Brief Communications

Cardiac and Lymphocytes Mitochondrias as Biomarkers of Pathology in the Chronic Indeterminate Phase of Chagas Disease.

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Abstract
The entrance of the T. cruzi in cardiomyocytes generates inflammatory processes that provokes damage in the mitochondria, organelles essential for the heart function. We have previously showed that they are involved in different manners in the acute or chronic phase of the T. cruzi infection; for all these they could be good marker of pathology and evolution of this cardiomyopathy. Having these in mind, we studied the mitochondria in heart and lymphocytes from mice infected with trypomastigotes of T. cruzi (50/mouse) Tulahuen strains (G1, n=10) and with the isolate SGOZ12 (G2 n:10) at 75 days post-infection (indeterminate chronic phase), analyzing the enzymatic activity of citrate synthase (CS) and complex III (CIII) by spectrophotometric methods. Control electrocardiograms were performed before infection and at the time of the study. We also studied an uninfected group mice NI, n:10). The ANOVA and Fisher test were used and p< 0.05 was considered significant. CS activity (umol / min.mg protein) decreased in myocardial mitochondria of G1 and G2 (G1:0.01±0.01, G2:0.01±1.7x10-3; NI:0.29±0.04) p<0.05; lymphocyte activity of G1 was similar to NI (0.42±0.21) and increased in G2 (0.44±0.09) p<0.05. CIII activity in cardiac mitochondria and limphocytes decreased in G1 and G2, cardiac mitochondria: (G1:0.01 ± 2.8x10-4; G2:0.11±3.1x10-3) p<0.05 NI (0.17±0.03); lymphocytes: G1:0.01±8.3x10-4, G2: 0.12±2.9x10-3, NI:0.33±0.06 p<0,05. The electrocardiograms showed that 50% of G1 and 65% of G2 did not present alterations, but in both there was a reduction in heart rate at 100% of mice. These results indicate that mitochondria are early markers of pathology in the chronic indeterminate phase and that their alterations are present even with normal electrocardiograms. The correlation found in the changes in the enzyme activity of the cardiac and limphocytes mitochondria allows to propose that with a simple blood colection we could infer about the myocardium mitochondria involvement in the chronic indeterminate phase of this disease.

Infection with *Trypanosoma cruzi* causes American Trypanosomiasis or Chagas disease, one of the most important determinants of congestive heart failure and sudden death in Latin America. *T. cruzi* infection has been divided into successive acute and chronic phases [World Health Organization, 2007]. The acute phase is often characterized by a patent parasitaemia that allows the parasite to spread throughout the host. The chronic phase, on the other hand, affects mainly the heart (cardiac form) and occurs in approximately 30% of infected people. Between the acute and the cardiac chronic form of the disease there is a period, ranging from a few months to decades (chronic indeterminate form) [Macedo, 1999]. This form was thought to be symptomless, but more sensitive tests have demonstrated that patients in this stage may present significant abnormalities [Ribeiro and Rocha, 1998]. According to cardiologists, this period could be key in knowing which patient develops the cardiac form of infection [Elizari,1999].

The pathogenesis of chronic chagasic cardiopathy is still under discussion; there is considerable evidence that inflammatory infiltrates and their mediators have a direct effect on cardiac cells. The entrance of the parasite in the target cells provokes a focal mononuclear inflammation and the cell lysis [Andrade, 1999; Texeira et al., 2006]; these pathological lesions are detected in every chagasic patient, present in the heart of 94.5% of the deaths, and directly related to the persistence of the parasite and the pathogenesis of the disease [Texeira et al., 2006].

Previous works of our lab have demonstrated cardiac mitochondria disorders in the acute and chronic phase of the *T. cruzi* infection [Baez et al 2008, 2011] of different order according to the parasite strain that infected the host and other authors have also shown lymphocytes mitochondria involvement in these chagasic phases [Wen et al 2006].

Giving the importance of the chronic indeterminate period in the future development of cardiopathy, in the present work we studied the structure and function of cardiac and lymphocytes mitochondria produced by different *T. cruzi* strains, in order to determine their involvement in the pathophysiologic mechanism of chronic chagasic myocardiopathy.

