Enalapril in Combination with Benznidazole Reduces Cardiac Inflammation and Creatine Kinases in Mice Chronically Infected with Trypanosoma cruzi

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Abstract. The protozoan Trypanosoma cruzi triggers an inflammatory process in mammalian heart causing events such as fibrosis, changes in the architecture and functionality in this organ. Enalapril, an angiotensin II-converting enzyme inhibitor, is a drug prescribed to ameliorate this heart dysfunction, and appears to exert a potential role in immune system regulation. Our aim was to evaluate the chronic cardiac inflammatory parameters after therapeutic treatment with enalapril and benznidazole in C57BL/6 mice infected with the VL-10 strain of T. cruzi. After infection, animals were treated with oral doses of enalapril (25 mg/kg), benznidazole (100 mg/kg), or both during 30 days. Morphometric parameters and levels of chemokines (CCL2, CCL5), IL-10, creatine kinases (CKs), and C-reactive protein were evaluated in the heart and serum at the 120th day of infection. Enalapril alone or in combination with benznidazole did not change the number of circulating parasites, but reduced cardiac leukocyte recruitment and total collagen in the cardiac tissue. Interestingly, the combination therapy (enalapril/benznidazole) also reduced the levels of chemokines, CK and CK-MB, and C-reactive proteins in chronic phase. In conclusion, during the chronic experimental T. cruzi infection, the combination therapy using enalapril plus benznidazole potentiated their immunomodulatory effects, resulting in a low production of biomarkers of cardiac lesions.

INTRODUCTION

Trypanosoma cruzi is a flagellated protozoan causative of the human Chagas disease. Glycoproteins molecules from this parasite trigger the release of varying levels of inflammatory cytokines during the mammalian host invasion, depending on the protozoan genetic background.1 During the acute phase, the inflammatory response is designed to eliminate parasites whereas during the chronic phase, it is rather controlled in such a way that the immune response is targeted to maintain the parasite at check preventing excessive tissue damage, fibrosis formation, and cardiac loss of function.2-4

Since the presence of T. cruzi is the switch for inducing and maintaining the inflammatory process running in the heart tissue, three cardinal therapeutic strategies are proposed: 1) an antiparasitic drug, where benznidazole is considered a standard treatment but still ineffective to those symptomatic individuals in chronic stage of Chagas cardiopathy5; 2) drugs that act directly on the cardiac dysfunction avoiding progressive failure of the organ—for example, diuretics, digitals, β-blockers, and angiotensin-converting enzyme (ACE) inhibitors6-7 and, more recently, 3) drugs that act in controlling the over-reactivity of the immune system avoiding severe destruction in the heart during chronic stage of the infection—for example, statins and ACE inhibitors.8-12

ACE inhibitors have been shown to be efficacious not only as therapies against hypertension and protection against left ventricular hypertrophy but also in the regulation of the immune system related to distinct diseases, including the T. cruzi-induced pathologies.9,13-15 Their actions are based on the angiotensin II, a key factor in the renin–angiotensin system that plays an essential role in the regulation of blood pressure, and that also interferes in the cardiac inflammatory activity through the activation of the nuclear factor kappaB (NF-κB) and in the production of inflammatory cytokines and chemokines.1,16

In the context of the experimental T. cruzi infection, the ACE inhibitor captopril was previously demonstrated to be able to decrease the delayed-type hypersensitivity response against parasite in acute myocarditis.8 Later, another ACE inhibitor (enalapril) was shown to ameliorated experimental acute myocarditis by decreasing leukocytes infiltration and circulating levels of chemokines (CCL5), tumor necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ).9 In addition, the authors of this study also observed a direct anti-T. cruzi activity both in vitro and in vivo, likely via inhibition of the parasite’s cytochrome-P450 pathway.

In this proposed study, C57BL/6 mice were infected with the VL-10 strain of the T. cruzi and treated during the acute phase for 30 days with enalapril, with benznidazole, or with both drugs in combination and inflammatory aspects observed after 120 days of infection, in particular the production of circulating chemokines, creatine kinases (CKs), and C-reactive proteins.

