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# Mitochondrial dysfunction in skeletal muscle during experimental Chagas disease

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ABSTRACT

*Trypanosoma cruzi* invasion and replication in cardiomyocytes and other tissues induce cellular injuries and cytotoxic reactions, with the production of inflammatory cytokines and nitric oxide, both sources of reactive oxygen species. The myocyte response to oxidative stress involves the progression of cellular changes primarily targeting mitochondria. Similar alterations could be taking place in mitochondria from the skeletal muscle; if that is the case, a simple skeletal muscle biopsy would give information about the cardiac energetic production that could be used as a predictor of the chagasic cardiopathy evolution. Therefore, in the present paper we studied skeletal muscle mitochondrial structure and the enzymatic activity of citrate synthase and respiratory chain complexes I to IV (CI–CIV), in Albino Swiss mice infected with *T. cruzi*, Tulahuen strain and SGO Z12 and Lucky isolates, along the infection. Changes in the mitochondrial structure were detected in 100% of the mitochondria analyzed from the infected groups: they all presented at least 1 significant abnormality such as increase in their matrix or disorganization of their cristae, which are probably related to the enzymatic dysfunction.

When we studied the Krebs cycle functionality through the measurement of the specific citrate synthase activity, we found it to be significantly diminished during the acute phase of the infection in Tulahuen and SGO Z12 infected groups with respect to the control one; citrate synthase activity from the Lucky group was significantly increased ($p < 0.05$). The activity of this enzyme was reduced in all the infected groups during the chronic asymptomatic phase ($p < 0.001$) and return to normal values (Tulahuen and SGO Z12) or increased its activity (Lucky) by day 365 post-infection (p.i.). When the mitochondrial respiratory chain was analyzed from the acute to the chronic phase of the infection through the measurement of the activity of complexes I to IV, the activity of CI remained similar to control in Tulahuen and Lucky groups, but was significantly augmented in the SGO Z12 one in the acute and chronic phases ($p < 0.05$). CII increased its activity in Tulahuen and Lucky groups by day 75 p.i. and in SGO Z12 by day 365 p.i. ($p < 0.05$). CIII showed a similar behavior in the 3 infected groups, remaining similar to control values in the first two stages of the infection and significantly increasing later on ($p < 0.0001$). CIV showed an increase in its activity in Lucky throughout all stages of infection ($p < 0.0001$) and an increase in Tulahuen by day 365 days p.i. ($p < 0.0001$); SGO Z12 on the other hand, showed a decreased CIV activity at the same time.

The structural changes in skeletal muscle mitochondria and their altered enzyme activity began in the acute phase of infection, probably modifying the ability of mitochondria to generate energy; these changes were not compensated in the rest of the phases of the infection. Chagas is a systemic disease, which produces not only heart damage but also permanent skeletal muscle alterations.



## 1. Introduction

The causative agent of Chagas disease is the intracellular protozoan parasite *Trypanosoma cruzi*; it affects approximately 20 million people (Moncayo and Silveira, 2009; WHO, 2007) and represents a serious health problem in Central and South America (Biolo et al., 2010). Chagas disease also denotes an increasing challenge for clinicians in the United States (Bern et al., 2007) and some European countries (Reesink, 2005) due to the continuous immigration of people from disease-endemic regions (Polo-Romero et al., 2011).

There are 3 stages in Chagas disease: the acute phase, with a local inflammatory lesions that appears at the site where the metacyclic trypomastigotes enter and the parasite spreads throughout the host organism (Prata, 2001; Umezawa et al., 2002); the cardiac chronic phase in which the diversity and severity of the symptoms range from a mild electrocardiographic alteration to sudden death due to cardiac

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dysrhythmias, varying in different patients and in different regions (Storino and Milei, 1994). In this stage of the disease, the heart is the organ most commonly involved and the dysrhythmias, branch blocks and cardiac heart failure are common symptoms of the 30% of patients that develop chagasic cardiomyopathy (Andrade, 1999; Storino and Milei, 1994). Patients however may also evolve into the digestive form or both cardiac and digestive forms together. Between the acute and the cardiac chronic phases exists a period called the chronic indeterminate stage, which is generally symptomless and may last for 10 to 20 years (Macêdo, 1999; Paglini-Oliva et al., 2012; Ribeiro and Rocha, 1998).

*Trypanosoma cruzi* entrance and replication in the cardiomyocytes cause cellular damage and a cytotoxic reactions, with inflammatory cytokines and nitric oxide production, both of them being source of reactive oxygen species (ROS) in the acute (Cardoni et al., 1997) and cardiac chronic phases of the infection (Talvani et al., 2004). A similar inflammatory response has been described for the chronic indeterminate phase. These responses appear to control parasite reproduction, but it may also have toxic effects upon host cellular components (Ueda et al., 2002).

Previous works from our laboratory (Báez et al., 2008, 2011, 2013) have demonstrated that the myocyte response to oxidative stress involves cellular changes, primarily targeting mitochondria (Long et al., 2004) and modifying therefore the energy supply; this bioenergetic dysfunction could be involved in the genesis and progression of heart failure (Guzmán Mentesana et al., 2010; Marin-García and Goldenthal, 2008; Tsutsui, 2006). We and other authors (Báez et al., 2008, 2011, 2013; Garg et al., 2003; Vyatkina et al., 2004), have demonstrated different structural and functional alterations in cardiac mitochondria isolated from mice infected with different *T. cruzi* strains throughout all stages of the experimental infection (Báez et al., 2013). We also reported the presence of the parasite in the skeletal muscle of these mice (Báez et al., 2013).

