Altered Cardiomyocyte Function and *Trypanosoma cruzi* Persistence in Chagas Disease

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Abstract. Chagas disease, caused by the triatominae *Trypanosoma cruzi*, is one of the leading causes of heart malfunctioning in Latin America. The cardiac phenotype is observed in 20–30% of infected people 10–40 years after their primary infection. The cardiac complications during Chagas disease range from cardiac arrhythmias to heart failure, with important involvement of the right ventricle. Interestingly, no studies have evaluated the electrical properties of right ventricle myocytes during Chagas disease and correlated them to parasite persistence. Taking advantage of a murine model of Chagas disease, we studied the histological and electrical properties of right ventricle in acute (30 days postinfection [dpi]) and chronic phases (90 dpi) of infected mice with the Colombian strain of *T. cruzi* and their correlation to parasite persistence. We observed an increase in collagen deposition and inflammatory infiltrate at both 30 and 90 dpi. Furthermore, using reverse transcriptase polymerase chain reaction, we detected parasites at 90 dpi in right and left ventricles. In addition, we observed action potential prolongation and reduced transient outward K^+ current and L-type Ca^{2+} current at 30 and 90 dpi. Taking together, our results demonstrate that *T. cruzi* infection leads to important modifications in electrical properties associated with inflammatory infiltrate and parasite persistence in mice right ventricle, suggesting a causal role between inflammation, parasite persistence, and altered cardiomyocyte function in Chagas disease. Thus, arrhythmias observed in Chagas disease may be partially related to altered electrical function in right ventricle.

INTRODUCTION

Trypanosoma cruzi, the etiologic agent of Chagas disease, is an important cause of cardiac diseases in Latin America.¹ The onset of Chagas disease is divided in two stages: an acute phase, with high levels of *T. cruzi* in the bloodstream, and a chronic phase, in which the symptoms are observed 10–40 years after the infection, and approximately 20–30% of patients manifest heart conditions including, but not restricted to, heart hypertrophy, heart failure, and cardiac arrhythmias.^{2,3} Changes in heart function is connected to a set of cellular and molecular changes in cardiac tissue such as disrupted myofibrils, increased myocyte apoptosis,⁴ collagen deposition,⁵ oxidative stress,⁶ altered cellular metabolism,⁷ reduced cellular contractility,⁸ and disturbance of electrical properties of the heart.^{9,10} There are distinct hypotheses to explain the molecular mechanisms of altered electrical and contractility properties of the heart during Chagas disease. One of the most accepted explanations claims that chronic cytokine production in the heart is responsible for most of the observed cardiac alterations.^{2,11}

Clinical studies and animal models have shown that right and left ventricles are compromised during the time course of Chagas disease.^{3,8–10,12–15} It is already known that right ventricular function is dysregulated during Chagas disease, however, few studies have pointed out which molecular mechanisms are responsible for right ventricular myocyte dysfunction.^{16,17}

Herein, using a murine model of Chagas disease, we studied whether the changes in electrical properties of the isolated right ventricular myocyte in acute and chronic phases of the disease are correlated with tissue fibrosis, inflammatory infiltrate, and parasite persistence in the heart.

MATERIALS AND METHODS

Mice. Mice were maintained at the Federal University of Minas Gerais (UFMG), Brazil, in accordance with National Institutes of Health guidelines for the care and use of animals. Experiments were performed according to approved protocols from the Institutional Animal Care and Use Committee at UFMG and Federal University of São Paulo.

Mice and parasites. Eight-week-old male C57BL/6 mice were intraperitoneally infected with 50 bloodstream trypomastigote forms of Colombian *T. cruzi* strain,¹⁸ which was maintained by serial passages in mice at the Centro de Pesquisas René Rachou/Fundação Oswaldo Cruz (Belo Horizonte, Brazil).

Histomorphometric analyses. The mice were killed, and hearts were carefully removed and fixed in formalin (10% w/v in isotonic saline). Sections (5 μ m thick) were stained and processed for light microscopic studies and morphometric analysis. Hematoxylin and eosin (HE) was used to count the number of parasite nests/field and quantify inflammatory infiltrates. Picrosirius staining followed by polarized-light microscopy was used to visualize and analyze collagen.¹⁹ Thickness of the right ventricle wall was measured using a magnifier in three different points per ventricular wall from each mouse. All staining was performed in paraffin-embedded heart sections mounted on glass slides. The images were digitized through a JVC TK-1270/JGB microcamera to perform the analysis and transferred to an analyzer (Kontron Elektronik, Munchen, Germany).

