Use of clomipramine as chemotherapy of the chronic phase of Chagas disease

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SUMMARY

Chagas infection is a major endemic disease affecting Latin American countries. The persistence of *Trypanosoma cruzi* generates a chronic inflammatory reactivity that induces an immune response directed to the host's tissues. The effectiveness of the treatment in the chronic phase is still unsatisfactory due, amongst other reasons, to the collateral effects of the drugs used. We investigated the effect of clomipramine, a tricyclic antidepressant that, when used as a treatment of *T. cruzi*-chronically infected mice, inhibits trypanothione reductase, an exclusive and vital enzyme of *T. cruzi*. Clomipramine improved survival ($P<0{\cdot}05$) by diminishing the parasite intensity as demonstrated by PCR studies in the heart and skeletal muscle, and significantly prevented the evolution to fibrosis of the inflammatory infiltrates. Clomipramine could be a good candidate for the treatment of chronic Chagas disease.

Key words: Chagas disease, treatment, trypanothione reductase, clomipramine, cardiac chronic stage.

INTRODUCTION

Chagas disease or American trypanosomiasis is a major public health problem in Latin America. It has been estimated that 8% of the Argentinean population is infected, from which 300 000 would suffer cardiac or digestive injuries (Moncayo, 1999). The clinical manifestations and epidemic characteristics of the disease vary greatly between each endemic region. The infection is broadly spread, mainly among rural areas across the entire Latin American continent; however, the migrational rural–urban movements which occurred in Latin America between 1970 and 1980 changed the epidemic pattern of the disease and transformed it into an urban infection, with the proportion of cases with Chagas disease even greater than those with HIV and hepatitis B and C (World Health Organization, 2010). Additionally, the constant migration from endemic countries is causing the 'globalization' of the disease to places where specific serological blood and organ controls are uncommon, such as the USA and Europe (Kirchhoff *et al*. 1993; Schmunis, 2007).

Chagas disease is a very complex zoonosis with an unclarified physiopathology. It is the result of a chronic infection with *Trypanosoma cruzi* and is characterized in humans by irreversible injuries in the cardiac muscle and the autonomous nervous system (Higuchi *et al*. 2003).

Nifurtimox and benznidazole are the only drugs accepted today by the World Health Organization for the treatment of Chagas disease (Rodrígues Coura and de Castro, 2002); both drugs have a trypanosomicidal effect at early stages of the disease and on patients younger than 14 years of age (benznidazole), causing death of the parasites (Morello, 1988).

Nifurtimox and benznidazole act by inhibiting the DNA and RNA and therefore inhibit protein synthesis in the parasite and increase macromolecule degradation (Goijman and Stoppani, 1985; Gonzalez and Cazzulo, 1989). The redox cycle of both drugs generates compounds that react with oxygen, which contribute to their trypanosomicidal effect but also to

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toxicity for the patient (Marr and Docampo, 1986; De Castro, 1993). Furthermore, resistance to these drugs has been related to the presence of and old yellow enzyme, described in trypanosomatides that is a NAD(P)H flavin oxidoreductase responsible of both drugs reduction (Murta *et al*. 2006) and by a NADH-dependent, mitochondrially localized, bacterial-like, type I nitroreductase (Wilkinson *et al*. 2008).

These pharmaceuticals are active in the acute stage of the infection (showing up to 80% of therapeutic success, defined as a parasitological cure indicated by negative findings in all immunological tests) (Cançado, 1999), as well as on the indeterminate form of the chronic stage (Sosa Estani and Segura, 1999). Unfortunately, these drugs present numerous adverse effects causing many patients to abandon the treatment. Some authors have even suggested a mutagenic action (Castro, 2006). However, the major problem in the treatment of this infection is the low effectiveness of the drugs when they are used in the cardiac chronic stage of Chagas disease, with a rate of up to 80% failure (Suasnábar *et al*. 2000).

The different effectiveness on the acute, chronic indeterminate or chronic stages of the infection has been proposed to be related to the result of inadequate pharmacokinetics between these compounds and the location of the parasites in the host tissues in the chronic stage of infection (Urbina, 2002). Therefore, there is a real need to have new and effective drugs that are safer and with low toxicity for the treatment of Chagas disease.

The search for new molecular anti-*T. cruzi* targets has focused mainly on metabolic pathways exclusive to the parasite. Trypanothione reductase (TR) in kinetoplastid parasites has been proposed as a molecular target since it acts in the reduction of peroxides, deoxyribonucleotide synthesis and ascorbate homeostasis, among other functions in the parasite (De Souza, 2002).

Trypanosoma cruzi contains low levels of glutathione but it does not have the enzyme glutathione reductase (GR), while the mammalian host does. In the parasite, TR is the enzyme responsible of the transformation of the oxide form $T(S)_2$ to the reduced form $T(SH)_2$. This indicates that it should be possible to inhibit TR in the parasite without affecting GR in the host (Tovar *et al*. 1998); therefore, the toxic effects of the drugs would be exclusive to the parasite and not to the host.