The skeletal muscle is a highly oxidative tissue that depends on mitochondria to provide the energy to perform its metabolic activities. Mitochondria represent approximately 39 to 47% of the total volume of the muscle fibers and provide 90% of the energy that skeletal muscle needs for its activity.

Some abnormalities in the function and structure of skeletal muscle fibers had been found with increasing frequency associated with Chagas disease and other pathologies (Iqbal and Hood, 2015).

Chagasic patients present increased glycolytic and reduced oxidative activity; some studies also demonstrated a decrease in type I and an increase in type II fibers (Montes de Oca et al., 2004) or vice versa (Ramirez-Archila et al., 2011).

The entrance of *T. cruzi* into myocardial cells generates an intense inflammatory process with cytokine and free radical production directly affecting mitochondria. In addition, the parasite remains in the host throughout the infection, inducing a chronic inflammatory process of different magnitude in relation to the parasite strain.

Inflammatory infiltrates are frequently observed when the parasite invades the skeletal muscle (Molina et al., 1987; Monteón et al., 1996; Ramirez-Archila et al., 2011) generating myositis, atrophy and necrosis of myofibrils according to the phase of the infection (Acquatella, 2008; Molina et al., 1987; Monteón et al., 1996).

It has been proposed that the functional and structural alterations of cardiac mitochondria during the evolution of *T. cruzi* infection would be involved in the pathophysiologic mechanism of chronic chagasic myocardiopathy (Báez et al., 2008, 2011, 2013) and that similar damage in skeletal muscle mitochondria could be found (Báez et al., 2011; Guzmán Mentesana et al., 2014; Marin-Garcia et al., 1999). If that would be so, a simple skeletal muscle biopsy would give information about the cardiac energetic production that could be used as a predictor of the evolution of the cardiopathy.

Taking this into account, our current study investigates the structure and function of skeletal muscle mitochondria in mice infected with different *T. cruzi* strains in the different stages of the experimental *T. cruzi* infection.

## 2. Materials and methods

### 2.1. Infection

Three month old female and male Swiss Albino mice weighing 30 ± 1 g (n = 120) were used as follows: 30 mice were inoculated, by intraperitoneal injection, with 50 trypomastigote forms of *T. cruzi*, Tulahuen strain, 30 mice with 50 trypomastigote forms of the Lucky isolate and 30 mice with 50 trypomastigote forms of the SGO Z12 isolate. The number of parasites/ml of blood was determined in each group using a Neubauer hemocytometer. A non-infected group (n = 30) was also studied. Parasitemias in all groups were determined in a Neubauer hemocytometer using blood samples obtained from the tail of the mice, twice a week, beginning seven days after the infection. The investigation was carried out according to the Guide for the Care and Use of Laboratory Animals (Tolosa de Talamoni et al., 2010).

### 2.2. Mitochondria isolation

Sections of skeletal muscles from hind legs (rectus femoris, sartorius and vastus medialis and lateralis muscles) were removed on days 35, 75 and 365 post-infection (p.i.), which correspond to the acute, chronic asymptomatic and chronic symptomatic stages of the experimental infection. 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*, 4 °C for 20 min and the supernatant transferred to a new tube. The pellet was resuspended in isolation buffer, homogenized, and centrifuged again at 10,000 *g*, 4 °C for 5 min. The supernatant was discarded and the pellet was resuspended in buffer and centrifuged at 10,000 *g*, 4 °C 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 °C.

### 2.3. Respiratory complex and citrate synthase activity

The enzymatic activity of the respiratory complexes I to IV (CI–CIV) and the citrate synthase was monitored by spectrophotometric methods as previously described (Báez et al., 2008, 2011, 2013; Jarreta et al., 2000; Trounce et al., 1996; Vyatkina et al., 2004) with slight modifications. Protein concentrations were calculated by Bradford assay (Bradford, 1976). All assays were performed in 1 ml final volume with 30–40 mg (for complexes I and II), 20–30 mg (for complex III) and 15 mg (for complex IV) of mitochondrial protein, and the linear change in absorbance was measured for 3 min.

#### 2.3.1. CI (NADH-ubiquinone oxidoreductase)

The reaction mixture consisted of 10 mM Tris–HCl buffer, pH 8.0, 80 μM 2,3-dimethoxy-5-methyl-6-decyl-1,4-benzoquinone (DB), 1 mg/ml BSA, 0.25 mM KCN. After incubating the mitochondria in the reaction mixture at 30 °C for 10 min., oxidation of NADH (200 μM) was monitored at 340 mM (e 8 $mM^{-1}$ $cm^{-1}$).

#### 2.3.2. CII (succinate-ubiquinone oxidoreductase)

Mitochondria were incubated in 1 M potassium phosphate buffer, pH 7.0, containing 0.1 ml succinate phosphate 0.1 M. After addition of assay mixture consisting of 50 μM 2,6-dichlorophenolindophenol (DCPIP), 5 μl EDTA 1 mM, 10 μl of Triton X-100 1%; 50 μl of DB. All the components were mixed. The reduction of DCPIP in association with CII catalyzed DB reduction was measured at 600 nm (e 20.5 × $10^6$ $M^{-1}$ $cm^{-1}$).

*2.3.3. CIII (ubiquinol-cytochrome c oxidoreductase)*

Mitochondria were suspended in 50 mM Tris–HCl buffer, pH 7.4, containing 1 mM EDTA, 250 mM sucrose, 2 mM KCN and 50 μM oxidized cytochrome c. After the addition of 80 μM reduced DB ($DBH_2$), the reduction of cytochrome c was measured at 550 nm (e 19.0 $mM^{-1}$ $cm^{-1}$).