Reverse transcriptase polymerase chain reaction. Total RNA was isolated from 50 mg of dissociated left and right ventricular myocytes (see below) from *T. cruzi* infected and control mice, and homogenized in 500 μ L of Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. For the reverse transcriptase polymerase chain reaction (RT-PCR), purified RNA (2 μ g) was used to synthesize complementary DNA (cDNA) using a Superscript III (Invitrogen) following the manufacturer's instructions. cDNA was amplified in a PCR using GoTaqGreen Master Mix (Promega, Madison, WI) and 18S ribosomal primers (forward: 5'-TTGTTTGTTGATTCCGTCA-3';

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reverse: 5'-CCCAGAACATTGAGGAGCAT-3'). The amplification program was performed in a Eppendorf Mastercycler[®] Nexus (Eppendorf AG, Hamburg, Germany), which included an initial denaturation at 95°C for 2 minutes, followed by 30 cycles of 95°C for 1 minute, 58°C for 1 minute, and 72°C for 30 seconds, with a final extension step at 72°C for 5 minutes. Amplification products were analyzed by electrophoresis in a 2% agarose gel and visualized after staining with GelRed (Biotium Inc., Hayward, CA). A positive result for PCR was the presence of a 200-bp band specific for *T. cruzi*.

Ventricular myocyte isolation. Right and left ventricular cardiomyocytes from age-matched mice were enzymatically isolated as previously described with few modifications.⁸ In brief, the heart was mounted on a homemade Langendorff system, perfused for 5 minutes with calcium-free solution containing (in mM) 130 NaCl, 5.4 KCl, 0.5 MgCl₂, 0.33 NaH₂PO₄, 3 pyruvate, 22 glucose, and 25 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) (pH set at 7.4). Afterward, the heart was perfused for 10–15 minutes with a solution containing 1 mg/mL collagenase type II (Worthington Biochemical Corp., Lakewood, NJ). The digested heart was then removed from the cannula, and the right and left ventricles was separated and cut into small pieces. Single cells were isolated by mechanical titration and stored in Dulbecco's modified Eagle's medium (Sigma Chemicals Co., St. Louis, MO). Only calcium-tolerant, quiescent, rod-shaped myocytes showing clear cross striations were studied. The isolated cardiac myocytes were used within 4–6 h after enzymatic dispersion.

Electrophysiology. Whole-cell voltage- and current-clamp recordings were obtained using an EPC-9.2 patch-clamp amplifier (HEKA Electronics, Lambrecht [Pfalz], Germany).²⁰ After attaining the whole-cell configuration, 2–5 minutes were allowed to the pipette solution to equilibrate with cellular interior. The experiments were carried out at room temperature (24–27°C). The recording electrodes had resistances of 1–2 MΩ. Current recordings were low-pass filtered (2.9 kHz) and digitized at 5–10 kHz before being stored in a computer. Myocytes showing series resistance (*R_s*) larger than 10 MΩ were not used in the analysis. *R_s* compensation was used at 40–70%.

To record action potentials (APs), inwardly rectifying K⁺ current (*I_{K1}*) and transient outward K⁺ current (*I_{to}*), the pipette solution was filled with (in mM) 130 K-aspartate, 20 KCl, 10 HEPES, 2 MgCl₂, 5 NaCl, and 5 EGTA, pH set to 7.2 with KOH. We used Tyrode's as bath solution containing (in mM) 140 NaCl, 5.4 KCl, 1 MgCl₂, 1.8 CaCl₂, 10 HEPES, and 10 glucose (pH set at 7.4). After measuring AP and *I_{K1}*, cardiac myocytes were bathed with recording solution containing 100 μM of Cd²⁺ to block L-type Ca²⁺ current (*I_{Ca,L}*).

We recorded AP until steady-state conditions were achieved. After that 30–50 APs were measured (paced at 1 Hz), and we used the last recorded AP to perform the analysis. We measured the overshoot amplitude, maximal rate of depolarization, and duration at 10%, 50%, and 90% of AP repolarization. *I_{K1}* was recorded from a holding potential of –40 mV, and then stepped to a set of membrane test potentials (1.5 seconds duration) every 15 seconds between –130 and –40 mV with increments of 10 mV. *I_{to}* was elicited by depolarization steps from –40 to 70 mV (3 seconds duration) from a holding potential of –80 mV every 15 seconds. We used a 50 ms pre-pulse from –80 to –40 mV to inactivate Na⁺ channels but still were able to record *I_{to}*.²¹ For measurements of *I_{Ca,L}*, recording pipettes were filled with internal solution containing (in mM) 120 CsCl, 20 tetraethylammonium chloride (TEA-Cl), 5 NaCl, 10 HEPES, 5 Ethylene glycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), pH set to 7.2 with CsOH. *I_{Ca,L}* was recorded in the presence of 1.8 mM extracellular Ca²⁺. Membrane potential was first stepped from a holding potential of –80 mV to –40 mV for 50 milliseconds (to inactivate Na⁺ channels), and then stepped to different membrane voltages between –40 and 50 mV (300 milliseconds duration). We corrected for junction potential errors (~10 mV) when measuring *I_{K1}* and *I_{to}*.

