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Mitochondrial involvement in chronic chagasic cardiomyopathy

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ABSTRACT

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. Here we studied the structure and function of cardiac mitochondria in chronic chagasic myocardiopathy. Cardiac mitochondrial structure and enzyme activity of citrate synthase and complexes I to IV of the respiratory chain were studied in albino Swiss mice infected with *Trypanosoma cruzi* (Tulahuen strain or SGO Z12 isolate) on 365 days post-infection (dpi). The presence of parasites in cardiac and skeletal muscle was also investigated. The activity of complexes I to IV was altered in different ways, according to the strain employed ($P < 0.0001$), in relation to the cristae disorganisation and the parasite persistence found in the Tulahuen group, and the chronic inflammatory process described in both groups; citrate synthase activity also increased in both infected groups. Changes in mitochondrial structure were detected in 89% of Tulahuen- and 58% of SGO Z12-infected mice. In this paper we demonstrate that parasite persistence and inflammation are likely to be involved in the structural and functional alterations in cardiac mitochondria from chronically *T. cruzi*-infected mice, demonstrating that the parasite strain determines different mitochondrial changes in chagasic cardiopathy.

1. Introduction

Heart failure is a highly prevalent chronic disease that results in varying degrees of functional alteration. Among the different aetiologies, chagasic cardiopathy appears to carry the worst prognosis.[1] It is produced by the intracellular protozoan parasite *Trypanosoma cruzi*, and has become the most frequent cause of heart failure and sudden death, as well as the most common cause of cardioembolic stroke in Latin America.[2,3]

The acute phase of the disease may progressively evolve into different chronic clinical forms: the indeterminate form, in which positive serologic tests are present for a long period without clinical manifestations, and the cardiac form, which is an inflammatory cardiomyopathy that develops in over 30% of infected people.[4] A significant number of these patients develop a dilated cardiopathy with fatal outcome. The remainder, i.e. those with cardiopathies

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of different prognosis, have a physiopathology that is still not well understood.[5]

The pathogenesis of chronic chagasic cardiopathy is still a matter under intense discussion; there is considerable evidence that the inflammatory process is a significant effector of cardiac damage.[6–8]

Inflammatory cytokines are produced in the chronic stage of Chagas disease, probably in response to parasite presence. Mononuclear cells increase their cytokine production (increased plasma levels of TNF-α and IFN-γ).[9,10] C-reactive protein and IL-6 are also considered to be potential markers of *T. cruzi*-induced myocardial damage.[11–13] These cytotoxic reactions are a common source of reactive oxygen species (ROS), which have a direct effect on cardiac cellular structure and function, and chronic exposure to these species may be related to heart remodelling and failure. They can also damage cardiac mitochondria, modifying the energy supply; this bioenergetic dysfunction could be involved in the genesis and progression of heart failure.[14,15]

Previous works from our laboratory (Universidad Nacional de Córdoba, Argentina) have demonstrated different structural and functional alterations in cardiac mitochondria isolated from mice infected with different *T. cruzi* strains in the acute stage.[16] Other authors have also demonstrated cardiac mitochondrial alterations in the acute and chronic phases of the infection.[17,18]

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. Taking this into account, our current study investigates the structure and function of cardiac mitochondria produced by different *T. cruzi* strains, in order to determine their involvement in the pathophysiologic mechanism of chronic chagasic myocardiopathy.

2. Materials and methods

2.1. Infection

Thirty 3-month-old female and male albino Swiss mice weighing 30 ± 1 g were used. Ten mice were inoculated with 50 trypomastigote forms of *T. cruzi*, Tulahuen strain (Tulahuen), and ten mice with 50 trypomastigote forms of *T. cruzi*, SGO Z12 isolate (SGO Z12), by intraperitoneal injection. 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. The principal differences between the parasite strains employed are that Tulahuen is a strain that has widely been used in different laboratories and has a well-defined behaviour, whereas the SGO Z12 isolate was obtained from a patient from an endemic area and has only been used and described in our laboratory.[16,19,20]

Parasitaemias in all groups were determined in a Neubauer haemocytometer using blood samples obtained from the tails of the mice once a week, beginning 7 days post-infection (dpi). The investigation was carried out according to the *Guide for the Care and Use of Laboratory Animals*.[21]