*2.3.4. CIV (cytochrome c oxidase)*

Mitochondria (2 μg protein) were permeabilized in 10 mM Tris–HCl, pH 7.0, 25 mM sucrose, 120 mM KCl, and 0.025% n-dodecyl-b-D-maltoside, and 50 μM reduced cytochrome c added. The oxidation of cytochrome c was measured at 550 nm (e 19.0 $mM^{-1}$ $cm^{-1}$).

*2.3.5. Citrate synthase*

The mitochondrial pellet was added to 100 mM Tris–HCl buffer, pH 8, 0.3 mM acetyl CoA, 100 μM 5,5-dithio-bis-2-nitrobenzoic acid (DTNB). The reaction was initiated by 0.5 mM oxaloacetate. Citrate synthase-catalyzed reduction of acetyl CoA with oxaloacetate in conjunction with DTNB reduction was monitored at 412 nm (e 13.6 $mM^{-1}$ $cm^{-1}$).

*2.4. Histopathological studies*

Sections of skeletal muscles of hind legs (rectus femoris, sartorius and vastus medialis and lateralis muscles) were removed from infected mice on days 35, 75 and 365 p.i., fixed in buffered (pH 7.0) 10% formaldehyde, and embedded in paraffin. Each section of skeletal muscle was cut horizontally into 5 μm sections from the apex to the auricles. The sections were stained with hematoxylin–eosin. A total of 50 slices from each group were analyzed with a 40× objective.

*2.5. Electron microscopy studies*

A 1 mm square section of skeletal muscle from the hind leg was fixed immediately after extraction in a Karnovsky solution (4% formaldehyde and 1.5% glutaraldehyde in 0.1 M cacodylate buffer) for at least 2 h at room temperature. Then the tissues were washed 3 times in cacodylate buffer and postfixed in 1% osmium tetraoxide for 1–2 h. After dehydration in a graded acetone solution (50%, 70% and 90%), the inclusion of samples was performed in epoxy composite mixture of araldite 506 (48.5%), dodecenilsuccinico anhydride (48.5%), dibutilftalato (0.5%) and accelerator dimetilaminobenceno (2.5%). Ultrathin cuts were stained with uranyl acetate and lead citrate and examined in a Zeiss electron microscope. In order to evaluate the changes on mitochondrial morphology observed in the different experimental groups (five micrographs for each mouse), a 4° classification was used:

Grade 1: normal structure.

Grade 2: normal size with dilated cristae.

Grade 3: normal size and/or altered shape. Intact membrane with few cristae. mitochondrial swelling.

*2.6. Statistical analysis*

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

## 3. Results

*3.1. Parasitemia*

Parasitemias were analyzed to assess the infection rate. The Tulahuen and SGO Z12 infected groups showed their highest parasitemia levels by day 14 p.i., with the levels of the SGO Z12 group being significantly lower ($p < 0.01$) than those presented by the Tulahuen group. Parasitemias were negative from day 42 onwards in the Tulahuen-infected mice and from day 49 onwards in the SGO Z12-infected mice. The group infected with the Lucky isolate presented the highest parasitemia levels by day 28 p.i., and became negative by day 49 p.i. (see Fig. 1).

*3.2. Mitochondrial respiratory complexes and citrate synthase activity*

In order to determine the mitochondrial function, we analyzed the Krebs cycle via the enzymatic activity of citrate synthase (matrix) and the respiratory chain complexes I to IV (cristae) in skeletal muscles from non-infected and infected mice (Tulahuen, SGO Z12 and Lucky) on different moments of the infection. As can be observed in Table 1, citrate synthase enzyme activity from Tulahuen and SGO Z12 mice was significantly decreased compared to the non-infected group ($p < 0.05$) at the beginning of the infection (acute and chronic indeterminate phases) and was similar to this control group by day 365 p.i. The behavior of this enzyme in the Lucky infected group was different:

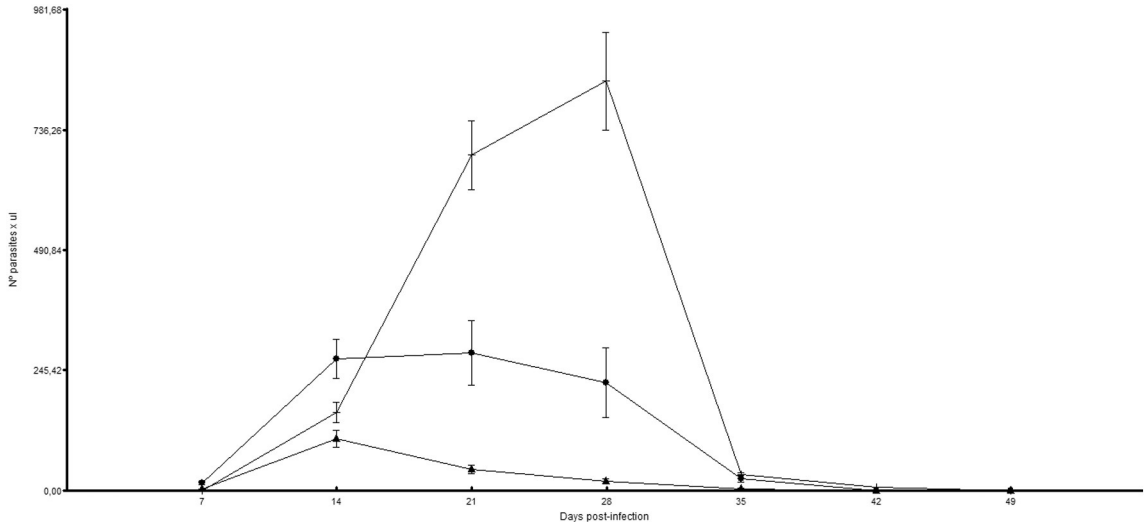


**Fig. 1.** Parasitemia evolution of mice infected with Tulahuen strain (n = 30, ●), SGO Z12 (n = 30, ▲) and Lucky (n = 30, +) isolates.