In the search for a new, more effective treatment for Chagas disease with fewer adverse effects, a 'screening' was conducted testing 500 drugs (Hamond *et al*. 1984), proving that phenothiazines and related compounds such as clomipramine and thioridazine, among others (see Fig. 1), were trypanosomicidal, not only for *T. cruzi*, but also for *Leishmania donovani* (Rivarola and Paglini-Oliva, 2002; Khan and Omar, 2007). These are drugs commonly used as psychiatric

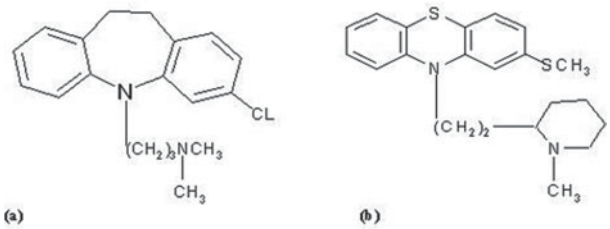

Fig. 1. Chemical structure of clomipramine (a) and thioridazine (b).

treatments and also as anti-emetics or antihistamines. Among their different biological actions, they have powerful antifungal, antibacterial and anti-plasmid activity. These drugs also showed anti-tumour, anti-inflammatory and anthelmintic activities and are useful against malaria (Amaral *et al*. 2001). Additionally, they interact with membranes or their components, with cellular proteins and with dopaminergic receptors; they also inhibit Mg^{2+}-dependent ATPase activity, increase membrane fluidity and present a strong anti-calmodulin activity (Paglini-Oliva and Rivarola, 2003).

In our laboratory we have studied the effect of some phenothiazines and tricyclic antidepressants (Rivarola *et al*. 2001, 2005; Bazán *et al*. 2008; Gobbi *et al*. 2010) on *T. cruzi* infection; clomipramine demonstrated a trypanosomicidal effect was probably due to this anti-calmodulin action (Roufogalis *et al*. 1983). Clomipramine is a tricyclic antidepressant that possesses an ethylene group on the 10th position, instead of a sulphur, and a chlorine in the 3rd position. In studies conducted on mice infected with *T. cruzi*, we demonstrated that if clomipramine is used in the acute or in the asymptomatic chronic phase, it modifies the natural evolution and prevents cardiopathy (Rivarola *et al*. 2001, 2005; Bazán *et al*. 2008).

In general, control programmes have centred their budgets and strategies towards the elimination of the vector insects and to treat infected patients in the acute stage; however, patients with chronic Chagas disease will also need treatment, having in mind that, during this phase, the parasite has been detected frequently (Dias *et al*. 2002).

Therefore, in the present work we used a murine model infected with *T. cruzi* Tulahuen strain to determine the effectiveness of clomipramine in the cardiac chronic stage of Chagas disease, analysing survival, histopathology, serology and the presence of the parasite by PCR on cardiac and skeletal muscle tissue.

MATERIALS AND METHODS

Mice

In total, 220 adult male and female Albino Swiss mice were used in this experiment; all were the same age, and had an average weight of 30 ± 1 g.

Drugs

Clomipramine from Sigma Chemicals was used.

Parasites

Trypomastigote forms of *T. cruzi* that belonged to the Tulahuen strain, designed as TINF/CL/1945/Tulahuen (according to the WHO classification based on the recommendations from the Satellite Reunion of the International Symposium to commemorate the 90th anniversary of the Discovery of Chagas Disease). This strain was isolated in 1945 from *Triatoma infestans* found in Chile and belongs to the *T. cruzi* TcIIe group (Miles *et al*. 1977).

The infection was conducted by inoculating 50 parasites per mouse via an intraperitoneal injection with heparinized blood obtained from mice used to maintain the parasite strain. This number of parasites was determined using a Neubauer haemocytometer and was sufficient to reproduce the acute and chronic stages (in their cardiac and indeterminate forms) of the experimental infection (Paglini-Oliva *et al*. 1987; Bustamante *et al*. 2003).

Experimental design

The treatment with clomipramine (5 mg kg^{-1} daily, during 60 days by an intraperitoneal injection) was administered to the animals once they were considered to be in the cardiac chronic stage of the experimental infection. To determine if the animals had developed a chronic myocardiopathy, electrocardiograms were conducted in the mice before the infection and at 90, 180 and 270 days post-infection (d.p.i.); the presence of electrocardiographic alterations (such as intraventricular or atrioventricular blockades) was considered a characteristic of the cardiac chronic stage. The electrocardiograms were performed while the animals were under anaesthesia with Ketalar HCl (Parke Davis) 10 mg kg^{-1}. A Fukuda Denshi (FD 16 Model) electrocardiograph was used, with electrodes adapted for mice, a paper speed of 50 mm s^{-1} and a calibrated amplitude of 1 mV = 10 mm; the animals were placed inside a Faraday cage. Bipolar and unipolar limb leads were determined in each case. The number of parasites per μL of blood was registered before the treatment, from the first week of infection until no parasites were detected.The animals were divided into the following groups: non-infected: 20 mice of the same age as the infected ones; and infected: 200 mice each infected with 50 trypomastigote forms of *T. cruzi* from the Tulahuen strain. As stated previously, an electrocardiographic study was performed in these mice at 90 d.p.i. to further classify them as: asymptomatic: without electrocardiographic alterations; and chronic: animals that presented with electrocardiographic alterations. The latter were subdivided in two groups: untreated: 25 *T. cruzi*-infected mice left untreated; and treated: 30 *T. cruzi*-infected mice treated with clomipramine (Clo) 5 mg kg^{-1} per day during 60 days by an intraperitoneal injection.

The treatment began 90 d.p.i. when the mice presented electrocardiographic alterations compatible with the chronic phase of the infection.

Treatment effectiveness was evaluated on the different groups on 180, 270 and 360 d.p.i. by means of histopathology, serology, PCR in skeletal and cardiac muscle and survival.