2.2. Mitochondria isolation

Hearts were removed on 365 dpi, which corresponds to the chronic phase of the experimental infection, obtaining both ventricles.[22] 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* at 4 °C for 20 min, and the supernatant transferred to a new tube. The pellet was re-suspended in isolation buffer, homogenized and centrifuged again at 10 000 *g* at 4 °C for 5 min. The supernatant was discarded and the pellet was re-suspended in buffer and centrifuged at 10 000 *g* at 4 °C for 10 min (twice = purification). The mitochondrial pellet was re-suspended in isolation buffer (tissue/buffer ratio, 1:1, w/v), and the aliquots stored at −80 °C.

2.3. Respiratory complexes and citrate synthase activity

Respiratory complexes I to IV and citrate synthase activities were monitored individually by spectrophotometric methods as previously described[16,18] with slight modifications (see below for details). Protein concentrations were calculated using the Bradford assay.[18,23–25]

Individual assays for each complex and citrate synthase were performed in 1 ml final volume with 30–40 mg (for complexes I and II), 20–30 mg (for complex III and citrate synthase) and 15 mg (for complex IV) of mitochondrial protein. The linear change in absorbance was measured for 3 min. Assays were performed as follows.

2.3.1. Complex I (NADH-ubiquinone oxidoreductase)

Mitochondria were incubated in the reaction mixture [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] at 30 °C for 10 min. Oxidation of NADH (200 μM) was monitored at 340 nm (ε 8 mM^{-1} cm^{-1}).

2.3.2. Complex II (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 [50 μM 2,6-dichlorophenolindophenol (DCPIP), 5 μl EDTA 1 mM, 10 μl Triton X-100 1%; 50 μl DB] all the components were mixed. The reduction of DCPIP, in association with complex II-catalysed DB reduction, was measured at 600 nm (ε 20.5×10^6 mM^{-1} cm^{-1}).

2.3.3. Complex III (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 oxidised cytochrome *c*. After the addition of 80 μM reduced DB (DBH_2), reduction

of cytochrome *c* was measured at 550 nm (ε 19.0 mM^{-1} cm^{-1}).

2.3.4. Complex IV (cytochrome c oxidase)

Mitochondria were permeabilised in 10 mM Tris-HCl, pH 7.0, 25 mM sucrose, 120 mM KCl and 0.025% n-dodecyl-β-D-maltoside, and 50 μM reduced cytochrome *c* were added. The oxidation of cytochrome *c* was measured at 550 nm (ε 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-catalysed reduction of acetyl CoA with oxaloacetate in conjunction with DTNB reduction was monitored at 412 nm (ε 13.6 mM^{-1} cm^{-1}).

2.4. Histopathological studies

The heart and the skeletal muscle samples from the posterior limbs of the mice were removed on 365 dpi, fixed in buffered (pH 7.0) 10% formaldehyde and embedded in paraffin. Each sample was cut into 5 μm sections (in the case of the hearts, the cuts were horizontally from the apex to the auricles). The sections were stained with haematoxylin and eosin. Fifty slices from each group were analysed with a 40× objective.

2.5. Electron microscopy studies

A section 1 mm^2 from the tip of the left ventricle 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. The tissues were then washed three times in cacodylate buffer and post-fixed in 1% osmium tetroxide for 1–2 h. After dehydration in a graded acetone solution (50, 70 and 90%), the sample inclusion was performed in a mixture of araldite 506 epoxy composite (48.5%), dodecenyl succinic anhydride (48.5%), dibutyl phthalate (0.5%) and dimethyl amino benzene accelerator (2.5%). Ultrathin cuts were stained with uranyl acetate and lead citrate and examined on a Zeiss electron microscope.

In order to evaluate the changes of mitochondrial morphology observed in the different experimental groups (five micrographs for each mouse), a four-degree 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; Grade 4, mitochondrial swelling.

The three-dimensional studies were carried out using the Femtoscan program (Advanced Technology Center, Moscow, Russia).

2.6. Statistical analysis

Results are shown as mean ± standard error. The data obtained were analysed by ANOVA (Fisher test), MANOVA (Hotelling's test corrected by Bonferroni) and χ^2 test for categorical variables. Axiovision 3.0 software (Carl Zeiss MicroImaging GmbH, Germany) was used to quantify mitochondria. The significance level was set at $P < 0.05$ for all cases.