MATERIALS AND METHODS

Animals, parasites, and infection. Ten-week-old male C57BL/6 mice were bred and maintained at the Center of Animal Science from the Universidade Federal de Ouro Preto (UFOP), Brazil. Mice (N = 10) were infected with 100 blood trypomastigote forms of T. cruzi (VL-10 strain). These parasites were maintained through successive passages in Swiss mice at the Laboratory of Immunobiology of Inflammation, UFOP, and parasitemia was determined daily, by optic microscopy analysis of 5 μL samples after tail bleeding, according to Brener.17 Animals were euthanized 120 days postinfection and blood and heart tissues were collected for immune and biochemical assays and histology.

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All procedures were conducted in accordance with the guidelines issued by the Brazilian College of Animal Experimentation and had previously been approved by the Ethic Committee in Animal Research at UFOP (protocol CEUA no. 081/2012).

**Pharmacological treatment.** Enalapril (Pharlab, Minas Gerais, Brazil) and benznidazole, the (N-benzy1-2-(2-nitro-1H-imidazol-1-yl)acetamide (LAFEPE, Recife/PE, Brazil), were commercially acquired and diluted in water suspended in 0.05% w/v of methyl cellulose at the appropriate concentrations for the indicated doses. The drugs were given orally by gavage (0.1 mL) once daily during 30 consecutive days, starting 24 hours after the *T. cruzi* infection. Animals were divided in five distinct groups: 1) 10 mice infected with *T. cruzi* treated with vehicle, 2) 10 mice infected with *T. cruzi* and treated with enalapril (25 mg/kg/day), 3) 10 infected mice treated with benznidazole (100 mg/kg/day), 4) 10 infected mice treated enalapril (25 mg/kg/day) plus benznidazole (100 mg/kg/day) administered 1 hour after the first drug, and 5) 10 uninfected animals (control group) that received vehicle following the same regimen of the animals under other treatments.

**Immunohistochemistry.** Blood from anesthetized mice was collected from the orbital plexus (0.8 mL) on the 120th day of infection, before euthanasia, and serum was used to determine the circulating levels of interleukin 10 (IL-10), regulated on activation, normal T cell expressed and secreted (CCL5/RANTES), and monocyte chemotactrant protein (CCL2/MCP-1). IL-10, CCL2, and CCL5 chemokines (PeproTech, São Paulo, Brazil) were then quantified by enzyme-linked immunosorbent assay as recommended by manufacturer. In brief, flat-bottom 96-well microtiter plates (Thermo Scientific Nunc, San Jose, CA) were coated with 100 μL/well of the appropriate monoclonal antibodies for 18 hours at 4°C and then washed with phosphate-buffered saline (PBS) buffer (pH 7.4) containing 0.05% Tween 20 (wash buffer). Nonspecific binding sites were blocked with 200 μL/well of 1% bovine serum albumin in PBS. Plates were washed, and 100 μL/well of samples were added followed by incubation for 18 hours at 4°C. Later, 100 μL/well of the appropriate biotinylated detection antibodies diluted in blocking buffer containing 0.05% Tween 20 were added and incubated for 1 hour at room temperature. Streptavidin–horseradish peroxidase was added and, after incubation and washing, 100 μL/well of the chromogen substrate o-phenylenediamine (Sigma, St. Louis, MO), diluted in 0.03 M citrate buffer containing 0.02% 30 v/v H2O2, was added for 30 minutes followed by incubation in the dark at room temperature. The reaction was stopped by the addition of 50 μL/well of 1 M H2SO4 solution and plates were read at 492 nm in a spectrophotometer (EMax; Molecular Devices, Sunny Vale, CA). All samples were measured simultaneously in duplicate.