Histopathology

Cardiac and skeletal muscle samples were fixed in buffered (pH 7·0) 10% formaldehyde and embedded in paraffin. Samples from both tissues were cut horizontally (5 μm thick) and stained with Haematoxylin-Eosin (H&E); the cardiac samples were also stained with Gömöri trichrome stain. In total, 50 cuts from each group were analysed; 30 areas in each cut were examined with a 20× objective. The areas with inflammatory infiltrates and fibrosis were quantified with the AxioVision 3.0 program.

Serology

The ELISA test was used employing serum dilutions of 1/20. The specifications from the Wiener Recombinant 3.0. ELISA kit were followed. The cut-off was set at 0·22 (Sánchez Negrette *et al*. 2008).

PCR in skeletal and cardiac muscle

Skeletal and cardiac muscle samples were obtained from the mice 365 d.p.i. The mice had previously been immunosuppressed with cyclophosphamide, as explained below, and afterwards a *T. cruzi*-specific PCR was performed to detect the parasite.

Immunosuppression

The effectiveness of treatment in the immunocompromised mice was also investigated at 360 d.p.i. For this purpose mice were treated with cyclophosphamide to induce immunosuppression (Cyclo) with a 200 mg kg^{-1} per day dose administered during 5 days by intraperitoneal injection of mice infected with *T. cruzi* that were treated or untreated. After this 1 g of skeletal and cardiac muscle was taken and PCR was performed in both tissues for the detection of *T. cruzi*. This technique was performed in the following steps:

(1) DNA isolation: Skeletal and cardiac muscle samples were incubated with 1·2 mL of xylene at 55 °C for 5 min to remove the paraffin. The samples were then washed twice with absolute ethanol to remove the xylene and centrifuged at

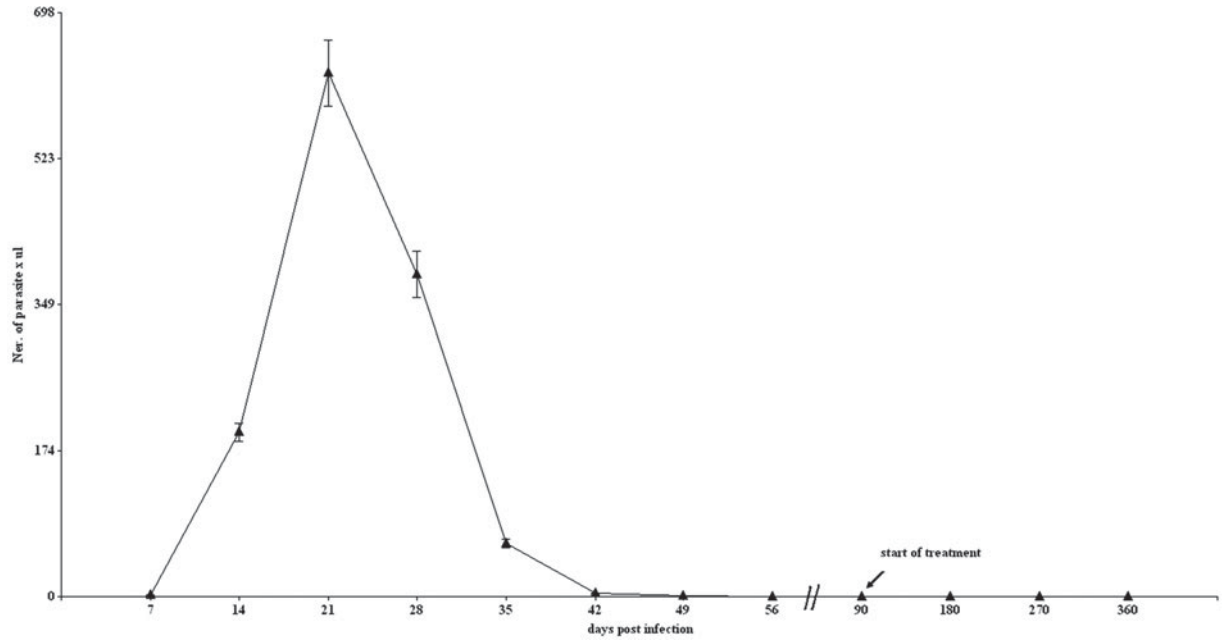

Fig. 2. Mean parasitaemia levels presented by *Trypanosoma cruzi*-infected mice (n = 200) until the end of the experiments.

maximum speed for 5 min with an Eppendorf centrifuge 5415 R. The tissue was then hydrated with 70% ethanol, re-suspended in 500 μL of STE buffer with 3 μL of Proteinase K (Sigma Aldrich) (20 mg mL^{-1}), and incubated overnight. Thereafter, the DNA was extracted by the phenol/chloroform technique (Jiménez Arce *et al*. 2007). As a positive control, *T. cruzi* DNA from cultured parasites was used. The parasites were re-suspended in TE buffer (pH 8) and boiled for 5 min. The DNA was extracted using the phenol–chloroform technique as with the paraffin-embedded samples.

(2) PCR for TCZ and GADPH segments: To amplify the highly repetitive nuclear DNA segment from *T. cruzi*, the TCZ1 and TCZ2 oligonucleotides (Invitrogen) were used (TCZ1: 5′-CGAGCTTTGCCCACACGGGTGCT-3′; TCZ2: 5′-CCTCCAAGCGGATAGTTCAGG-3′). PCR amplifications were performed in a final volume of 50 μL, containing 10 mM of PCR buffer (5×), 10 mM of each primer, 1 nM of each of the 4 deoxynucleotide triphosphates and 1·25 Units of platinum Taq DNA polymerase (Invitrogen). Finally, 5 μL of isolated DNA sample were added. The reaction mixtures were subjected to 30 cycles of amplification in a programmable thermal cycler (Eppendorf). During each cycle the samples were incubated at 94 °C for 3 min, cooled to 63 °C for 1 min and heated to 72 °C for 1 min; a final elongation step was allowed at 72 °C for 3 min (Moser *et al*. 1989).