Materials and methods

Infection

Albino Swiss female and male mice weighing 30 ± 1 g (n = 90) were used as follows: 10 mice were inoculated, by intraperitoneal injection, with 50 trypomastigote forms of T. cruzi, Tulahuen strain (Tulahuen), and 10 mice with 50 trypomastigote forms of the SGO Z12 isolate (SGO Z12), which was obtained from a patient from an endemic area. The number of parasites/ml of blood was determined in each group using a Neubauer haemocytometer. A non-infected group (n = 10) was also studied. All the experiments were carried out 75 days post infections; this time corresponds to chronic

indeterminate phase of the infection.

Mitochondria isolation
Hearts were removed on days 75 post infection (pi), which correspond, obtaining both ventricles. They were washed and suspended in ice-cold isolation buffer (5 mM HEPES, pH 7.2 containing 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 0.5% BSA (fatty acid-free), tissue/ buffer ratio, 1:10 w/v) and immediately homogenized. Homogenates were centrifuged at 1500 g, 4ºC for 20 min and the supernatant transferred to a new tube. The pellet was resuspended in isolation buffer, homogenized, and centrifuged again at 10,000g, 4º C for 5 min. The supernatant was discarded and the pellet was resuspended in buffer and centrifuged at 10,000g, 4ºC for 10 min (twice = purification). The mitochondrial pellet was resuspended in isolation buffer (tissue/buffer, 1:1 ratio, w/v), and the aliquots stored at -80 ºC.

For biochemical assays, whole-blood homogenates were prepared in 10 mM potassium phosphate (pH 7.4) containing 30 mM KCl and a protease inhibitor cocktail (1:1 v/v).

Mitochondria were isolated as described [Wen et al, 2006]. Briefly, blood aliquots were homogenized in equal volumes of ice-cold 10mM Hepes buffer, pH 7.2, containing 420 mM mannitol, 140 mM sucrose, 2 mM EGTA, and 2 mg/ml BSA (fatty acid free) and subjected to sequential centrifugation at 800 and 8100g, each for 15 min at 4 °C. The pelleted mitochondria were washed, resuspended in isolation buffer, and stored at –80 °C. Protein concentration was determined by the Bradford method. Samples of venous blood wre collected with and without K3EDTA (1.5 mg/ml of blood) in order to obtain plasma and serum.

Respiratory complex and citrate synthase activity
The activities of the respiratory complex (CIII) and the citrate synthase were monitored by spectrophotometric methods previously described [Trounce et al., 1996; Jarreta et al., 2000; Vyatkina et al., 2004] with slight modifications. Protein concentrations were calculated by Bradford assay [Bradford, 1976].

Electrocardiograms (ECG)
Electrocardiogram (ECG) tracings were obtained with a digital electrocardiographic unit (CardioCom – Model CC12DER MCP) under Ketamine ClH (Ketalar, Parke Davis, Warner Lambert Co., USA) anaesthesia (10 mg/ kg), before infection and during the chronic indeterminate.

form of the experimental disease. In order to follow the evolution of the cardiopathy and to study the conduction alterations in the chronic infection, ECGs were also performed at 135 days p.i.
The electrocardiographic tracings were obtained with six standard leads (dipolar leads DI, DII, DIII and unipolar leads aVR, aVL, aVF), recording at 50 mm/s with amplitude set to give 1 mV/10 mm. Data were then transferred to a computer for further analysis. Wave durations (seconds) were calculated by CardioCom software after cursor placement.

Statistical analysis
Data are the result from five independent randomized experiments. Results are shown as mean ± standard error. The obtained data were analysed by ANOVA and multiple comparison by Fisher Test; and Chi-square test for categorical variables. Axiovision 3.0 program was used to quantify mitochondria. The significance level was set at $p < 0.05$ for all cases.

Mitochondrial citrate synthase and respiratory complex III activity of myocardium and lymphocyte es
In order to evaluate the mitochondrial function, we analyzed the enzymatic activity of the mitochondrial citrate synthase (matrix) and in the respiratory chain complex III (cristae) in the myocardium and lymphocytes from uninfected and Tulahuen and SGO Z12 infected mice, 75 days pi. As can be observed in Figure 1 the citrate synthase activity (µmoles min -1/mg de prot) from the Tulahuen (0,01±0,01) or the SGOZ12 mice ((0,01±1.7 x 10 -3) in mitochondrias isolated from cardiac muscle , was diminished when compared with the non infected group (0,43±0,20) . The citrate synthase activity from mitochondrias of lymphocytes in the same moment from Tulahuen infected mice was diminished when compared to non infected and the SGO Z12 ones was significantly higher, $p<0.05$ (See **Figure 1**).