**Histopathology and polarized microscopy of picrosirius red-stained collagen.** For the analysis and quantification of inflammatory infiltrate and collagen, fragments of cardiac tissue were stained by hematoxylin and cosin (H&E). In brief, tissue fragments were fixed in 10% buffered formalin solution, dehydrated, cleared, and embedded in Paraplast (Sigma). Blocks were cut into 4-mm-thick sections and stained by H&E for inflammation assessment. Twenty fields from each of the H&E-stained section were randomly chosen at 40× magnification, giving a total of 74,931 μm²—the equivalent area of 50 fields of analyzed myocardium. Images were obtained through a Leica DM 5000 B microchamber (version 2.4.0 R1; Leica Application Suite, Milton Keynes, United Kingdom) and processed by software Leica Qwin (V3) image analyzer.

The inflammatory process was evaluated by the number of cellular nuclei observed in the infected heart tissue and compared with the background from cardiac cellular nuclei observed in the noninfected mice. Heart sections were also stained with picrosirius red (S2365; Poly Scientific, Bay Shore, NY) to visualize collagen content in the heart tissue at 120 days postinfection. Polarized micrographs were obtained via Leica DM 5000 B microchamber equipped with a color camera and utilizing NIS Elements Software (Nikon Instruments Inc., Melville, NY). The collagen defined by the polarized micrographs was then measured on ImageJ (National Institutes of Health, Bethesda, MD).

**Determination of the plasma CKs and C-reactive protein.** CK, CK myocardial band (CK-MB) and C-reactive protein were measured in the plasma from different groups of infected mice by an Olympus AU640 autoanalyzer (Olympus, Hamburg, Germany) using the manufacturer’s appropriate reagents. Calibration and quality control of the equipment were performed according to the recommended protocol.

**Statistical analysis.** The statistical analysis of the results were carried out using a Shapiro–Wilk test to verify the normality among data and one-way analysis of variance test followed by a Bonferroni’s multiple comparison test and Wilcoxon rank sum test for multiple comparisons through the GraphPrism v.5 (GraphPad Software, Inc., La Jolla, CA). Results represent mean ± standard error of the mean (SEM) of 6–10 survivors per experimental group and differences with a P value of < 0.05 were considered statistically significant.

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**FIGURE 1.** Parasitemia and percentage of surveillance in mice infected with the VL-10 strain of *Trypanosoma cruzi*. C57BL/6 mice were infected with VL-10 strain (100 parasites/mouse) of *T. cruzi* and (A) the parasitemia was evaluated until 30 days of infection and (B) the surveillance observed till 120 days postinfection, when animals were euthanized. Data are shown as a mean of 10 animals ± standard error of the mean (SEM).

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RESULTS

Parasitemia and survival curves. During the first 30 days of infection, there was no difference observed in the prepatent period or in the peak of parasitemia among groups receiving enalapril, benznidazole, or both drugs in combination (Figure 1A). After 5 days of continuous negativation in the parasitemia curve, the evaluation was interrupted. During and after the acute phase, a survival curve was plotted showing low mortality induced by VL-10 in isogenic C57BL/6 mice. There was observed 50% of survival rate in untreated animals and 60% in those treated with enalapril, benznidazole, or both drugs in combination (Figure 1B) at 120 days of postinfection.

Inflammatory infiltrate and collagen profile in cardiac tissue at 120 days of infection. The production of circulating IL-10 and proinflammatory chemokines was elevated at 120th day in mice after infection with the VL-10 strain of *Trypanosoma cruzi*. In agreement with these findings, the evaluation of the cardiac tissue demonstrated a high number of inflammatory cells associated with infected animals (Figure 2A). However, when compared with the infected group treated with vehicle, animals treated with benznidazole, enalapril, or with combinatory therapy (enalapril plus benznidazole) displayed drastically reduced leukocyte infiltration into the cardiac tissue despite an absence of difference among these groups.

In parallel, an evaluation of total, type I and type II, collagens was done in this same cardiac tissue. The analysis revealed a massive increase of all types of collagen in infected animals when compared with those that remained uninfected (Figure 2B). The different therapeutic strategies were able to reduce the total collagen when compared with the *T. cruzi*-infected group. However, using the VL-10 strain of *T. cruzi*, there was no difference among benznidazole, enalapril, or the combinatory therapy in terms of levels of type I and II collagens in the chronic phase.

