Benznidazole prevents endothelial damage in an experimental model of Chagas disease

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\textbf{A B S T R A C T}

Objectives: To evaluate the effect of benznidazole on endothelial activation in a murine model of Chagas disease.

Methods: A low (30 mg/kg/day) and a high (100 mg/kg/day) dose of benznidazole were administered to mice infected with \textit{Trypanosoma cruzi} during the early phases of the infection. The effects of the treatments were assessed at 24 and 90 days postinfection by evaluating the parasitaemia, mortality, histopathological changes and expression of ICAM in the cardiac tissue. The blood levels of thromboxane A\textsubscript{2}, soluble ICAM and E-selectin were also measured. \textit{T. cruzi} clearance was assessed by the detection of parasite DNA in the heart tissue of infected mice.

Results: Benznidazole decreased the cardiac damage induced by the parasite, and amastigote nests disappeared at 90 days postinfection. Both doses cleared the parasite from the cardiac tissue at 24 and 90 days postinfection. In addition, benznidazole decreased the thromboxane levels and normalized the plasma sICAM and E-selectin levels by 90 days postinfection.

Conclusions: Early administration of benznidazole at a dose as low as 30 mg/kg eradicates \textit{T. cruzi} from cardiac tissue. Additionally, benznidazole prevents cardiac damage and modulates endothelial activation as part of its antichagasic activity.

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1. Introduction

Chronic Chagas disease is one of the most devastating causes of cardiac failure (Barbosa et al., 2011; Vilas Boas et al., in press). Although extensive public health measures to control vectors and prevent blood transmission have decreased the incidence and prevalence of this disease, current therapies do not possess high efficacies. Additionally, human migration has provided a source of the disease in non-endemic countries, increasing the risk of blood-borne or congenital transmission in regions where systematic blood testing is not performed (Munoz et al., 2009). Thus, understanding the mechanisms of current therapies and identifying other therapeutic strategies will help to improve the treatment of Chagas disease, particularly during the chronic phase of the disease.

One of the pathophysiologic mechanisms involved in chronic chagasic cardiomyopathy (CCC) is the presence of microvascular abnormalities and ischemia, events that are related to endothelial activation or dysfunction due to thromboxane A\textsubscript{2} (TXA\textsubscript{2})-mediated platelet aggregation (Constans and Conri, 2006; Keller et al., 2003; Marin-Neto et al., 2007; Rossi et al., 2010). TXA\textsubscript{2} is produced by both the parasite and the host (Ashton et al., 2007; Rossi et al., 2010). Activation of the TXA\textsubscript{2} receptors increases the expression of adhesion molecules (Daniel et al., 1999; Ishizuka et al., 1998) such as intercellular adhesion molecule-1 (ICAM-1 or CD54); vascular cell adhesion molecule-1 (VCAM-1 or CD106) and E-selectin (Kobayashi et al., 2007). The expression of these molecules is increased in the \textit{T. cruzi}-infected myocardium and could participate in the establishment of the chagasic infection by interaction with the \textit{T. cruzi} trypomastigotes (Huang et al., 1999).
Benznidazole reduces cardiac abnormalities in a chronic murine model of Chagas disease (Garcia et al., 2005), mainly related to its trypanocidal activity. However, the therapeutic scheme employed in that study was administered over a long period of time, and the benznidazole dose used corresponds to the maximum effective dose reported for mice (benznidazole 100 mg/kg/day). The effect of benznidazole on endothelial function has not yet been explored. Recent reports show that benznidazole modulates pro-inflammatory cytokines and NO* release in macrophages by inhibiting NF-κB (Manarin et al., 2010). Because NF-κB is capable of inducing ICAM-1 expression, benznidazole may also modulate the inflammatory response elicited by the parasite (Piaggio et al., 2001).

Therefore, benznidazole could protect the host's endothelial environment during a chronic T. cruzi infection. Herein, we determined the effect of low dose, short duration benznidazole therapy on cardiac abnormalities. Using a chronic Chagas murine model, we investigated the effect of the therapy on endothelium adhesion molecules and cardiac histopathology at 90 days post-infection (d.p.i.). Our results suggest that a low dose of benznidazole decrease the expression of the adhesion molecules and improves the histopathology of the cardiac tissue. This study provides evidence of an efficacious low-dose therapy that could decrease the risk of adverse dose-related events.