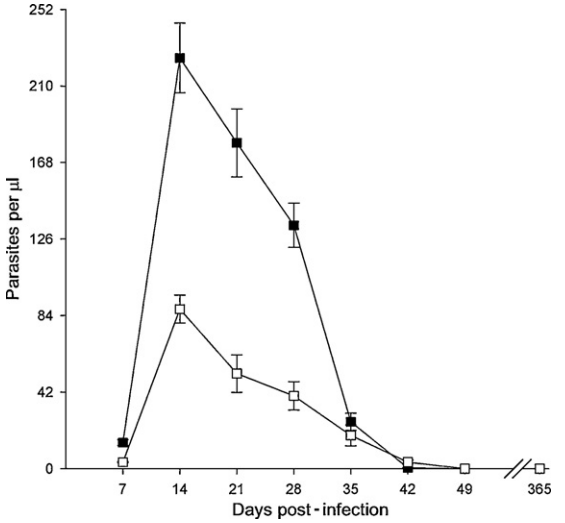

Figure 1. Parasitaemia evolution up to 365 days post-infection. Black squares: Tulahuen-infected mice ($n = 10$); white squares: SGO Z12-infected mice ($n = 10$).

3. Results

3.1. Parasitaemia

Parasitaemias were analysed to assess the infection rate. Both infected groups showed their highest parasitaemia levels by 14 dpi, with the levels of the SGO Z12 group being significantly lower ($P < 0.01$) than those presented by the Tulahuen group. Parasitaemias were negative from day 42 onwards in the Tulahuen-infected mice and from day 49 onwards in the SGO Z12-infected mice (see Figure 1).

3.2. Cardiac histopathological studies and mitochondrial ultrastructural analysis during chronic Trypanosoma cruzi infection

Hearts from the Tulahuen-infected group extracted at 365 dpi presented fibre fragmentation, whereas hearts from the SGO Z12-infected group showed fibre fragmentation and mononuclear inflammatory infiltrates (lympho-monocitary cells) of 10 cells or fewer each (Figure 2).

The structural results were analysed using a four-grade classification of mitochondrial damage. At 365 dpi, 89% of the mitochondria from the Tulahuen-infected mice presented at least one significant abnormality (grade 2 and 3), such as an increase in their matrix or disorganization in their cristae, compared with the non-infected group (Figure 2). Similar alterations were detected in 58% of the mitochondria (grade 2 and 3) from the SGO Z12-infected group, showing a considerable reduction in their diameter ($P < 0.05$) and a significant reduction of the area occupied by mitochondria ($P < 0.01$) (See Figure 2 and Table 1). Figure 3 shows a three-dimensional view of cardiac