**Table 1**
Mitochondrial enzymatic activity (μmol $min^{-1}$ $mg^{-1}$ protein) of skeletal muscle obtained from non-infected (n = 30), Tulahuen-infected (n = 30), Lucky-infected (n = 30) and SGO Z12-infected (n = 30) mice at 35, 75 and 365 days post-infection.

| Enzymatic activity (μmol $min^{-1}$ $mg^{-1}$ protein) | Non-infected | Tul 35 d.p.i. | Tul 75 d.p.i. | Tul 365 d.p.i. | Lucky 35 d.p.i. | Lucky 75 d.p.i. | Lucky 365 d.p.i. | Z12 35 d.p.i. | Z12 75 d.p.i. | Z12 365 d.p.i. |
|---|---|---|---|---|---|---|---|---|---|---|
| Citrate synthase ($p < 0.05$) | $1.47 \pm 0.05$ | $0.64 \pm 0.05^{*}$ | $0.08 \pm 0.02$ | $1.94 \pm 0.87^{*}$ | $1.75 \pm 0.05^{*}$ | $0.90 \pm 0.02^{*}$ | $2.95 \pm 0.87^{*}$ | $0.07 \pm 0.05^{*}$ | $0.02 \pm 0.02^{*}$ | $1.40 \pm 0.87^{*}$ |
| Complex I ($p < 0.05$) | $0.03 \pm 0.16$ | $0.03 \pm 0.16$ | $0.01 \pm 0.06$ | $0.01 \pm 0.08$ | $0.02 \pm 0.16$ | $0.17 \pm 0.06$ | $0.10 \pm 0.08$ | $0.39 \pm 0.16$ | $0.01 \pm 0.06$ | $0.26 \pm 0.08^{*}$ |
| Complex II ($p < 0.05$) | $2.1 \times 10^{-7} \pm 1.1 \times 10^{-7}$ | $3.3 \times 10^{-8} \pm 1.1 \times 10^{-7}$ | $7 \times 10^{-8} \pm 7 \times 10^{-8}$ | $2.1 \times 10^{-8} \pm 9.7 \times 10^{-8}$ | $3 \times 10^{-7} \pm 1.1 \times 10^{-7}$ | $5.8 \times 10^{-7} \pm 6.8 \times 10^{-8*}$ | $2.7 \times 10^{-7} \pm 9.7 \times 10^{-8*}$ | $4.5 \times 10^{-9} \pm 1.1 \times 10^{-7}$ | $1.6 \times 10^{-8} \pm 6.8 \times 10^{-8}$ | $8.0 \times 10^{-9} \pm 9.7 \times 10^{-8}$ |
| Complex III ($p < 0.0001$) | $0.01 \pm 0.01$ | $0.01 \pm 0.01$ | $0.10 \pm 0.02$ | $0.50 \pm 0.08^{*}$ | $0.05 \pm 0.01$ | $0.04 \pm 0.02$ | $0.22 \pm 0.08^{*}$ | $0.05 \pm 0.01$ | $2.1 \times 10^{-3} \pm 0.02^{*}$ | $0.13 \pm 0.08^{*}$ |
| Complex IV ($p < 0.0001$) | $0.41 \pm 0.20$ | $1.07 \pm 0.20$ | $0.42 \pm 0.17$ | $1.17 \pm 0.12^{*}$ | $1.59 \pm 0.20^{*}$ | $1.48 \pm 0.17^{*}$ | $1.13 \pm 0.12^{*}$ | $0.19 \pm 0.20$ | $0.14 \pm 0.17$ | $0.14 \pm 0.12^{*}$ |

The results are expressed as mean ± standard error unless otherwise e indicated. (Asterisks (*) indicate significant differences. d.p.i.: days post-infection.).

it remained similar to non-infected values in the acute phase, it significantly decreased later on (chronic indeterminate stage) and presented an important increase in the cardiac chronic phase (see Table 1).

It is interesting to remark that during the chronic indeterminate phase (75 days p.i.) the infection with any parasite strain/isolate caused a significant decrease of the citrate synthase activity when compared with non-infected group ($p < 0.01$).

CI activity remained similar to non-infected values throughout the infection in the Tulahuen and Lucky infected groups, but it was significantly increased both in the acute and chronic phases in the SGO Z12 one ($p < 0.05$). CII enzyme activity increased in the Tulahuen and Lucky groups by day 75 p.i. and in the SGO Z12 by day 365 p.i. ($p < 0.05$).

CIII showed a similar behavior in the three infected groups, remaining similar to non-infected values in the first two stages of the infection and significantly increasing its activity afterwards ($p < 0.0001$). CIV showed an increase in its specific activity in the three phases of the infection in the Lucky infected group ($p < 0.0001$). This increment was also detected in the Tulahuen group but only by day 365 p.i. ($p < 0.0001$); SGO Z12 on the other hand, presented a significantly decreased CIV activity in the chronic phase of the infection (see Table 1).

### *3.3. Skeletal muscle histopathological studies and mitochondrial ultrastructural analysis during the evolution of T. cruzi infection*

Skeletal muscle from Lucky and Tulahuen infected mice presented inflammatory infiltrates and amastigotes nest by day 35 p.i; SGO Z12 infected mice also showed inflammatory infiltrates (Fig. 2B, C, D). During the chronic indeterminate phase the inflammatory infiltrates persisted (Fig. 2F, G, H). By the chronic stage, 365 days p.i., the skeletal muscle from the Lucky and SGO Z12 groups demonstrated inflammatory infiltrates and proliferation of satellite cells; the Tulahuen infected one presented not only inflammatory infiltrates but also amastigote nests (Fig. 2J, L, K).