2. Materials and methods

2.1. Animals

Adult male Balb/c mice (20–25 g) were obtained from the animal facility at the Faculty of Medicine, University of Chile. The mice were maintained on ad libitum Purina Laboratory Chow (5001) and water. All of the animal housing and handling procedures followed the “Guide for the Care and Use of Laboratory Animals” from the National Institutes of Health, USA (National Research Council (U.S.), Committee for the Update of the Guide for the Care and Use of Laboratory Animals, et al., 2011), and all protocols were approved by the Institutional Ethical Committee at the Faculty of Medicine, University of Chile.

2.2. Parasites and infection model

Animals were randomly infected with 500 blood trypomastigotes of the T. cruzi strain Dm28c by intraperitoneal (i.p.) injection. After infection, the animals were randomly divided into groups of 8 individuals to receive the different treatments or to be allocated in the infected or non-infected control groups for each of the observation periods (24 or 90 d.p.i.). The T. cruzi infections were confirmed by direct microscopic visualization of circulating trypomastigotes in the peripheral blood (Bustamante et al., 2007; Huang et al., 2002).

2.3. Benznidazole treatments

Mice were treated with 30 or 100 mg/kg/day benznidazole for 20 days starting on the second d.p.i. The drug was suspended in aqueous 1% methylcellulose and administered by oral gavage. The control mice received only the vehicle. The pharmacologic effect on acute and chronic cardiac disease was confirmed by histopathologic analysis of heart tissue from animals euthanized at 24 or 90 d.p.i. Hearts from animals that received both benznidazole treatments and from controls were examined (Bustamante et al., 2007; Huang et al., 2002). Euthanasia was performed by the i.p. administration of 150 mg/kg ketamine (Drug Pharma Invetec, Chile) and 30 mg/kg of xylazine (Laboratorios Alfasan, Argentina).

2.4. Cardiac tissue preparation

For histopathologic analysis, a heart sample from each euthanized animal was fixed in 10% formaldehyde in 0.1 M phosphate buffer (pH 7.3) for 24 h and then prepared by routine hematoxylin–eosin staining. The slides were qualitatively analyzed at 40× magnification to detect T. cruzi amastigote nests and inflammation in the myocardium (Caldas et al., 2008; Duaso et al., 2010). Twenty fields from each slide were randomly chosen, and a representative field is shown in Fig. 2.

2.5. Expression of endothelial adhesion molecules

Standard immunoperoxidase techniques were used on a heart sample from each euthanized animal to determine the expression levels of endothelial adhesion molecules. ICAM antibodies (rat-anti-mouse 1:1000 dilution v/v, Santa Cruz Biotechnology, USA) were used for this purpose. Primary antibodies were applied to each section overnight at 4 °C. A biotinylated anti-rat IgG at a 1:50 dilution v/v (Vector Laboratories, USA) was used as the secondary antibody. The immunostaining was performed with a horseradish peroxidase-labeled streptavidin biomix kit (RTU-Vectorstain kit, Vector Laboratories, USA) following the manufacturer’s directions and using diaminobenzidine as the chromogen. The sections were counterstained with Mayer’s hematoxylin (DAKO, Denmark) and mounted with Entellan® Mounting Medium (Merck-Millipore, Germany). The immunohistochemical controls were prepared by replacing the primary antibodies with phosphate buffered saline. Sections were examined with a Leitz Orthoplan® light microscope, and images were captured digitally with a Canon® PCT258 camera. A representative field is presented in the Fig. 4.

2.6. Plasma levels of endothelial dysfunction markers

The plasma levels of mouse sICAM-1 and sE-selectin were determined by ELISA using Quantikine® Kits (R&D Systems, USA) according to the manufacturer’s protocols. TXA2 was measured indirectly by quantifying its stable metabolite, Thromboxane B2 (TXB2), using the TXB2-EIA® Kit (Cayman Chemical, USA) according to the manufacturer’s protocols. All measurements were performed on plasma samples from animals euthanized after 24 and 90 d.p.i. and from both benznidazole treatment groups and controls.