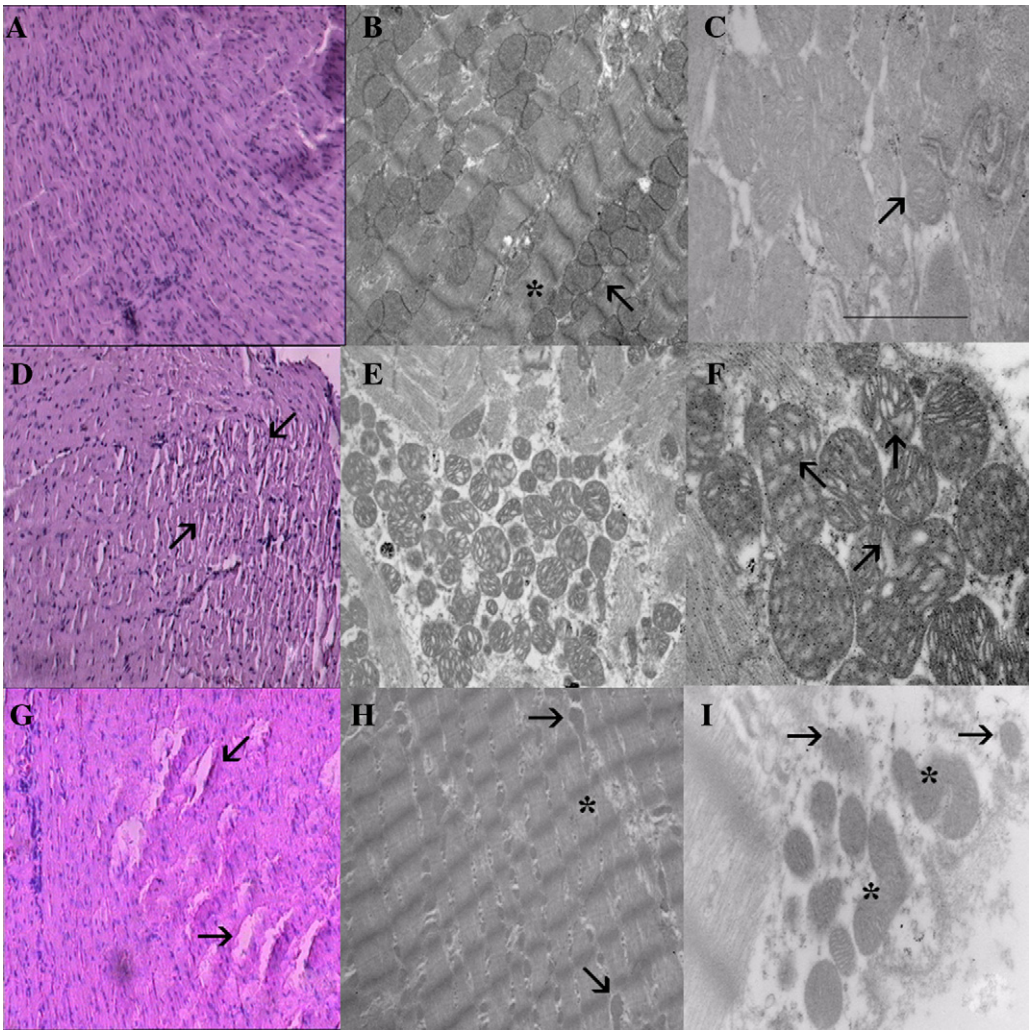

Figure 2. (A) Cardiac histological sections from non-infected mice, 100×. (B) Cardiac ultrastructure from non-infected mice in which sarcomere (asterisk) and mitochondria (arrow) can be observed, 10 000×. (C) Cardiac ultrastructure from non-infected group in which mitochondria (arrow) can clearly be observed, 27 800×, scale bar = 2.2 μm. (D) Cardiac histological sections from Tulahuen-infected group (50 trypomastigotes of *Trypanosoma cruzi* per animal), 365 days post-infection (dpi) showing fibre fragmentation (arrow), 100×. (E) Ultrastructure of cardiac mitochondria from Tulahuen-infected group showing separate cristae with increase of matrix volume, 10 000×. (F) Ultrastructure of cardiac mitochondria from Tulahuen-infected group, 365 dpi; modifications of the internal cristae can be observed (dilation) (arrows), 27 800×. (G) Hearts from the isolate SGO Z12-infected group (50 trypomastigotes of *T. cruzi* per animal), 365 dpi, showing mononuclear cell infiltrates and fragmentation of muscle fibres (arrow), 100×. (H) Ultrastructure of cardiac tissue from the isolate SGO Z12-infected group, 365 dpi, showing sarcomere (asterisk) and mitochondria (arrow), 10 000×. (I) Ultrastructure of cardiac tissue from the isolate SGO Z12-infected group showing small mitochondria (arrow) with different morphology (asterisks), 27 800×.

Table 1
Results of ultrastructural analysis of cardiac mitochondria of non-infected ($n = 5$ micrographs per mouse), Tulahuen-infected ($n = 5$ micrographs per mouse) and SGO Z12-infected ($n = 5$ micrographs per mouse) mice at 365 days post-infection (dpi)

Measurements	Non-infected ($n = 5$)	Tulahuen 365 dpi ($n = 5$)	SGO Z12 365 dpi ($n = 5$)
Total area occupied by mitochondria (μm^2)	36 152.02	35 422.28	11 011.6
Average mitochondrial area (μm^2)	903.80 ± 76.48	885.55 ± 94.37	256.1 ± 39.30*
Mean diameter (μm)	6.64 ± 0.44	5.65 ± 0.4	3 ± 0.28*
Grade of alteration	80% grade 1	11% grade 1	41.9% grade 1
	20% grade 2	63% grade 2	46.5% grade 2
	0% grade 3	26% grade 3	11.6% grade 3
	0% grade 4	0% grade 4	0% grade 4
Total number of mitochondria in 5 micrographs per mouse	40	41	43
Average number of mitochondria per micrograph	8 ± 2.30	8.2 ± 0.55	8.6 ± 1.69

The results are expressed as mean ± standard error unless otherwise indicated.
* $P < 0.05$ within each row.