To verify the presence of DNA, the GADPH segment of each sample was amplified; the oligonucleotides (Invitrogen) sequences in this case were: 5′-GGAGTCAACGGATTTGGT-3′ (sense); 5′-GTGATGGGATTTCCATTGAT-3′ (antisense) (Markvardsen *et al*. 1995; Rossmanith *et al*. 1999). The reaction conditions in this case were: 4 min of initial denaturalization at 94 °C, followed by 30 cycles of 1 min at 94 °C, 1 min at 60 °C and 2 min at 72 °C; the final extension was conducted at 72 °C for 10 min. Five μL of the PCR products were subjected to electrophoretic fractionation on 1·6% agarose gels and then stained with ethidium bromide. The samples were considered positive when they presented a 200 bp band.

Survival

Survival was monitored weekly until the end of the experiments.

Statistical analysis

The data were analysed according to the nature of the variable under study (ANOVA, and multiple comparison tests and Chi Square test). A level of $P < 0{\cdot}05$ was considered significant.

RESULTS

Parasitaemia

Figure 2 shows the evolution of the parasitaemia levels in infected mice (n = 200). As can be observed, infected mice presented a peak in the blood parasite

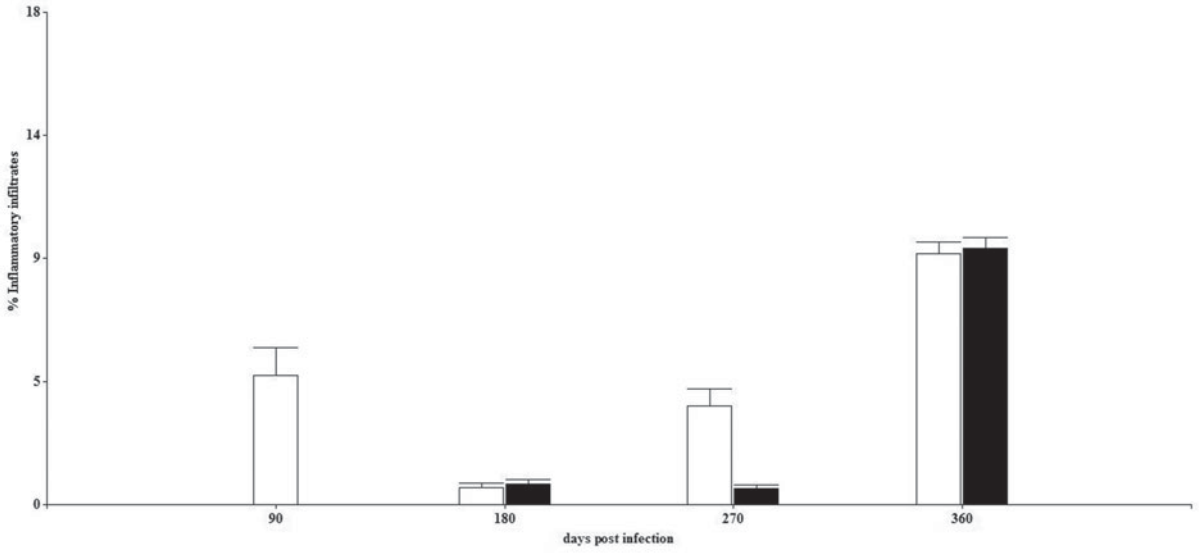

Fig. 3. Percentage of inflammatory infiltrates in cardiac tissues from *Trypanosoma cruzi*-infected mice along the experimental infection: (□) untreated; (■) treated with clomipramine 5 mg kg^{-1} per day during the chronic stage of the infection.

levels at 21 d.p.i., decreasing thereafter until no parasites could be observed by this method from the 49 d.p.i. until the end of the experiments (360 d.p.i.).

Histopathology

Cardiac muscle. By quantifying the percentages of areas with inflammatory infiltrates, it was observed that on 180 and 360 d.p.i. these percentages were not significantly different between treated and untreated mice (Fig. 3). However, on 270 d.p.i., the hearts (see Fig. 4A) from the treated mice presented significantly fewer infiltrates than the untreated ones (see Fig. 4B).

Skeletal muscle. The percentage of areas with inflammatory infiltrates in skeletal muscle samples is shown in Fig. 5. As can be observed, the percentage of areas with infiltrates is significantly minor ($P<0{\cdot}05$) in the samples from the infected mice treated with clomipramine; however on the 360 d.p.i. the percentage was minor in the untreated mice (see Fig. 4C and D).

Fibrosis on cardiac muscle. The percentage of areas occupied with fibrosis was higher ($P<0{\cdot}05$) in the cardiac muscle samples (Fig. 6) from the untreated mice than in those from the treated mice along the entire duration of infection, as can be observed in Fig. 4E.

Serology

Despite the fact that the titres of anti-*T. cruzi* antibody in the mice treated with clomipramine (5 mg kg^{-1}per day for 60 consecutive days) were not different from those presented by the untreated group throughout the 360 days that the experiments lasted, the antibody titres from the treated group significantly diminished ($P<0{\cdot}05$) from the beginning of the treatment until the end of the experiments.

