazole treatment significantly reduces the parasite burden or symptoms in the established chronic phase of the disease (Marin-Neto et al., 2009). The recently published results of the BENEFIT clinical trial showed that benznidazole treatment of patients with established Chagas cardiomyopathy was able to reduce (although only transiently) blood parasite levels but did not significantly reduce cardiac clinical deterioration through 5 years of follow-up (Morillo et al., 2015). Pre-clinical studies have demonstrated the beneficial effect of etiologic treatment on reducing tissue damage (Garcia et al., 2005; Bahia et al., 2012; Caldas et al., 2014) and on electrocardiographic alterations (Caldas et al., 2013). Others have demonstrated that benznidazole treatment was able to induce a mild improvement in systolic dysfunction in dogs chronically infected with *T. cruzi* (Santos et al., 2012). However, the authors showed that this improvement in systolic heart function in benznidazole-treated dogs was not accompanied by prevention of growth of the cardiac chambers, including the left atrial volume, a parameter that may also be evaluated to assess left ventricular diastolic cardiac function. Considering these antecedents, the present investigation was undertaken to better elucidate whether trypanocidal therapy with benznidazole in the chronic phase of Chagas disease would be effective in preventing or reducing the left ventricular diastolic dysfunction and tissue damage as well as in reducing the parasite burden immediately and for one year post-treatment.

2. Methods

2.1. Parasite

The Berenice-78 *T. cruzi* strain (*T. cruzi* II), isolated by xenodiagnosis in 1978 (Lana and Chiari, 1986) from the first reported human case of Chagas disease, was used in this study

2.2. Experimental animals

Thirty 4-month-old mongrel dogs from the Kennel of the Ouro Preto Federal University (UFOP), Minas Gerais State, Brazil, were used in this study. The animals were fed with commercial chow and water ad libitum. Before the study, the animals were dewormed and vaccinated against several infectious diseases. All procedures and experimental protocols were conducted in accordance with the guidelines of the COBEA (Brazilian College of Animal Experimentation) and with the approval of the Ethics Committee for Animal Experimentation of UFOP (Protocol number 2008/08). The infected animals were inoculated intraperitoneally with 4000 blood trypomastigotes per kg of body weight and then were divided into two experimental groups: (i) 11 dogs that were treated with benznidazole at 7.0 mg/kg bid (Q12) for 60 days and (ii) 11 dogs that were maintained as untreated controls. An additional 8 animals were maintained as a non-infected/healthy control group.

2.3. Drug and treatment scheme

The drug benznidazole (Bz; *N*-benzyl-2-nitro-1-imidazolacetamide) was synthesized at LAFEPE, Pernambuco, Brazil. The treatment scheme was previously described by Guedes et al. (2002). In all chronic-stage therapeutic schemes, oral treatment was initiated 120 days post-infection.

2.4. Assessment of parasite clearance

The parasite load was monitored by blood PCR and blood culture assays performed before treatment and in the 1st, 6th and 12th months post-treatment. Additionally, quantitative real-time PCR (qPCR) was performed on heart tissue samples from the animals euthanized in the 1st and 12th months post-treatment.

2.4.1. Blood PCR

For the PCR assays of the blood, 10 mL of blood was collected from each animal during follow-up evaluations in the 4th month of infection (before treatment) and at the 1st, 6th and 12th months post-treatment. The blood samples were mixed with an equal volume of a 6 M guanidine hydrochloride-0.2 M EDTA solution and were stored for two weeks at room temperature, followed by boiling for 15 min before DNA was extracted from 200 μL aliquots taken from each sample (Guedes et al., 2002). PCR amplification was performed in a total volume of 20 μL 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 Chemical Co.); 1 μL of Taq DNA polymerase (Invitrogen, USA); 20 pmol of S35 (5′-AAATAATGTACGGG(T/A)GAGATGCATGA-3′) and S36 (5′-GGGTTCGATTGGGGTTGGTGT-3′) primers; and 2 μL of DNA for each sample (Ávila et al., 1991). The reaction mixture was subjected to 35 cycles of amplification in an automatic thermocycler (Biocycler). The temperature profile was as follows: denaturation at 95 °C for 1 min (with a longer initial time of 5 min at 95 °C), 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. The PCR products were visualized by 6% polyacrylamide gel electrophoresis, followed by silver staining. All DNA extraction steps and reaction mixtures used for PCR were monitored and compared with positive and negative controls. The PCR analysis was considered negative after three failed DNA extractions for a given sample.

2.4.2. Blood culture

Parasite detection was performed by culturing 10 mL blood samples collected in parallel with the blood used for the DNA extraction and PCR amplification. Blood culture assays were performed for treated and control/untreated dogs, as described by Guedes et al. (2002). The cultures were maintained at 28 °C, homogenized weekly, and examined monthly for up to 120 days for parasite detection.

2.4.3. Quantitative real-time PCR

DNA extraction from the left atrium was performed at 1 and 12 months after treatment using a Wizard® Genomic DNA Purification