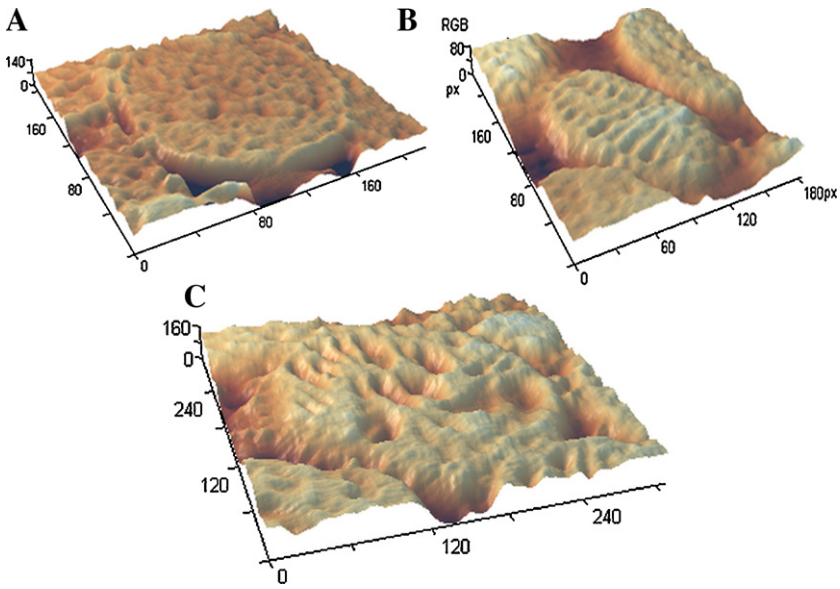

Figure 3. Three-dimensional view of cardiac mitochondria obtained from (A) non-infected, (B) SGO Z12-infected and (C) Tulahuen-infected mice. A clear reduction in the size of the mitochondria can be observed in the SGO Z12-infected group and an increase in the matrix and disorganisation of the cristae in the Tulahuen-infected group. The measurements were made in pixels.

mitochondria obtained from non-infected, SGO Z12-infected and Tulahuen-infected mice. A clear reduction in mitochondrial size can be observed in the SGO Z12-infected group and an increase in the matrix and disorganization of the cristae in the Tulahuen-infected group.

Figure 4A shows an amastigote nest in the skeletal muscle obtained from the Tulahuen-infected group and Figure 4B shows inflammatory infiltrates surrounding a vascular structure from the skeletal muscle of SGO Z12-infected mice. At the same time no parasites were detected in the hearts by this technique in either of the groups analysed.

3.3. Mitochondrial respiratory complexes and citrate synthase activity

In order to assess mitochondrial function, we analysed the enzymatic activity of the mitochondrial respiratory chain complexes I to IV (cristae) and citrate synthase (matrix) in the myocardium from uninfected, Tulahuen-infected and SGO Z12-infected mice, at 365 dpi. As can be observed in Table 2, at 365 dpi, the citrate synthase enzyme activity from the SGO Z12-infected mice was increased compared with the control group ($P < 0.05$); there were no significant differences between parasite strains (the increase in the Tulahuen group was not significant).

Complex I activity significantly decreased in the SGO Z12-infected group compared with the control group ($P < 0.05$); in the Tulahuen-infected group the diminution in the activity of this complex was not significant. Complex II enzyme activity decreased in both infected groups by 365 dpi ($P < 0.05$), this decrease was significantly higher in the SGO Z12-infected group. Complex III enzyme activity significantly diminished with infection with either parasite strain ($P < 0.001$). Complex IV showed an increase in its