Current–density relationships were fitted using the following equation:

$$I_V = G_{\max} \times \frac{(V_m - E_x)}{1 + \frac{\exp(V_m - V_{0.5})}{S}} \quad (1)$$

where *G_{max}* is the maximal conductance; *V_m* the membrane potential; *E_x* the electrochemical potential equilibrium for ion x; *V_{0.5}* the potential in which channels are half activated; *S* the slope factor.

Statistical analysis. In all experiments, we used 3–5 different hearts. All results are expressed as mean ± standard error of the means, and the number of cells is given as *n*. Differences between groups were determined using one-way analysis of variance, followed by Bonferroni post hoc test. Differences were considered significant when *P* < 0.05.

RESULTS

Chagas disease causes an increased progression in collagen deposition in both right and left ventricles.^{9,22,23} In a previous study, we have shown that Colombian strain of *T. cruzi* causes marked collagen accumulation in the mice left ventricle.⁹

FIGURE 1. Typical histological sections (10 μm, Picrosirius staining) of right ventricular tissue. (A) Representative images in (i) control, (ii) 30, and (iii) 90 days postinfection (dpi). (B) Progressive collagen deposition during the time course of Chagas disease. *Control × 30 dpi and #control × 90 dpi, *P* < 0.05.

FIGURE 2. Typical histological sections (50 μm, stained with hematoxylin and eosin) of right ventricular tissue. (A) Representative images in (i) control, (ii) 30, and (iii) 90 days postinfection (dpi). (B) Sustained inflammatory infiltrate during *Trypanosoma cruzi* infection during the time course of Chagas disease. *Control × 30 dpi and #control × 90 dpi, $P < 0.05$.

As shown in Figure 1A, using Picrosirius staining technique, we detected progressive collagen deposition in right ventricle of infected mice. As depicted in Figure 1B at 30 and 90 days postinfection (dpi), we observed increased collagen deposition ~120% and ~198%, respectively. Next, we examined the presence of inflammatory cells in the right ventricle using HE staining. Figure 2A shows typical images of inflammatory cell infiltration in right ventricle. At 30 dpi, an intense inflammatory infiltrate in the right ventricle is noticed when compared with control (Figure 2A and B), which was still observed at 90 dpi indicating a set of chronic inflammation. In addition, we investigated the *T. cruzi* persistence at 90 dpi in the chagasic heart. Using microscopic techniques it was not possible to detect *T. cruzi* nests or isolated parasites in right ventricle (Figure 3A). However, using RT-PCR, we detected ribosomal *T. cruzi* mRNA in both right and left isolated cardiomyocytes at 90 dpi (Figure 4). Interestingly, in this later condition, we verified 23% reduction in the ventricular wall thickness (Figure 3B) in agreement with other study.²⁴

There is compelling evidence in the literature that during the chronic phase of Chagas disease the cardiac tissue presents significant changes in electromechanical properties.^{2,3} However, there is no study correlating inflammation, parasite persistence, and electrical properties of isolated right ventricular myocytes during chagasic cardiomyopathy.⁹ Thus, we used isolated right ventricular myocytes and patch-clamp technique to study the impact of Chagas disease on plasmatic membrane electrical properties.

First, we studied the AP waveform. Figure 5A shows typical AP recordings from isolated cardiac myocytes in control, at 30 dpi, and at 90 dpi. Figure 5B summarizes the results. *Trypanosoma cruzi* infection increased time to action potential repolarization (APR) at both, 30 and 90 dpi. AP duration was increased at 10%, 50%, and 90% of repolarization. Interestingly, as seen in Table 1, no significant

changes in the maximal rate of AP depolarization and overshoot were observed, suggesting no important changes in transient Na^+ current.