Parasite presence in tissues from infected mice as detected by PCR

Figure 7 shows the amplification products from the tissues obtained from the mice immunodepressed with cyclophosphamide (200 mg kg^{-1} per day). As can be observed, the PCR was positive on cardiac and skeletal muscles from both the treated and the untreated mice; however, the bands corresponding to the tissues from the treated mice (lanes 2 and 5 in the figure) were less intense than those observed in the untreated mice (lanes 3 and 4).

Survival

Figure 8 shows that at 90 d.p.i. just less than 30% of the infected mice had survived. The treatment was started, and then the untreated group presented a 10% survival rate by 360 d.p.i. On the other hand, the treated group reached 360 d.p.i. at with a significantly higher percentage ($P<0{\cdot}05$) (see Fig. 8).

DISCUSSION

Taking into account the hypothesis that parasite persistence is involved in the genesis of chagasic myocardiopathy, and considering the several studies that prove that *T. cruzi* still remains within the host in the chronic stage of the infection (Rivarola *et al.* 2005; Bazán *et al.* 2008), an anti-parasitic treatment

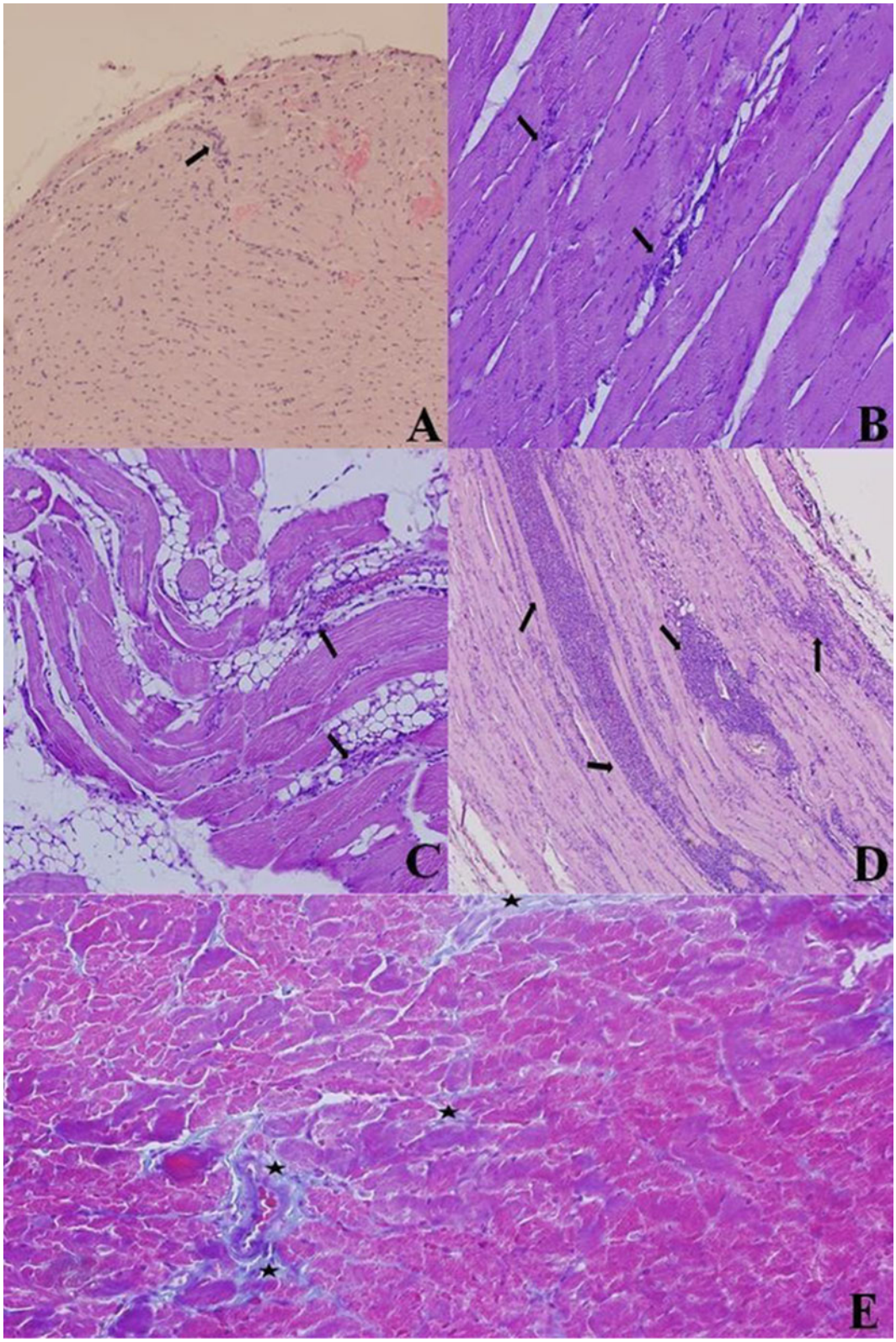

Fig. 4. Histological sections from *Trypanosoma cruzi*-infected mice. (A) Cardiac tissue from an untreated mouse, 270 d.p.i. The arrow shows a small inflammatory infiltrate. H&E 20×. (B) Cardiac tissue from a treated mouse, 270 d.p.i. Arrows show inflammatory infiltrates. H&E 20×. (C) Skeletal muscle tissue from untreated mice, 360 d.p.i. Small inflammatory infiltrates can be observed (arrows). H&E 20×. (D) Skeletal muscle tissue from a treated mouse, 360 p.i. Severe inflammatory infiltrates are shown (arrows). H&E 10×. (E) Cardiac tissue from untreated mice, day 360 d.p.i. Fibrosis areas are marked with stars. GS 40×.