2.7. TaqMan real time PCR for detection of Trypanosoma cruzi

Heart samples extracted from euthanized animals from each experimental group at 24 or 90 d.p.i. were homogenized in 1 mL of 1.18% KCl, and the total genomic DNA was isolated using a Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer’s instructions. The DNA was quantified by measuring its absorbance at 280 nm using an EpochTM Micro-Volume System spectrophotometer (Bio-Tek, USA). A Real Time PCR TaqMan assay designed by our group was used to quantify parasite DNA. Primers TecT 4-Fw (5′-GACAACAAGTGTGATGAC-3′), TecT 1-Rev (5′-AAGAATTTCGCGAGCTCTTG-3′) and the TecT-1 probe (5′-56FAM-ATCGCCGATGTCGACCCGTC-3′) were designed to amplify a T. cruzi satellite-DNA sequence of 84 bp. As an endogenous control, we designed the Mus-F (5′-GCAAAAGCTGCAACTCTCAGA-3′) and Mus-R (5′-TACACGTGACCCGATGTCGACCCGTC-3′) primers coupled with the MM-1 probe (5′-CAL Fluor Orange 560-AAAGCATTCGCTCCG-BHQ-1-3′) to amplify a sequence of 67 bp of the GAPDH gene of Mus musculus. The primers and probes were designed using the Primer Express 3.0 Software (Applied Biosystems, USA). The PCR reactions were carried out in an ABI7300 real-time thermocycler (Applied Biosystems, USA). The reaction mixture had a final volume of 20 μL containing 50 ng of genomic
DNA, 10 μL of SensiMix II Probe® Kit (Bioline, UK), 200 nM of primers, 500 nM of ROX and 100 nM of the TcSt-1 probe or 200 nM of the MM-1 probe. For both TaqMan assays, the thermal cycles consisted of a polymerase activation step carried out at 95 °C for 10 min (one cycle) and a two-step amplification phase: 95 °C for 15 s and 55 °C for 45 s (40 cycles). The fluorescence was measured at the end of each amplification cycle. Standard calibration curves were constructed for each pair of primers and their respective probe using serial dilutions of pure T. cruzi DNA (in a background of murine DNA). An efficiency of 91% was obtained for the T. cruzi TaqMan assay (between 10 ng/μL and 1 fg/μL of T. cruzi DNA as the sensitivity range), and a 99% efficiency was obtained for the murine TaqMan assay (between 4 ng/μL and 10 pg/μL of murine gDNA as the sensitivity range). With this information, we were able to quantify the parasite burdens of the heart tissue samples obtained from infected mice. The data are expressed as the ratio of T. cruzi DNA to murine DNA.

2.8. Statistical analysis

The results represent the mean ± SD from 3 independent experiments. The one- and two-way ANOVA analysis and Tukey’s post hoc test were performed when required. The log rank test was performed to analyze survival, and the non-parametric Mann–Whitney test was used for the q-PCR analysis. Statistical significance was defined as p < 0.05.

3. Results

To assess whether an early, short course with benznidazole could provide protection from the development of endothelial alterations in the chronic stage of Chagas disease, mice were treated with the maximum effective dose reported (100 mg/kg) or with a lower dose (30 mg/kg) of benznidazole. Both treatments decreased the level of parasitaemia and significantly increased the survival rate of the mice (Fig. 1A and B). When comparing the two treatments, there was a significant difference only at day 12 post-infection (p < 0.05, data not shown). It is interesting that the Balb/c mice in our model developed a parasitaemia profile with two maximum peaks separated by three days (12 and 15 d.p.i.). We have consistently observed this phenomenon even after using a high parasite inocula (Bryan et al., 2010; Faundez et al., 2008). There were no differences in the degree of increased survival between the two doses of benznidazole assayed (Fig. 1C and D).

Parasitaemia became undetectable at 24 days post-infection in all experimental groups. At this point, the hearts from the infected control mice (n = 4) showed an intense and generalized inflammatory infiltrate, several amastigote nests, edema and cardiac disorganization (Fig. 2A). However, the 30 mg/kg dose of benznidazole diminished the number of inflammatory infiltrates, although there were still several amastigote nests and focal inflammatory infiltrates present (Fig. 2B). As expected, the hearts of mice treated with the 100 mg/kg dose of benznidazole showed no differences from the non-infected hearts (Fig. 2C). During the chronic phase of the infection (90 d.p.i.), cardiac disarrangement and focal inflammatory infiltrates were observed in control infected mice that were consistent with persistent low-level inflammation, indicating chronic cardiomyopathy (Fig. 2D). Notably, we observed an improvement in the cardiac histology of hearts from mice treated with either the low or high dose of benznidazole (Fig. 2E and F). This effect could be directly attributed to the trypanocidal activity of benznidazole and its elimination of the source of chronic cardiac inflammation. Therefore, treatment with benznidazole beginning as early as the second day post infection can prevent chronic cardiac damage. Benznidazole at a dose of 100 mg/kg was able to cure the infection early and thus limit the effect of the parasite on the cardiac tissue, as confirmed by the absence of parasitaemia and cardiac damage during the acute phase (Figs. 1C and 2C). However,
the most interesting finding is that a dose of 30 mg/kg of benznidazole was able to erase the signs of cardiomyopathy by 90 days post infection (Fig. 2E), although low levels of parasitaemia (Fig. 1A) and focalized inflammatory infiltrates were present in the cardiac tissue at day 24 (Fig. 2B).