When the skeletal muscle mitochondrial structure was analyzed, 71% of the mitochondria from the Tulahuen group (Fig. 3D) and 100% of the mitochondria from the SGO Z12 and Lucky groups (Fig. 3B, C) presented at least one significant abnormality, such as an increase in their matrix or disorganization in their cristae, in the acute phase of the infection (35 days p.i.) (see Table 2). Later on (75 and 365 days p.i.), all the mitochondria from the infected groups were altered (Fig. 3F–L) (see Table 2).

## 4. Discussion and conclusion

The globalization of Chagas disease, due to migration from endemic countries to North America, Europe, Asia and Oceania has created a new epidemiological, economic, social and political concern in non-endemic countries (Coura and Borges-Pereira, 2010; Schummis, 2007). The main issue in these non-endemic countries is the risk of transfusional and congenital transmission, while infected triatomines are still the common way of transmission in endemic countries. The determinants of Chagas disease come from several, complex and different factors: quantity of parasites that provoke the infection, *T. cruzi* lineages, specific histotrophic receptors of the host, and patient's initial immune response, among others (Andrade et al., 2006; Bustamante et al., 2003a,b; Coura, 1988; Zingales et al., 2009); despite this, all infected individuals present a symptom or symptomless acute phase that will evolve into the chronic indeterminate phase and about 30% to severe forms of heart disease (WHO, 2011).

For that, there is a real need to identify markers of the beginning and evolution of the tissues damages in order to prevent or reduce the severity of the disease.

We have previously demonstrated that parasite persistence and inflammation are likely to be involved in the structural and functional alterations in cardiac mitochondria from acute, chronic indeterminate and chronically *T. cruzi* infected mice, demonstrating that in chagasic

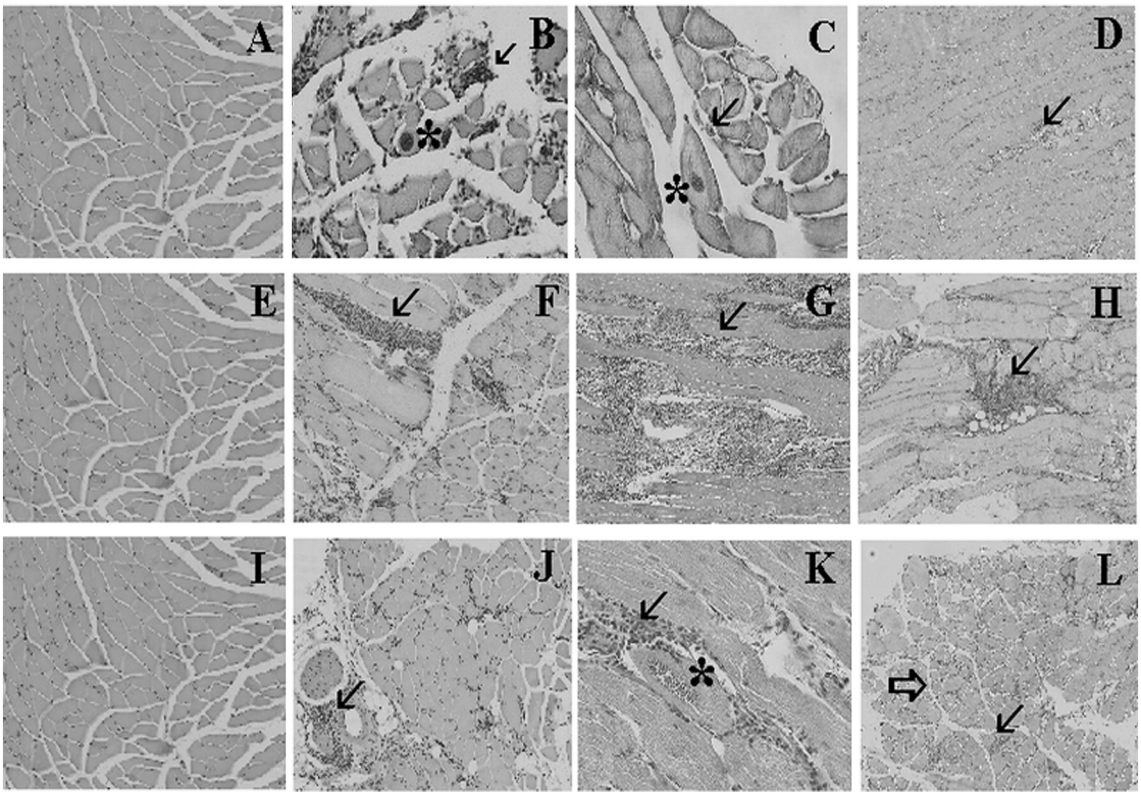


**Fig. 2.** Skeletal muscle histological sections from the different groups: (A, E, I) non-infected mice. 200×. (B) Lucky-infected group, 35 days p.i. 400×. (C) Tulahuen-infected group, 35 days p.i. 600×. (D) SGO Z12-infected group, 35 days p.i. 100×. (F) Lucky-infected group, 75 days p.i. 100×. (G) SGO Z12-infected group, 75 days p.i. 100×. (H) Tulahuen-infected group, 75 days p.i. 100×. (J) Lucky-infected group, 365 days p.i. 400×. (K) Tulahuen-infected group, 365 days p.i. 600×. (L) SGO Z12-infected group, 365 days p.i. 100×. ↙: inflammatory infiltrates; *: amastigote nests; ⇨: satellite cell proliferation.