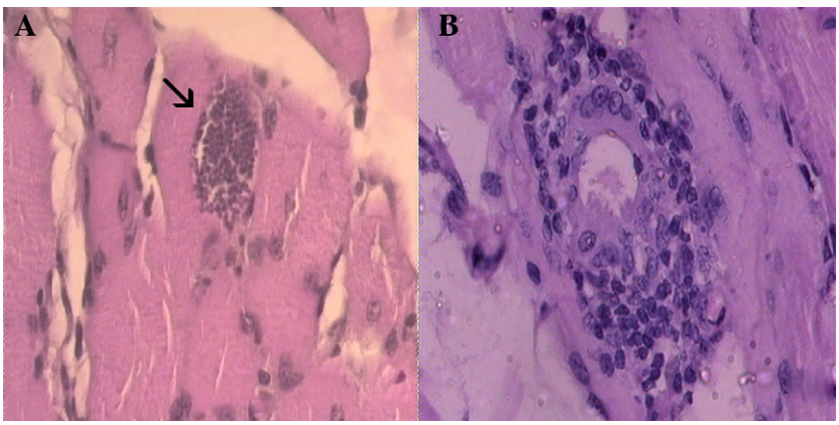

Figure 4. (A) Skeletal muscle from Tulahuen-infected mice (365 days post-infection) showing an amastigote nest (arrow), 400×. (B) Skeletal muscle from SGO Z12-infected mice showing perivasculitis, 400×.

Table 2
Mitochondrial enzymatic activity (μmoles min^{-1} mg^{-1} protein) of hearts obtained from non-infected ($n = 10$), Tulahuen-infected ($n = 10$) and SGO Z12-infected ($n = 10$) mice at 365 days post-infection

Enzymatic activity (μmoles min^{-1} mg^{-1} protein)	Non-infected	Tulahuen	SGO Z12	*P*-value
Citrate synthase	290 ± 40 (A)	360 ± 70 (A,B)	470 ± 60 (B)	A vs B 0.05
Complex I	40 ± 20 (B)	20 ± 10 (A,B)	10 ± 1 (A)	A vs B 0.05
Complex II	$1.1 \times 10^{-6} \pm 5.7 \times 10^{-8}$ (C)	$4.1 \times 10^{-7} \pm 2.8 \times 10^{-4}$ (B)	$9.2 \times 10^{-8} \pm 2.4 \times 10^{-4}$ (A)	A vs B vs C 0.05
Complex III	170 ± 30 (B)	40 ± 10 (A)	60 ± 20 (A)	A vs B 0.001
Complex IV	110 ± 4.4 (A)	1030 ± 110 (C)	590 ± 110 (B)	A vs B, A vs C 0.01 C vs B 0.05
Comparison of all variables (Hotelling's test corrected by Bonferroni)	(A)	(B)	(C)	A vs B vs C 0.0001

The results are expressed as mean ± standard error unless otherwise indicated.

specific activity during chronic infection in both Tulahuen- and SGO Z12-infected groups ($P < 0.01$), with the values from the Tulahuen group being significantly higher than those presented for the SGO Z12 group ($P < 0.05$). Results are summarized in Table 2.

Despite the fact that only complexes II and IV were found to be different between the parasite strains when the variables were compared one to one (ANOVA by Fisher's test), when we took into consideration all the variables together (Hotelling's test corrected by Bonferroni), both strains were found to be significantly different ($P < 0.0001$).

4. Discussion

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.[14] Cardiac mitochondria are also targets for several endogenous and exogenous offenses that might affect their function[26]; for example, the cardiopathy induced by the hepatitis C virus increases the number of mitochondria in transgenic mice[26] and the human septic shock provokes myocardial contractile dysfunction and derangement of mitochondrial cristae.[27]

In addition, myocarditis is described as an inflammatory process that affects the myocardium in response to various chemical, physical or infectious agents. In undeveloped countries, the infectious aetiology is the most frequent. The inflammatory process can occur during or after a variety of infectious diseases, by either direct invasion, production of toxins or as a consequence of immunologically mediated mechanisms.[28,29] Chronic chagasic cardiopathy is the consequence of one of these infectious diseases. The common result of all myocarditis, regardless of aetiology, is heart failure.

In the present paper we demonstrated the presence of the parasite in the chronic stage of the chagasic infection in the form of *T. cruzi* nests in the skeletal muscle samples from the Tulahuen-infected mice. The samples from the SGO Z12-infected group presented inflammatory infiltrates in both tissues. These results confirm the persistence of a chronic cardiac inflammatory process, which has a direct effect upon cardiac mitochondria.