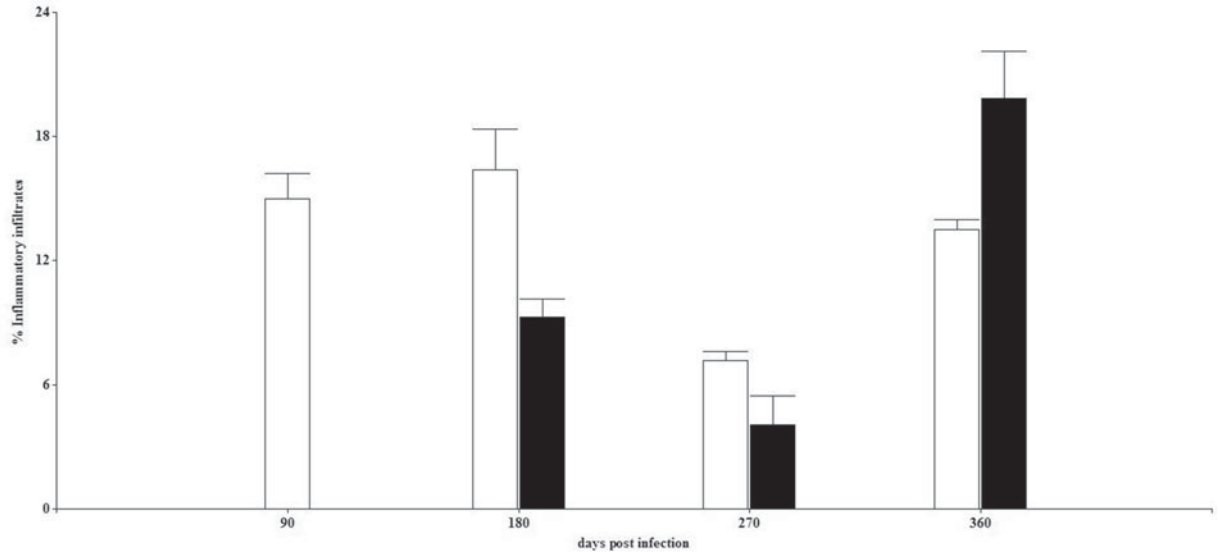

Fig. 5. Percentage of inflammatory infiltrates in skeletal muscle samples from *Trypanosoma cruzi*-infected mice along the experimental infection: (□) untreated; (■) treated with clomipramine 5 mg kg^{-1} per day during the chronic stage of the infection.

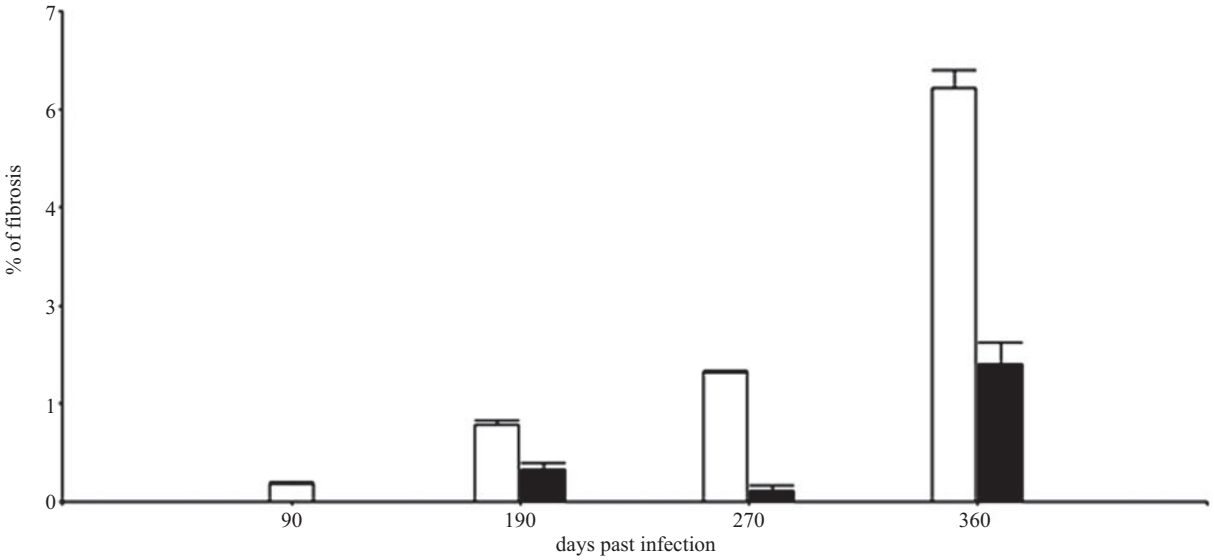

Fig. 6. Percentage of fibrosis in cardiac tissues from *Trypanosoma cruzi*-infected mice along the experimental infection: (□) untreated; (■) treated with clomipramine 5 mg kg^{-1} per day during the chronic stage of the infection.

at this stage, which will reduce the parasite load even though not able to totally eliminate it, would be fundamental to prevent the evolution of cardiopathy and, consequently, to diminish the severity of the cardiac damage (Tarleton, 2001; Bustamante *et al*. 2007).