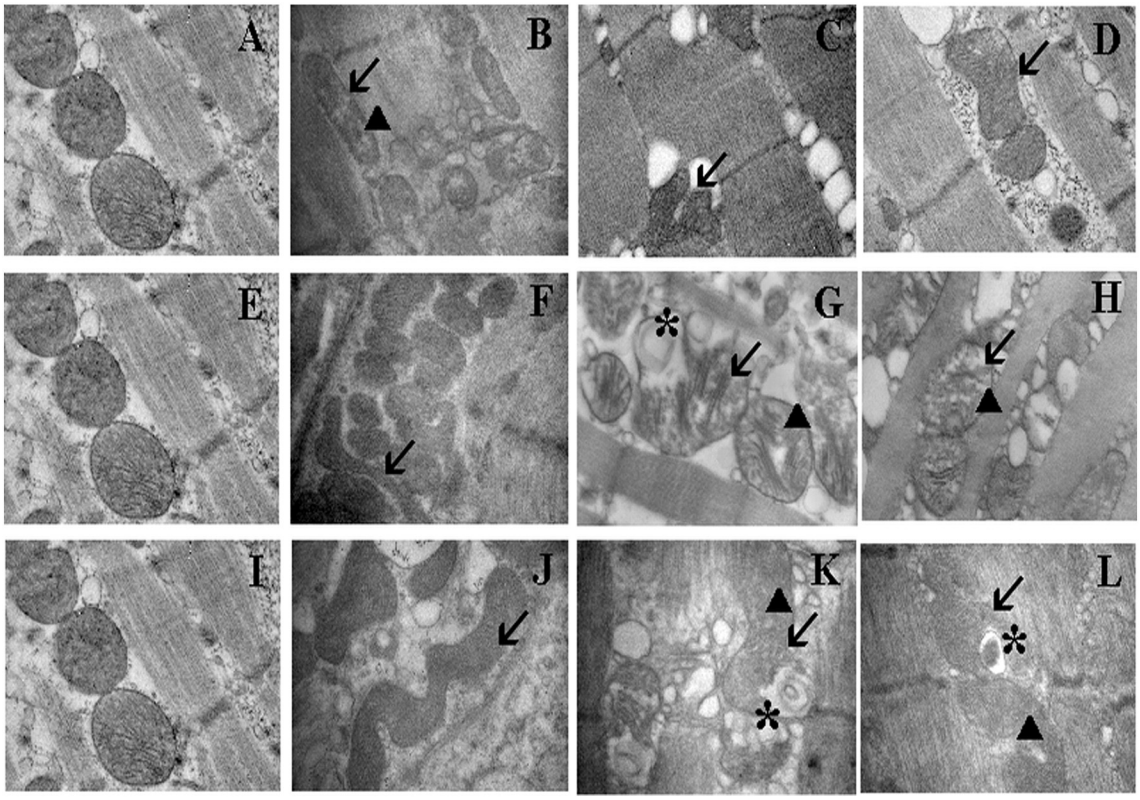


**Fig. 3.** Ultrastructural studies of skeletal muscle mitochondria from the different groups: (A, E, I) non-infected mice 28,700×. (B) Lucky-infected group, 35 days p.i., 27,800×. (C) SGO Z12-infected group, 35 days p.i., 27,800×. (D) Tulahuen-infected group, 35 days p.i., 27,800×. (F) Lucky-infected group, 75 days p.i., 27,800×. (G) SGO Z12-infected group, 75 days p.i., 27,800×. (H) Tulahuen-infected group, 75 days p.i., 27,800×. (J) Lucky-infected group, 365 days p.i., 27,800×. (K) SGO Z12-infected group, 365 days p.i., 27,800×. (L) Tulahuen-infected group, 365 days p.i., 27,800×. ↙: heterogeneous mitochondria; *: vacuolization; ▲: separate cristae.

**Table 2**
Measurements of the morphological features of mitochondria for groups. Non-infected (n = 30 micrographs per mouse), Tulahuen (n = 30 micrographs per mouse), Lucky (n = 30 micrographs per mouse) and SGO Z12 isolation (n = 30 micrographs per mouse) at 35, 75 and 365 d.p.i. (days post-infection).

| Measurements | Non-Infected | Tul 35 d.p.i. | Tul 75 d.p.i. | Tul 365 d.p.i. | Lucky 35 d.p.i. | Lucky 75 d.p.i. | Lucky 365 d.p.i. | Z12 35 d.p.i. | Z12 75 d.p.i. | Z12 365 d.p.i. |
|---|---|---|---|---|---|---|---|---|---|---|
| Total area occupied by mitochondria ($\mu m^2$) | 812.54 | 959.12 | 164.53 | 1181.56 | 918.42 | 637.98 | 932.97 | 626.65 | 440.22 | 846.16 |
| Average mitochondrial area ($\mu m^2$) $P < 0.0001$ | 822.83 ± 111.31 | 1161.57 ± 145.49 | 137.35 ± 14.38 | 1167.61 ± 186.66 | 704.52 ± 112.18 | 500.15 ± 42.48 | 676.39 ± 83.87 | 529.47 ± 60.38 | 360.17 ± 26.16 | 924.09 ± 108.81 |
| Mean diameter (μm) $P < 0.05$ | 90.67 ± 6.57 | 101.18 ± 8.77 | 29.09 ± 1.50 | 92.48 ± 7.12 | 74.57 ± 5.21 | 61.76 ± 2.45 | 75.33 ± 4.17 | 72.82 ± 5.08 | 58.90 ± 2.43 | 88.17 ± 4.97 |
| Grade of alteration | 83% G 1 | 29% G1 | 0% G1 | 0% G1 | 0% G1 | 0% G1 | 0% G1 | 0% G1 | 0% G1 | 0%G1 |
| | 17% G 2 | 67% G2 | 18% G2 | 19% G2 | 4% G2 | 36% G2 | 37% G2 | 28% G2 | 22% G2 | 25%G2 |
| | 0% G 3 | 4% G3 | 82% G3 | 81% G3 | 96% G3 | 64% G3 | 62% G3 | 78% G3 | 78% G3 | 74%G3 |
| Total number of mitochondria | 25 | 24 | 169 | 47 | 23 | 73 | 43 | 14 | 69 | 32 |
| Average number of mitochondria per micrograph $P < 0.05$ | 5.00 ± 1.48 | 4.80 ± 1.02 | 33.80 ± 6.06 | 9.40 ± 1.21 | 4.60 ± 1.03 | 14.60 ± 2.48 | 6.40 ± 0.75 | 2.80 ± 0.37 | 13.80 ± 3.07 | 8.60 ± 1.44 |