Inflammatory mediators in the chronic phase of experimental infection. Soluble inflammatory mediators are pivotal agents during the inflammatory response induced by parasites. There was an increase in the level of circulating CCL2/MCP1 (Figure 3A) as well as of circulating CCL5/RANTES (Figure 3B) in mice infected with the VL-10 strain of *T. cruzi* in comparison with those uninfected. Benznidazole and enalapril alone were not capable of reducing the release of these chemokines, but the combinatorial therapy reduced the levels of both inflammatory mediators in the plasma of infected animals during the chronic phase. In addition, the regulatory IL-10 cytokine was elevated in infected mice and in those treated with enalapril with or without combination with benznidazole (Figure 3C), when compared with uninfected animals.

Determination of the cardiac biochemical parameters in the chronic *T. cruzi* infection. The low, but persistent inflammation in C57BL/6 mice caused by the VL-10 strain of *T. cruzi* during 120 days was able to elevate the plasma level of the CK (Figure 4A), CK-MB (Figure 4B) and C-reactive protein (Figure 4C). The administration of benznidazole or enalapril reduced the plasma level of C-reactive protein. The combinatory therapy using enalapril plus benznidazole was capable to

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Inflammatory cells and collagen in heart tissue in the chronic phase of *Trypanosoma cruzi* infection. Cellular infiltration in animals infected with VL-10 strain of *T. cruzi* (100 parasites/animal) were quantified (A) and area of total type I and type II collagens represented (B), using picrosirius red staining. Data are shown as a mean of 5 to 6 animals ± standard error of the mean (SEM) and * means difference (*P* < 0.05) when compared with the same collagen from other groups and when compared with total collagen from the *T. cruzi*-infected group.
reduce the levels of CK, CK-MB and C-reactive protein, all markers of cardiac dysfunction in mice.

**DISCUSSION**

The responsiveness of immune system is capable to define the course of the acute and chronic *T. cruzi* infection in mammalian. The initial inflammatory response triggered, mainly, by the glycoproteins and other molecules from this protozoan promotes a self-perpetuation of this inflammation, often culminating in a cellular destruction. However, this prominent immune response mediated by innate cells and inflammatory cytokines (e.g., IFN-γ, IL-12, TNF-α, and CCL2), antibodies, and other molecules (e.g., nitric oxide) are necessary to repress parasite replication and invasion. This acute stage is followed by a life-long chronic condition where the cellular immune response limits parasite proliferation despite being unable to eradicate the infection, which leads to a sustained and chronic inflammatory process.

The genetic characteristics of the parasites or hosts are pivotal aspects for the establishment of a diffuse cellular infiltration composed mainly of mononuclear cells and lymphocytes. This pattern will contribute to the pathogenesis of cardiac lesions because of 1) the disruption and displacement of local vascular beads and the cardiac myofibrils, 2) the collagen deposition in the heart followed by fibrosis, and 3) the remodeling and loss of functionality of the heart in human and in experimental models. Therefore, the elimination of the parasites is an obvious interest of chemical therapy because of the persistent antigenic stimulation caused to the mammalian host.