Studies conducted on the biochemistry of *T. cruzi* have allowed the identification of parasite enzymes such as trypanothione reductase, different from those presented by the host, which elevates them to the category of possible targets for chemotherapy. Trypanothione reductase is a flavoprotein that maintains trypanothione in its reduced state, leading to a diminution in free radical levels and maintaining reduced intracellular conditions (Krauth-Siegel and Comini, 2008). Clomipramine, currently used in clinical treatments as an antidepressant, has been found to be also capable of inhibiting trypanothione reductase irreversibly (Benson *et al*. 1992) by peroxidase/H_2O_2/phenothiazine and related compounds systems. Trypanothione reductase inactivation depended on the time of incubation with the drug, the peroxidase nature and concentration. Production of cation radicals by tricyclic drugs via their peroxidation was essential for enzyme inactivation. Tricyclic drugs are direct inhibitors of trypanothione reductase (Benson *et al*. 1992). The actions of peroxidase-activated tricyclic compounds

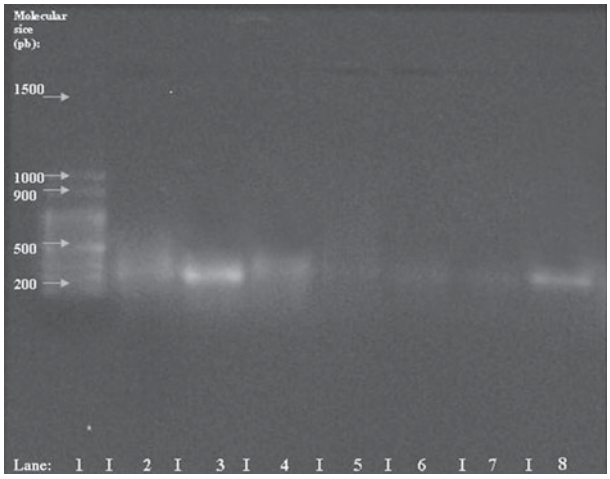

Fig. 7. *Trypanosoma cruzi* TCZ segment amplification by PCR in tissues from treated and untreated mice 360 d.p.i. and immunodepressed with cyclophosphamide. Lanes: (1) Molecular ladder. (2) Cardiac tissue from a mouse treated with Clo. (3) Cardiac tissue from a mouse left untreated. (4) Skeletal muscle from an untreated mouse. (5) Skeletal muscle from a mouse treated with Clo. (6) Skeletal muscle. (7) Cardiac muscle. (8) *T. cruzi* DNA.

through three different peroxidases were studied by Gutierrez-Correa *et al*. (2001): horseradish peroxidase, myeloperoxidase and a myoglobin, a heme protein which is an oxygen stabilizer in striated muscle. Their results support the hypothesis that cation radicals produced by peroxidation of tricyclic drugs irreversibly inhibit the *T. cruzi* enzyme.

Jones *et al*. (2010), making a comparison to identify potential differences in the sensitivity of trypanothione reductases from *Trypanosoma brucei* and *T. cruzi* to 10 potential inhibitors, demonstrated that clomipramine inhibits competitively by occupying at least part of the oxidized trypanothione-binding region (Benson *et al*. 1992). These studies explain the potential beneficial effects of clomipramine and other tricyclic compounds in modifying the evolution of experimental Chagas disease.

In this work, mice chronically infected with *T. cruzi* from the Tulahuen strain were treated with clomipramine to evaluate its effect upon this late stage of the experimental infection. To verify the effectiveness of the treatment, parasitological, immunological and histopathological methods were employed.

Currently, there are no conclusive results to affirm that this disease is the result exclusively of an autoimmune process (Benoist and Mathis, 2001; Bonney and Engman, 2008). With the increasingly common use of more sensitive techniques, such as PCR, for the detection of parasites within the blood and tissues of infected individuals, and the observation that immune-suppressed patients show parasitaemia peaks, and even *T. cruzi* encephalitis (Sosa-Estani *et al*. 2009), the presence and persistence of the parasite along the different phases of the disease has became relevant to explain the development and evolution of the disease (Barbosa Marcon *et al*. 2011).

Here we verified the presence of *T. cruzi* in our model through the detection of parasite DNA on cardiac and skeletal muscle tissue until 360 d.p.i. (advanced chronic stage). These facts prove that the parasite, although hard to detect, is present in every phase of the infection (Bazán *et al*. 2008). These results indicate that skeletal muscle acts as a reservoir for the parasites that are later released into the blood, thus perpetuating the inflammatory process and the chronic cardiac damage (Zhang and Tarleton, 1999). These observations could contribute to explain the functional muscular incapacity described in many chagasic patients (Montes De Oca *et al*. 2007).

On the other hand, amastigote nests are infrequently observed in cardiac tissue; apparently myocardial cells would have the capacity to control intracellular multiplication of *T. cruzi* due to the presence of determined cytokines (Postan *et al*. 1999).

Clomipramine treatment did not completely eliminate the presence of *T. cruzi* from the host, as the parasites were detected in the DNA studies on samples of cardiac and skeletal muscle tissue. The parasite load was found to be significantly diminished, as was confirmed by the amplification of *T. cruzi* DNA on cardiac and skeletal muscle, that demonstrated stronger bands in the samples from the untreated mice; the parasite DNA would therefore be present in minor quantities in the treated animals, despite being detected in both groups.

When the immunological response was analysed by ELISA, a decline in the anti-*T. cruzi* antibody titres from the beginning of the infection towards the end of the experiment was detected in the treated animals in comparison to the untreated ones ($P<0{\cdot}05$). However, the antibody titres remained positive; this has to be attributed to the partial elimination of *T. cruzi* in the treated subjects discussed before. Other studies have found that parasite antigens persist for long periods in infected individuals, even after the parasitaemia load was reduced by the treatment (Fabro *et al*. 2007). This has also been found for benznidazole, nifurtimox or allopurinol tests, where the serology was positive many years after the treatment started (Segura *et al*. 1996). These findings allow us to propose that negative serology as a cure condition should be re-discussed.