To correlate the parasite load with the histological findings, the presence of parasite DNA in the blood and heart was assessed at 24 d.p.i. As observed in Fig. 3a, the T. cruzi DNA content of the heart tissue decreased depending on the benznidazole dose employed. Thus, 30 mg/kg of benznidazole decreased the T. cruzi DNA content by 90%, while the 100 mg/kg dose resulted in a 99.9% decrease. The 30 mg/kg dose of benznidazole decreased the parasite load in the blood by 24.5%, and the 100 mg/kg dose resulted in a 99.7% decrease (Fig. 3b). This decrease in T. cruzi DNA content remained steady into the chronic phase of infection (90 d.p.i., data not shown). These findings are in agreement with the microscopic observations described previously.

Endothelial activation is necessary for the establishment of chronic microvascular alterations in chronic cardiac Chagas disease. To study the effect of benznidazole on endothelial function, the expression of ICAM-1 was assayed in cardiac tissue from control mice and treated mice. The uninfected hearts did not show ICAM-1 immunoreactivity, but the T. cruzi-infected hearts showed an increased expression of ICAM-1 during the acute phase of infection (Fig. 4A) compared to the uninfected control (data not shown). Benznidazole decreased the ICAM-1 signal at both of the doses assayed (Fig. 4B and C). The level of ICAM-1 expression in the infected hearts persisted through 90 days postinfection and decreased after benznidazole treatment, although residual immunoreactivity was observed in mice that were given the 30 mg/kg dose (Fig. 4D–F). Thus, both treatments are effective at preventing increases in the expression of this adhesion molecule.

Additionally, the levels of several endothelial dysfunction markers, including TXA2 and the soluble forms of ICAM and E-selectin, were measured in plasma from infected, treated and non-treated mice. In T. cruzi-infected Balb/c mice, TXA2 levels were significantly elevated at 24 d.p.i. (Fig. 5A), and this increase was more dramatic
4. Discussion

Chronic Chagas cardiomyopathy is a clinical condition triggered and sustained by the presence of T. cruzi in the myocardium. This disease is accompanied by an abnormal immune response, leading to a chronic myocarditis which involves microvascular lesions. The microvascular contribution to heart alterations during chronic Chagas disease is well known (Lannes-Vieira et al., 2009b; Rossi and Ramos, 1996; Tanowitz et al., 2005, 2009). However, the endothelial effects of Chagas disease are just beginning to be studied. The association of platelet aggregation with ischemic lesions in the chagasic myocardium has already been described (Rossi and Bestetti, 1995), and the role of endothelin in chronic Chagas cardiomyopathy has been studied (Petkova et al., 2001). In addition, it has been reported that in the early stages of Chagas disease, there is a prothrombotic state (Herrera et al., 2003) that could be modulated by benznidazole therapy (Pinazo et al., 2011). This pro-aggregation state is facilitated by the release of TXA2 which participates in platelet activation, and by the expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin that increase endothelial susceptibility to infection (Dias et al., 2008). Ashton and colleagues (Ashton et al., 2007) demonstrated that the amastigote form of T. cruzi is capable of synthesizing TXA2. Thus, this may be one of the mechanisms by which T. cruzi can directly promote microvascular alterations.

One of the main concerns about the treatment of Chagas disease is the reduced efficacy of the classic antichagasic drugs nifurtimox and benznidazole during the chronic stage of infection. However, it is recommended that chronic Chagas patients are treated with benznidazole because the pathogenesis and evolution of Chagas disease suggests that parasite eradication could decrease the number of inflammatory foci, promote tissue regeneration and reverse fibrosis (Coura and Borges-Pereira, 2011). Although microvascular alterations have been related to the development of chronic chagasic cardiomyopathy (Rossi et al., 2010), there is little information about the effect of benznidazole on endothelial dysfunction.

Our results agreed with previous reports regarding the effect of benznidazole on parasitaemia and mortality in infected mice in a chronic model of infection (Fig. 1) (Bustamante et al., 2007). Unexpectedly, our data show that a dose as low as 30 mg/kg of benznidazole was as effective as the therapeutic antichagasic dose of 100 mg/kg that was used in previous studies (Bustamante et al., 2007).