More than one century after the original description of the Chagas disease and more than four decades after its introduction as a treatment, the “nitro derivative benznidazole” (N-benzyl-2-nitroimidazole acetamide) is still the most used trypanocidal drug against *T. cruzi*. Unfortunately, this drug...
has unsatisfactory efficacy and leads to undesired adverse reactions. Since the inflammatory response against the parasite persists for decades causing a silent cardiac damage in humans, a new consensus of benznidazole treatment has been proposed aiming the reduction or elimination of the parasite loads, which could cause less inflammation. In this way, administration of benznidazole during chronic phase of experimental T. cruzi infection prevents the development of a more severe form of cardiopathy, in part by the reduction of the parasite load, in part by affecting host immune regulation. Indeed, previous in vitro study, demonstrated that benznidazole acts downregulating NOSII gene promoting nitric oxide inhibition and also promotes macrophage NF-κB inhibition after stimulation with lipopolysaccharide and IFN-γ. Its immunomodulatory effect goes further through the observation that the benznidazole is able to inhibit inflammatory cytokines, autoantibodies, nitric oxide production, and also leukocyte recruitment into the heart during T. cruzi infection in human and experimental models. In these studies, the toxicity of the benznidazole overrides the cardiac beneficial effects since activation and/or misbalance of the host inflammatory response become detrimental to the heart function. Yet far from a cure for chronic stage of the infection, a novel therapeutic strategy has been proposed in which immunomodulation in the absence of immunosuppression could be beneficial to the host during chronic T. cruzi infection. In this context, well-known cardiovascular therapeutic options (e.g., ACE-inhibitors, β-blockers, statins) have gained new interest because of their more recently described modulatory properties over the release of inflammatory cytokines and chemokines, the cellular infiltration, and fibrosis leading to improvements in ventricular cardiac function in experimental and human T. cruzi studies.

Our group previously described, in an experimental model of acute infection by T. cruzi, a relevant role for the enalapril using the Colombian strain of the parasite whose characteristic feature is to cause high systemic and cardiac inflammation in C57BL/6 mice. In this study, the short treatment with 25 mg/kg/day of enalapril was capable to reduce circulating parasites, heart tissue inflammation and plasma IFN-γ, TNF-α, and CCL5/RANTES, suggesting a protective effect to this ACE inhibitor to the chronic stage of the infection. In this study, a similar treatment was performed using enalapril and/or the antiparasitic drug benznidazole that also might modulate immune response in experimental models of infection, however now using a different strain (VL-10) of the parasite, which allowed for the evaluation of the chronic phase of infection (120 days). Both enalapril and benznidazole were capable to induce a low leukocyte infiltration into cardiac tissue as well as to reduce the releasing of C-reactive protein levels in C56BL/6 mice contributing to a regulatory systemic response. However, when other parameters were evaluated such as circulating chemokines (CCL2 and CCL5) and markers of cardiac dysfunction (CK and CK-MB), a significant reduction was detected only in those animals treated with the combinatorial therapy (enalapril plus benznidazole). Clearly, the most important event during T. cruzi VL-10 infection occurs during the first 30 days of infection, when parasites were still in replication and the mice immune response was over reactive. These early therapeutic interventions given during acute events appear to be crucial in defining the course of the incipient pathological process in the infected heart tissue, which might predict future damage to this organ.

Chemokines CCL2/MCP-1 and CCL5/RANTES have been largely associated with the recruitment of monocytes and lymphocytes to inflammatory foci with the primary role to kill parasites and inevitably driving host tissues to the damage. But even so, both chemokines have been suggested as good biomarkers of inflammation since their expression in T. cruzi-infected heart tissue was detected from as far as 15th to the 240th day of infection. Indeed, the role of CCL5 in the recruitment of CCR5 plus leukocytes is reinforced by studies where CCR5-deficient mice became more susceptible to T. cruzi infection by the reduction of macrophages and T-cells migration into the heart, especially during early stages of the infection. In addition, a partial blockage of CC-chemokine receptor inhibitor (Met-RANTES) was shown to induce a reduction of the leukocyte influx modulated by T. cruzi followed by parasite load reduction and fibronectin deposition in the heart tissue. Besides, CCL2 was also associated with the control of parasite burden, as well as with the increase of inflammatory infiltration and angiogenesis process induced by T. cruzi, in particular by its essential role in recruiting monocytes to inflammatory foci.

In this chronic study using the VL-10 strain of T. cruzi, we conclude that enalapril and benznidazole given in combination were more effective in reducing 1) the plasma levels of CCL2 and CCL5, 2) the intensity of inflammatory infiltration, and 3) the reduction of deleterious effect in the heart, reflected by the low levels of CK and CK-MB in infected animals in a long-term infection in mice.

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