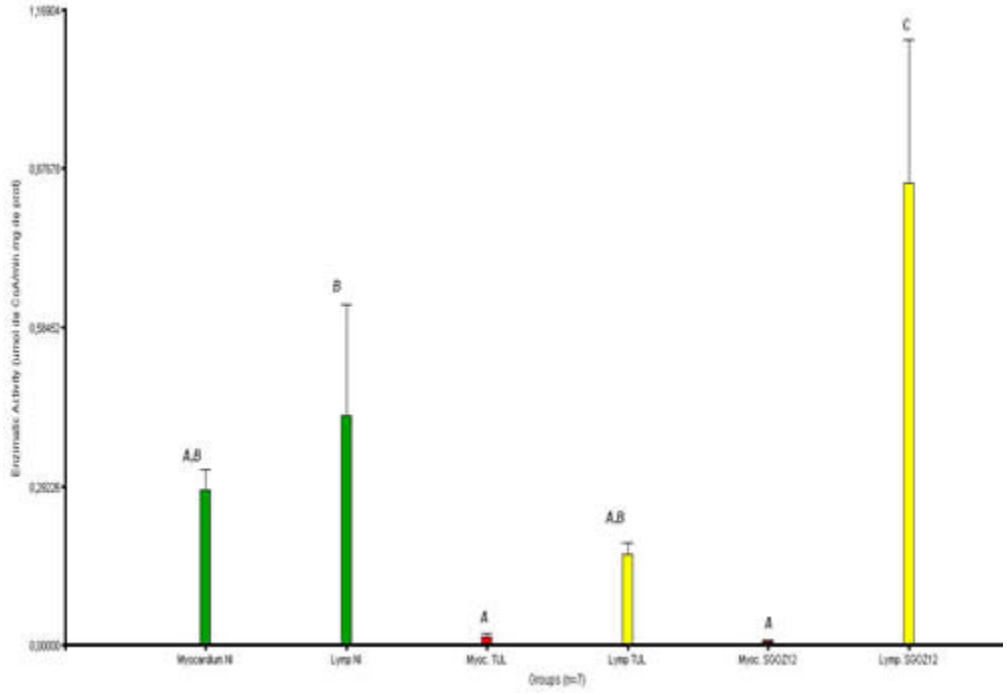

Figure 1. Enzymatic activity of citrate synthase in the myocardium and lymphocytes. The results shown correspond to mean ± ES (different letters indicate the Significant differences p <0.05). dpi: days post-infection

Figure 2 shows the respiratory chain complex III activity. As can be observed the activity of complex III was diminished in myocardium mitochondria as well in lymphocytes mitochondria, in a similar manner, when compared with the non infected group (p<0.01).