The results are expressed as mean ± standard error unless othe in %. n = 30.

cardiopathy the parasite strain determines different mitochondrial changes that are involved in the pathophysiology of heart failure and could be important predictors of the evolution of cardiopathy (Báez et al., 2008, 2011, 2013).

Additionally, few studies have shown that the skeletal muscle contractile activity is significantly reduced in patients during the acute stage of the disease (Montes de Oca et al., 2004; Ramirez-Archila et al., 2011) and that this could be reversed by regeneration of muscle fiber through satellite cells (Maldonado et al., 2004).

We have previously demonstrated the presence of *T. cruzi*, in the form of amastigote nests, in the skeletal muscle samples from Lucky- and Tulahuen-infected mice in the acute (Lo Presti et al., 2014) and chronic (Báez et al., 2011) phases of the infection. The skeletal muscle samples from the SGO Z12-infected group presented only inflammatory infiltrates. These results confirm the presence and persistence of the parasite throughout all stages of the infection and demonstrate that the skeletal muscle is a reservoir for the parasite. The inflammatory infiltrates described are probably the result of successful evasive strategies, such as the release of proteases that activate TGF-β, that allow the parasite to persist in host tissues throughout the infection (Araujo Jorge et al., 2008; Maya et al., 2010; Waghabi et al., 2009). Additionally, phagocytosis and apoptotic bodies originated from T cells or neutrophils induce a prostaglandin-dependent production of TGF-β in macrophages. All this provokes a diminished nitric oxide production and the INF-γ induced inflammatory response is attenuated; consequently, the parasite proliferates (Gutierrez et al., 2009). All of these changes that have no clinical evidence are generating cellular structural damage that can affect cardiac mitochondria either at or near the inflammatory formation (Báez et al., 2013; Tsutsui, 2006).

Skeletal muscle is a tissue affected by this disease due to its metabolic activity and is highly oxidative and dependent on the energy generated by mitochondria for all cellular functions, especially contractility. An important part of this energy is obtained from the oxidative phosphorylation, the beta-oxidation of fatty acids and the electron transport respiratory chain, all processes that take place in mitochondria.

Under normal physiological conditions, the main source of ROS is the mitochondrial electron transport chain, in which oxygen can activate superoxide radicals by non-enzymatic processes. This basal ROS generation occurs under normal metabolism when electrons are being transported from the electron transport chain to complexes I, III and IV. Evidence shows that semiquinones generated within complexes I and III are the main electron donors to oxygen molecules, providing a constant source of superoxide (Marin-García, 2005).

Mitochondrial ROS generation can be augmented in cells with an abnormal respiratory chain function, as described by other authors (Marin-García and Goldenthal, 2008) and sustained by the present results. Under these pathophysiological conditions, inflammatory mediators provoke an increase in ROS levels, which mainly affect mitochondrial function.

Increased levels of oxidative stress, associated with alterations in mitochondrial metabolic functions, have been shown in blood from patients with positive serology for Chagas disease (Wen et al., 2006). Studies on oxidant/antioxidant state in infected mice showed a moderate increase of antioxidant 3-nitrotyrosine in the skeletal muscle in the acute and chronic stages of the disease. Also during the acute phase, carbonylation increases in the skeletal muscle (Wen et al., 2008). The antioxidant enzymes catalase and glutathione peroxidase have been found to increase their activity in the skeletal muscle in 72 and 113% respectively in the acute phase; these changes however were normalized by the chronic stage (Wen et al., 2008). These authors found no changes in the activity of the enzymes superoxide dismutase and Mn-dependent superoxide dismutase which remained at normal levels in the skeletal muscle but altered in other tissues, such as the cardiac muscle and the blood.

Mitochondria can therefore neutralize ROS by forming antioxidant enzymes; in long-term aggression however, as in the chronic phase of Chagas disease, homeostatic control becomes insufficient, particularly if mitochondrial structure and function are damaged, as has been found in the present results.

In this paper we analyzed the effect of the infection with one strain of *T. cruzi* (Tulahuen) and two natural isolates (SGO Z12 and Lucky) upon the skeletal muscle and the structure and function of skeletal muscle mitochondria, in the acute, chronic asymptomatic and chronic symptomatic stages.

Inflammatory infiltrates were present in the skeletal muscle of the infected mice (with either strain/isolate) all along the experimental infection; furthermore, amastigote nests were found in the skeletal muscles form mice infected with Lucky or Tulahuen in the acute phase. This last group also showed amastigote nests by day 365 p.i., demonstrating the persistence of parasite in theses tissues even in the chronic phase of the *T. cruzi* infection (Bazán et al., 2012).