Every cell type, including cardiomyocytes, is capable of generating ROS; mitochondria, xanthine oxidase and NADPH oxidase are the major sources. 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. Mitochondrial ROS generation can be augmented in cells with an abnormal respiratory chain function, as described in the present results.[14] Under these physiopathological conditions, inflammatory mediators provoke an increase in ROS levels, which mainly affect mitochondrial function. Mitochondria from cardiomyocytes, however, can neutralise ROS by forming antioxidant enzymes, but when the aggression is so long, as in chronic chagasic myocarditis, homeostatic control becomes insufficient, especially when mitochondria structure and function are damaged, as found in the present results. It has been previously demonstrated that the oxidative stress caused by *T. cruzi* infection provokes a diminution in the activity of the enzymes from the respiratory chain, resulting in a decreased ability to produce energy in the myocardium in the acute phase of the infection.[16,30–32]

When we studied the mitochondrial respiratory chain in the chronic stage of the chagasic infection through the measurement of the specific activity of complexes I to IV, we found that the activity of these complexes was altered in different ways according to the strain used for the infection ($P < 0.0001$). These alterations in the respiratory complexes are related to the cristae disorganisation found in both infected groups, but more so in the Tulahuen-infected group. In addition, alterations are also related to the parasite persistence shown in Tulahuen, and chronic inflammatory process described in both groups analysed. Different results were obtained in the acute stage of the *T. cruzi* infection,[16] in which the inflammatory infiltrates were significantly more important, and the grades of alteration of mitochondria were higher.

When complex IV activity was determined, we found that it was significantly increased in both infected

groups when compared with the uninfected group ($P < 0.01$), with activity being significantly higher in the Tulahuen group. These results differ from those we obtained in the acute phase, when the activity of this complex was similar to that found in the non-infected group.[16]

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 increase in activity was detected in both infected groups, a result that might be related to the increase of the mitochondria matrix also observed in both groups. The alterations in the activity of this enzyme began in the acute phase[16] and persisted in the chronic phase of infection. Different results were found in our laboratory[33] and by other authors[14,34] in congestive heart failures of different aetiology, in which mitochondrial complex III was the seriously altered complex. We can assume that the other abnormalities found in the present paper can be achieved by *T. cruzi* infection.

The structural results showed mitochondrial damage, with 89% of the mitochondria from the Tulahuen-infected mice having at least one significant abnormality such as an increase in their matrix or a disorganisation of their cristae compared with the non-infected group. Similar alterations were detected in 58% of the mitochondria from the SGO Z12-infected group, showing, in addition, a significant reduction in their diameter ($P < 0.05$) and a significant reduction in the area occupied by mitochondria ($P < 0.01$).

Different results between parasite strains were also found by other authors, demonstrating that the evolution of the cardiopathy and the parasite strain determine the mitochondrial functional damage, which may explain the different clinical symptoms characterizing Chagas disease.[30–32,35–37]

Even though we have analysed only two parasite strains, the differences found here are important and can be attributed to different strains within a genetically diverse polymorphic parasite population; consequently, the pathogenicity can be associated with the diversity of parasite strains, stocks and isolates, which would explain the different cardiac symptoms that patients and experimental models present in the chronic phase.[37]

The mechanisms involved in the pathogenesis of Chagas disease and its progression to chronic cardiomyopathy are still under intense discussion. In this paper we demonstrate that parasite persistence and inflammation are likely to be involved in the structural and functional alterations in cardiac mitochondria from chronically *T. cruzi*-infected mice, demonstrating that in chagasic 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.

Authors' contributions: All authors were involved in the conception and design of the study, the analysis and interpretation of data, drafting the article and revising it critically for intellectual content and giving final approval of the version to be published. AB and MSLP carried out the experiments; GGM was responsible for the statistical analysis; PP and RF carried out ultrastructural and histological studies; PPO was responsible for the protocol design and follow-up of the experiments and contributed to the writing and revision of the manuscript. PPO is guarantor of the paper.

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Conflicts of interest: None declared.

Ethical approval: The Science and Technology Secretary of the Faculty of Medicine of the National University of Cordoba, Argentina, gave the ethical approval (Res 209/ 2009).

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