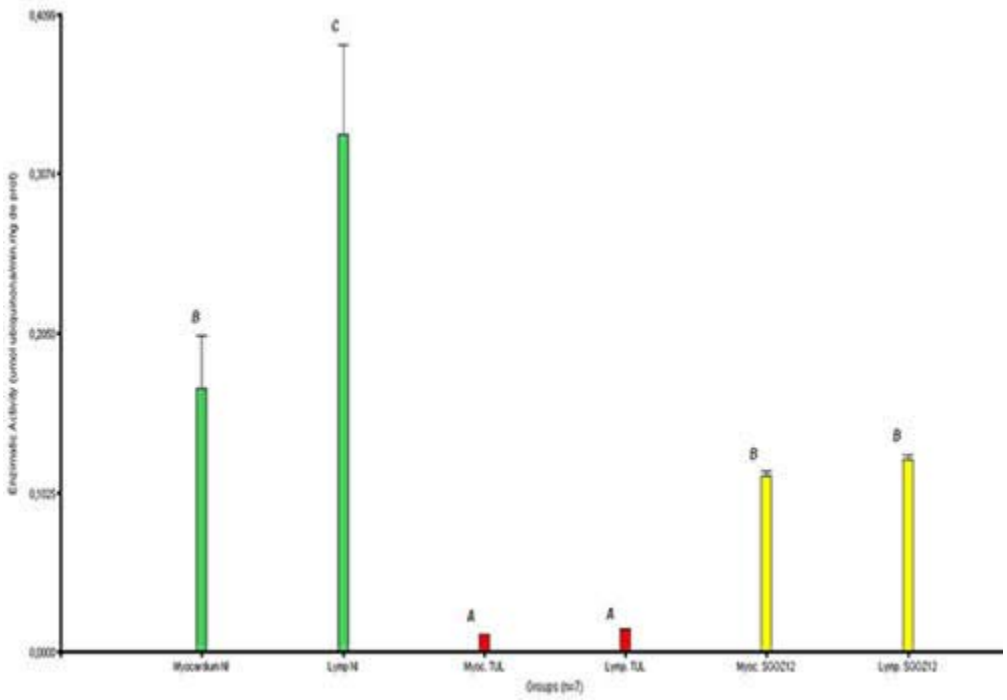

Figure 2. Enzymatic activity of complex III in the myocardium and lymphocytes. The results shown correspond to mean ± ES (different letters indicate the significant differences p<0.05) dpi: days post-infection

Electrocardiograms

Table 1 shows the electrocardiographic results from uninfected mice (**Table 1**) and mice infected with *T. cruzi*, Tulahuen strain or SGO-Z12 isolate, in the chronic indeterminate stage of the infection. As can be observed, 50% of the Tulahuen and 35% of the SGO-Z12 infected mice exhibit electrocardiographic alterations. Both infected groups showed lower cardiac frequency relative to the uninfected controls, and 33.33% of the mice infected with Tulahuen strain and 18.92% of those infected with SGO-Z12 isolate presented auricle–ventricle blockade (AVB), as shown by the prolonged PR segment ($P < 0.05$). Intra-ventricular blockades (IVB) were observed in 14% of the Tulahuen strain-infected mice and 6% of the SGO-Z12-infected mice, as shown by the prolonged Q–T interval ($P < 0.05$). Mice infected with SGO-Z12 also exhibited IVB associated with AVB (8%).

Groups	Cardiac frequency (beats/min)	Interval Q-T (s)	Segment PR (s)	Electric axis (grades)	% Of mice showed electrocardiographic abnormalities
Non infected (n=10)	633.57 ± 10.16 (a)	0.0298 ± 0.0004 (a)	0.0221 ± 0.0004 (a)	56.71 ± 3.34 (a)	10 (a)
Tulahuen 75 d.p.i. (n=10)	540.31 ± 14.55 (b)	0.028 ± 0.0011 (a)	0.03 ± 0.0015 (b)	24.38 ± 8.56 (b)	50 (b)
SGO-Z12 75 d.p.i. (n=10)	579.18 ± 12.12 (b)	0.0324 ± 0.0006 (b)	0.0267 ± 0.001 (c)	57.49 ± 4.09 (a)	35.14 (b)

Table 1. Electrocardiographic results from uninfected mice and mice infected with T. cruzi, Tulahuen strain or SGO-Z12 isolate, in the chronic indeterminate stage of the infection

Discussion

The study of the natural history of Chagas disease demonstrates that 30% of infected people, 10–30 years later, develop signs and symptoms of heart disease to different degrees, which constitutes the cardiac chronic phase of the disease [Elizari, 1999]. Between the acute and this cardiac chronic stage, a clinically silent and long period could be the key to knowing which patients will develop chagasic myocardiopathy and why 70% of infected patients will never develop heart disease [Elizari, 1999]. The study of this phase, called chronic indeterminate or asymptomatic stage, could therefore be important to determine the evolution of Chagas disease. Besides the crucial involvement of mitochondria in myocardial bioenergetic regulation, and in the balance of oxidant and antioxidant agents, suggests that these organelles are the centre of the pathophysiology of the failing heart [Marin Garcia et al 2008].

In the present work even in those mice without ECG alterations the mitochondrial function was modified. When we studied the mitochondrial respiratory chain through the measurement of the specific activity of complex III, we found that the activity of these complex was altered in different way according to the strain used for the infection, but always the activity was significantly reduced not in cardiac and lymphocytes mitochondria.

Enzymes of the Krebs cycle are located in the matrix of the mitochondria; when we studied this cycle functionality by measuring the citrate synthase activity, a significant decrease in the Tulahuen group was detected in cardiac and lymphocytes mitochondria. On the other hand citrate synthase activity of lymphocytes from SGOZ12 mice presented a significantly higher activity.

Present results demonstrates a clear involvement of cardiac and lymphocytes mitochondria in the chronic indeterminate phase of the chagasic infection, which is a silent period of the diseas and that results obtained from the studies of complex III activity allows to propose that with a simple blood extraction and studying lymphocytes mitochondria one could probably infer the cardiac muscle compromise and predict the evolution of the cardiopathy.

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