The APR is a complex interplay between distinct ionic currents, notably the diversity of voltage-dependent K^+ currents.^{9,25} To investigate the ionic basis to account for AP lengthening, we measured macroscopic outward and inward K^+ currents. Figure 6 shows the results for peak outward K^+ currents, which represent transient outward K^+ current component in cardiomyocytes (I_{to}).²⁶ Figure 6A depicts typical tracings for the outward K^+ current recorded in control, at 30 dpi, and at 90 dpi, respectively. Figure 6B summarizes the results. *Trypanosoma cruzi* infection provoked I_{to} reduction (about 50%) in membrane potentials from 0 to +70 mV. For example, current density (in pA/pF) at +50 mV was 31.9 ± 2.6 (control, $n = 17$), 15.1 ± 1.5 (at 30 dpi, $n = 15$, $P < 0.05$), and 16.9 ± 1.4 (at 90 dpi, $n = 12$, $P < 0.05$). To further investigate the participation of inwardly rectifying K^+ current (I_{K1}), we performed a series of experiments in isolated right ventricle cardiac myocytes during Chagas disease. The results are summarized in Figure 7. Figure 7A shows examples of I_{K1} recordings from control, at 30 dpi, and at 90 dpi. Figure 7B shows the result for sustained component of I_{K1} . Chagasic cardiomyopathy did not change I_{K1} in right ventricular myocytes.

It is well known that during the chronic phase of Chagas disease, electrical and mechanical remodeling of cardiac myocytes occurs.^{3,8-10} In ventricular cardiomyocytes, $I_{\text{Ca,L}}$ is a key player, integrating electrical with mechanical properties.²⁷ Thus, we evaluated whether $I_{\text{Ca,L}}$ was modified in right

FIGURE 3. Right ventricular parameters during *Trypanosoma cruzi* infection. (A) Number of *T. cruzi* nest/field in right ventricle in the acute phase. (B) Right ventricular thickness.

FIGURE 4. Reverse transcriptase polymerase chain reaction (RT-PCR) detection of *Trypanosoma cruzi* in mice cardiac tissue. PCR amplification of a 200-bp fragment represents a positive assay. LV = left ventricle; M = molecular weight marker (100 bp DNA ladder); NC = negative control (without sample); RV = right ventricle.

FIGURE 5. Prolonged action potential (AP) from right ventricular myocytes in Chagas disease. (A) Typical AP traces for (i) control, (ii) 30, and (iii) 90 days postinfection (dpi). Marks represent 0 mV. (B) Bar graphs comparing AP repolarization time at different repolarization levels. Control is marked with white bars, 30 dpi with light gray bars, and 90 dpi with dark gray bars. n = number of cells. *Control $\times$ 30 dpi and #control $\times$ 90 dpi, $P < 0.05$.

FIGURE 6. Reduced outward K⁺ current (I_{to}) in right ventricular myocytes. (A) Representative outward K⁺ current recordings from (i) control, (ii) 30, and (iii) 90 days postinfection (dpi). (B) Voltage dependence of I_{to} is plotted as current density (pA/pF) from -40 to +70 mV. Control is marked with white circle, 30 dpi with light gray circles, and 90 with dpi dark gray circles. Lines were fitted according to equation 1. n = number of cells. *Control $\times$ 30 dpi and #control $\times$ 90 dpi, $P < 0.05$.

ventricular myocytes during Chagas disease. Figure 8A represents recordings for $I_{Ca,L}$ measured in isolated right ventricular myocytes in control, at 30 dpi, and at 90 dpi. As shown in Figure 8B, *T. cruzi* infection significantly reduced $I_{Ca,L}$.

DISCUSSION

Chagas disease is a serious health problem, especially in Latin American countries. Despite of great efforts in the last century, many aspects of Chagas disease are still unknown.² Clinical studies have described impaired electromechanical properties in chagasic hearts.^{3,28,29} A number of hypotheses were proposed to better explain the clinical phenotype observed in chagasic patients. Two of them are generally accepted based on the relationship between parasite and host. The first puts forward a pivotal role of parasite's persistence to drive chronic inflammation in heart tissue as the major cause of cardiovascular pathology. The second accepted hypothesis postulates that an autoimmune response against self-antigens is responsible for the tissue damage observed in affected organs of chagasic individuals.

In our model, we observed the presence of *T. cruzi* nests in the right ventricular wall of the heart at 30 dpi. More important, at 90 dpi, using RT-PCR, we were able to detect the presence of *T. cruzi* in isolated cardiomyocyte in both right and left ventricles. These results firmly suggest that, in our model, parasite persistence is responsible for chronic inflammation. It is interesting to note, however, that most of the previous studies detected *T. cruzi* in cardiac tissue and not in isolated cardiomyocytes.^{30,31} Here, we detected the presence of *T. cruzi* in isolated cardiomyocytes, which

strongly indicates that parasites are present in cardiomyocyte cells. However, we cannot completely rule out the presence of infected cells isolated along with cardiomyocytes during the enzymatic dissociation of the heart.