Benznidazole treatment decreased the cardiac fiber disarrangement and inflammation produced by the parasite at 24 d.p.i. (Fig. 2), and this effect persisted at 90 d.p.i. In chronic models of Chagas disease, the late benznidazole treatment decreased the level of cardiac inflammation (Bustamante et al., 2007; Garcia et al., 2005). This...
also occurred when benznidazole was administered early in the course of the disease, although it did not resolve the inflammation in the chronic phase of the disease (Caldas et al., 2008). Nevertheless, our results indicate that early administration of benznidazole can decrease and even prevent inflammatory damage in chronically T. cruzi-infected hearts, at least with the T. cruzi strain used in this study. Most importantly, this effect was observed even at the low dose of 30 mg/kg.

These results are related to the expression of ICAM-1 in cardiac tissue and, consequently, with endothelial activation. The infected controls showed elevated levels of this adhesion molecule during the acute and chronic stage, whereas treated animals showed either low levels of ICAM-1 expression or no ICAM-1 expression (Fig. 4).

Endothelial activation could also be a consequence of increased parasite-induced thromboxane production (Ashton et al., 2007). Several studies reported high serum levels of soluble sVCAM-1, and CD44 (a fibronectin and hyaluronic acid ligand) during the acute phase of infection, whereas soluble P-selectin (sP-selectin or CD62P) was associated with chronic disease severity (Lannes-Vieira et al., 2009a; Laucella et al., 1999). During endothelial activation and in response to inflammatory inputs, the endothelium increases the expression of ICAM-1, VCAM-1, E-selectin and other molecules. These molecules all involve the IKK-β/IκB/NF-κB axis (Maier et al., 2012). The administration of benznidazole during the early stages of the disease normalized the thromboxane, sICAM and sE-selectin serum levels, and the effect persisted 60 days after the drug was administered (Fig. 5).

The main pathogenic input in the Chagas cardiomyopathy is parasite persistence. This is aligned with the observation that 100 mg benznidazole eradicated the parasite and normalized the endothelial markers assayed, as a consequence of reduced tissue injury and improvement of the immune function in the treated mice. However, as observed in Fig. 2, at 30 mg/kg/day parasites persist in the cardiac tissue, but endothelial markers, mainly ICAM-1, were almost undetectable. This findings support the idea that other mechanisms, independent of the trypanocidal activity of benznidazole may be involved. Indeed, it has been shown that benznidazole has anti-inflammatory properties because it can inhibit NF-κB (Manarin et al., 2010; Ronco et al., 2011). Consequently, the regulation of adhesion molecules that promote endothelial activation, leukocyte migration and other inflammatory processes (Morris et al., 2009) can be achieved through the effect of benznidazole upon NF-κB. It has been proposed that this inhibition of NF-κB activation occurs by blocking the IκB kinase (IKK) complex and p38 MAPK, (Manarin et al., 2010; Piaggio et al., 2001) two pathways that are involved in regulating the expression of adhesion molecules like ICAM and VCAM (Al-Mutairi et al., 2010; Huang et al., 2004; Wang and Dong, 2012). Thus, in chronic chagasic cardiomyopathy, benznidazole treatment could both eradicate the parasite and promote a protective endothelial environment.
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Indeed, fragment significantly of different aetiologies have shown that the plasma levels of sEL-1 and sICAM-1 are useful as markers of endothelial dysfunction progression and as risk factors for acute coronary syndromes and death (Gross et al., 2012; Maggio et al., 2012; Wijnstok et al., 2010).

In conclusion, a dose of benzimidazole as low as 30 mg/kg modulates endothelial activation, prevents cardiac damage and significantly decreases the parasite load in heart tissue when it is administered early in infection. Thus, it could be possible to achieve a therapeutic effect at a low benzimidazole dose, decreasing the risk of adverse reactions associated with this drug. In addition, soluble endothelial adhesion molecules may be useful markers to evaluate the progression of Chagas disease and the response to therapeutics. Further studies are needed to evaluate the utility of this approach in humans. We have demonstrated a direct relationship between anticaghashic therapy and its role in endothelial cell activation in the context of a chronic cardiomyopathy model of Chagas disease for the first time.

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Conflict of interest

None to declare.

Author contributions

All authors contributed to the conception and design of the study, laboratory analysis, data analysis and interpretation and the drafting of this manuscript. All of the authors contributed to, read and approved the manuscript.

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References


Wang, J., Dong, S., 2012. ICAM-1 and IL-8 are expressed by DEHP and suppressed by curcumin through ERK and p38 MAPK in human umbilical vein endothelial cells. Inflammation 35, 859–870.