The mitochondrial structural results, using a 3-grade classification of mitochondrial damage, showed that 100% of the mitochondria from the infected groups presented at least one significant abnormality, such as an increase in their matrix or disorganization in their cristae, throughout the evolution of the infection, abnormalities that are probably related to the enzymatic dysfunction. These results show that mitochondria are affected from the beginning of the infection and the production of energy by the organelle is probably also compromised. Similar mitochondrial alterations were described for the cardiac muscle in Tulahuen and SGO Z12 infected groups (Báez et al., 2008, 2011, 2013).

Additionally, we demonstrated functional changes in the mitochondria from the skeletal muscle of different degrees according to the parasite strain that infected the host and the moment of infection (alterations were observed however, from day 35 to day 365 p.i.).

When we studied the Krebs cycle functionality through the measurement of the activity of the enzyme citrate synthase in Tulahuen and SGO Z12 infected groups, we found it to be significantly reduced with respect to the control one, during the acute phase of infection (35 days p.i.); the activity of this enzyme however was significantly increased in the Lucky group. Later on the infection, during the chronic asymptomatic phase, the activity of this enzyme was reduced in all the infected groups, and by day 365 p.i. it returned to normal values (Tulahuen and SGO Z12 groups) or increased its activity (Lucky). These findings can be related to the different alterations found in the mitochondrial matrix described for the experimental groups, probably induced by the inflammatory infiltrates of different magnitude found in these groups, according to the different parasite strains and the stage of the infection. Other authors (Montes de Oca et al., 2004) found that the activity of the enzyme citrate synthase decreased, and proposed that these results are favoring anaerobic metabolism.

When the mitochondrial respiratory chain was analyzed from the acute to the chronic phase of the infection through the measurement of the specific activity of complexes I to IV, we found that the activity of CI remained similar to control throughout the infection in the groups infected with Tulahuen and Lucky; in the SGO Z12 group it significantly increased in the acute and chronic phases of the infection. CII presented a significant increase in the groups infected with Tulahuen and Lucky by day 75 p.i. and in the group infected with SGO Z12 by day 365 p.i. CIII showed a similar behavior in the three infected groups, remaining similar to control values in the first two stages of the experimental infection and significantly increasing later on. CIV in turn showed an increase in its specific activity in the three phases of the infection in the group infected with Lucky; in the group infected with Tulahuen this increase was detected by day 365 p.i. The SGO Z12 group on the other hand, presented a significant decrease in CIV activity by this time.

Different alterations in the complexes of the respiratory chain from cardiac mitochondria, related to the parasite strain that infected the host and to the phase of the infection, have been previously reported (Báez et al., 2008, 2011, 2013). All these results indicate that CIII is the mainly damaged complex in cardiac mitochondria. This is not only related to infectious cardiac diseases but also to different heart failure diseases (Guzmán Mentesana et al., 2014; Marin-García et al., 2013; Tsutsui, 2006; Tsutsui et al., 2001).

We have previously demonstrated (Guzmán Mentesana et al., 2014) that ROS levels are augmented in both cardiac and skeletal muscles and that mitochondria from both tissues are compromised in patients with heart failure. Heart failure is characterized by hemodynamic adaptations, sympathetic overdrive, activation of immune response with systemic inflammation and a catabolic state; consequently, enzymatic and mitochondria abnormalities are often found in the skeletal muscle which can lead to limitations in the movements (Kinugawa et al., 2005; Palaniyandi et al., 2010). Chagas disease is a cause of heart failure and the inflammatory infiltrates that are found in the heart and the skeletal muscle throughout the infection (Báez et al., 2008, 2011, 2013) can consequently increase ROS levels.

Protein modifications induced by oxidative stress have been found in the myocardium of *T. cruzi*-infected animals (Dhiman et al., 2008; Wen and Garg, 2004; Wen et al., 2004) and in the peripheral blood of seropositive chagasic patients (De Oliveira et al., 2007; Wen et al., 2006). This peripheral oxidative stress was associated with a poor glutathione antioxidant defense and an increased in myeloperoxidase activity and ROS production (Dhiman et al., 2012). *T. cruzi* infection also causes a significant fall in mitochondrial membrane potential; ROS-induced oxidation of mitochondrial membranes may also affect its potential and consequently the respiratory chain efficiency (Gupta et al., 2009).

The inflammatory pathology varies along the different stages of the infection. The mitochondrial ROS release due to electron transport chain dysfunction and enhanced release of electrons to molecular oxygen has been demonstrated to be the primary source of oxidative stress (Wen and Garg, 2008) causing a permanent aggression not only to the cardiac cells but also to the skeletal muscle cells as demonstrated in the present study.

Our results have shown a direct relationship between mitochondrial alterations and the progression of Chagas disease, a fact that is correlated with similar changes found in the heart (Báez et al., 2008, 2011, 2013). This allows us to relate the imbalance in Krebs cycle and oxidative phosphorylation with the onset and progression of pathology, demonstrating that mitochondria are involved in the physiopathology of Chagas disease.

The structural skeletal muscle mitochondrial changes and the altered enzyme activity begin in the acute phase of the infection, probably modifying the ability of mitochondria to generate energy; these alterations are not compensated in the rest of the phases of the infection. Chagas disease is a systemic infection, which causes not only heart damage but also permanent skeletal muscle alterations.

## Conflict of interest statement

The authors declare that they have no competing interests.

## Authors' contributions

BAL, RMN and P-OP participated in the study conception, design, case selection and experiments. BAL, RMN, SM and BPC carried out the data collection. P-OP, LMS, PP, MN and RHW performed the data analysis and writing of the manuscript. All the authors read and approved the final manuscript.

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