In the context of cellular and molecular mechanisms involved in the cardiovascular physiopathology of Chagas disease, most studies have been focused on the left side of the heart. However, many reports in the literature, in both humans and animal models, observed changes in the physiology of the right side of the heart, implying right ventricle as an important player in the development of chagasic cardiomyopathy.^{13,14,24} Various studies demonstrated right branch block, dilated right ventricular chamber, compromised right ventricular myocyte contractility, and thinning of the right ventricular wall even in the absence of left ventricle remodeling, indicating a primary involvement of right ventricle in chagasic cardiomyopathy. However, there is no clear consensus in the literature.¹⁵ Thus, apparently the initial and/or later involvement of right ventricular chamber in chagasic cardiomyopathy progression may depend on the genetic background of the host and/or strain of *T. cruzi* causing the infection.³² Clearly, more studies are necessary to draw a more conclusive opinion about this issue.

FIGURE 7. Inward K⁺ current (I_{K1}) is not altered in right ventricular myocytes. (A) Representative I_{K1} recordings from (i) control, (ii) 30, and (iii) 90 days postinfection (dpi). (B) Voltage dependence of I_{K1} is plotted as current density (pA/pF) from -130 to +40 mV. n = number of cells. Control is marked with white circle, 30 dpi with light gray circles, and 90 dpi with dark gray circles.

TABLE 1
AP parameters

	dV/dt (mV/ms)	Overshoot (mV)	Capacitance (pF)
Control	238.1 ± 14.6 (n = 20)	49.17 ± 1.52 (n = 20)	127.1 ± 7.5 (n = 29)
30 dpi	225.9 ± 17.2 (n = 20)	55.21 ± 2.24 (n = 20)	120.8 ± 6.4 (n = 30)
90 dpi	202.3 ± 11.8 (n = 19)	43.63 ± 1.72 (n = 19)	142.0 ± 7.8 (n = 24)

AP = action potential; dpi = days postinfection.

FIGURE 8. Altered L-type Ca^{2+} current ($I_{Ca,L}$) in Chagas disease. (A) Representative $I_{Ca,L}$ recordings from (i) control, (ii) 30, and (iii) 90 days postinfection (dpi). (B) Voltage dependence of $I_{Ca,L}$ is plotted as current density (pA/pF) from -40 to +50 mV. Control is marked with white circle, 30 dpi with light gray circles, and 90 dpi with dark gray circles. Lines were fitted according to equation 1. n = number of cells *Control $\times$ 30 dpi and #control $\times$ 90 dpi, $P < 0.05$.

Our results show that *T. cruzi* leads to significant alterations in AP waveform of right ventricular myocytes in acute and chronic phases. Put in perspective, we may suggest that analogous mechanisms cause AP changes in both right and left ventricle myocytes.^{9,10} We previously found increased levels of transforming growth factor beta (TGF- β) and tumor necrosis factor alpha (TNF- α) in the myocardium and blood-stream of infected mice during acute and chronic phases.^{8,9} TNF- α and TGF- β were previously demonstrated as modulators of ionic currents in cardiomyocytes. It is known that TNF- α downregulates I_{to} in cardiomyocytes, through a mechanism dependent on nitric oxide and/or superoxide production.³³ Thus, TNF- α could be responsible for I_{to} current density attenuation in right ventricular myocytes, which might explain the delay in the APR. Hence, reducing TNF- α in the myocardial tissue would restore AP profile in the right ventricular myocytes. However, this hypothesis still needs to be evaluated.

In this model, robust attenuation of $I_{Ca,L}$ was observed. In the context of right ventricular myocytes physiology, $I_{Ca,L}$ is critical to link electrical and mechanical functions. Thus, reduction of $I_{Ca,L}$ could be responsible for the uncoupling of electromechanical function in right ventricular myocytes, contributing to arrhythmogenesis, a common cause of death in chagasic patients. Indeed, an attractive mechanism, one would argue, is that by recovering $I_{Ca,L}$ current density would reduce cardiac arrhythmias propensity contributing, therefore, to a better prognosis in the chronic phase of chagasic cardiomyopathy.⁸ It is important to point out that there are other possible factors involved in altered electrical properties of right ventricular myocytes. Accordingly, *T. cruzi* infection increases expression of β -adrenergic¹⁶ and muscarinic receptors,³⁴ which are known to modulate ionic currents in ventricular myocytes.

Taking together, to our knowledge this study is the first to directly associate parasite persistence, inflammation, and electrical disturbances in the right ventricle of *T. cruzi*-infected mice. Thus, targeting key cytokines (e.g., TNF- α , TGF- β) may have clinical and therapeutic implications during the development of heart malfunction in Chagas disease.

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