According to the mixed theory for the physiopathology of the disease, the presence of the parasite would trigger an inflammatory, self-damaging process that would culminate in the progressive accumulation of fibrosis (Elias *et al*. 2003). The lower antibodies titres would mean a lesser number of parasites and therefore, a possibility of minor cardiac damage (fibrosis) in the treated animals. The extent

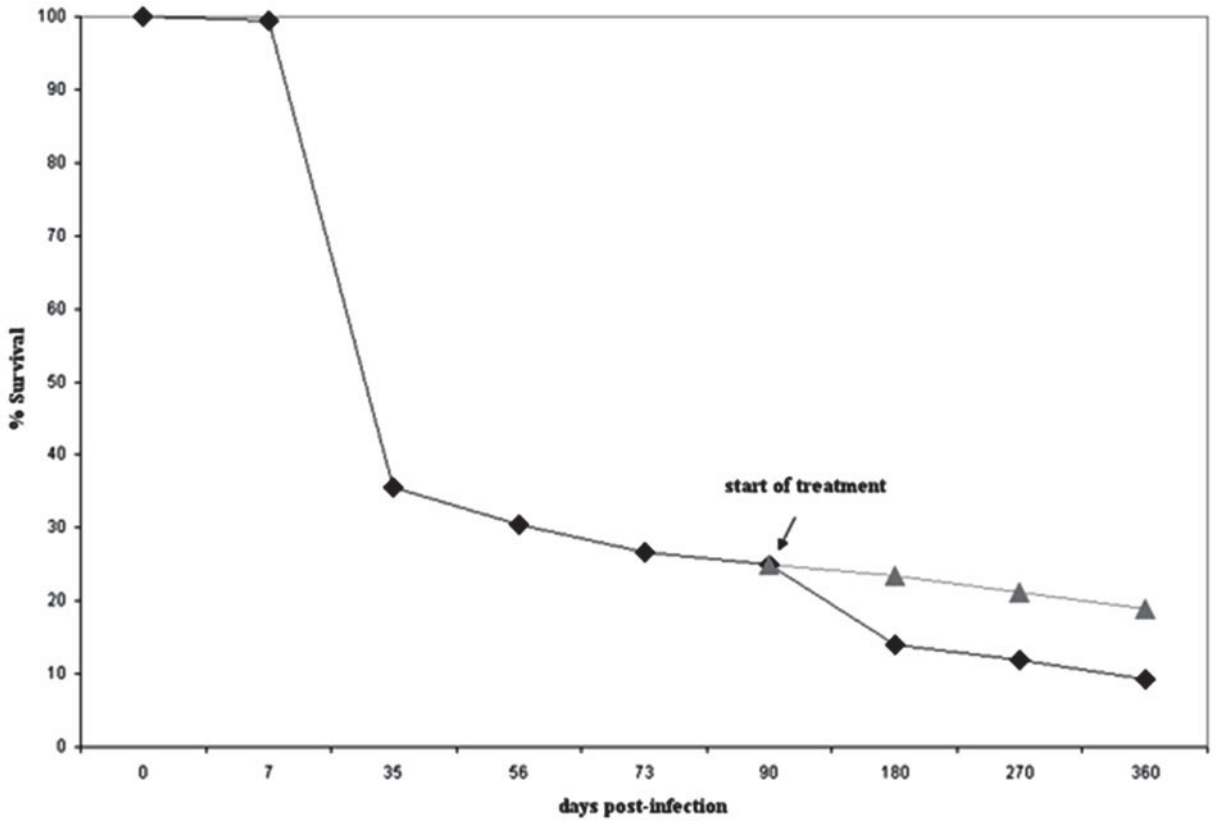

Fig. 8. Percentage survival of the *Trypanosoma cruzi*-infected mice throughout the experimental infection: (♦) untreated; (▲) treated with clomipramine 5 mg kg^{-1} per day during the chronic stage of the infection (the start of treatment is shown at 90 d.p.i.).

of inflammatory infiltrates in the cardiac muscles from either the treated or untreated groups was not different at 180 and 360 d.p.i., but was significantly less intense in the treated mice at 270 d.p.i.; this would explain the diminished fibrosis found in the treated mice, probably because, even though the inflammation was not reverted, the cardiac damage did not continue its course to culminate in fibrosis.

In the skeletal muscle, the inflammatory infiltrates were less intense ($P<0{\cdot}05$) in the treated animals at 180, 270 and 360 d.p.i.; by quantifying the areas with fibrosis in the cardiac muscle, we observed a significant diminution ($P<0{\cdot}05$) in the treated animals. This means that in our experimental model of mice treated in the chronic stage of the infection, there was no observable diminution in the inflammatory process in cardiac muscle in comparison to the untreated animals. There was, however, a lower percentage of fibrosis in the mice treated with clomipramine. This would explain the higher survival of the treated group ($P<0{\cdot}05$).

Taking all this into consideration, we can conclude that:

- The parasite remains in the host throughout the entire infection.
- An anti-parasitic treatment in the chronic stage of the infection is, therefore, pertinent.
- The use of more sensitive techniques is necessary to determine the presence of *T. cruzi* and the effectiveness of any treatment.
- Trypanothione reductase has gained relevance as a therapeutic target for Chagas disease, and clomipramine has proved to be trypanosomicidal at different stages of the infection, diminishing the parasite load.
- Even though it has been described that clomipramine provokes arrhythmias, this effect was not detected in our experimental model.
- This diminution in the parasite load would produce fewer injuries in the tissues, particularly the heart, and therefore would diminished mortality.
- According to the obtained results, clomipramine is a good candidate for the treatment of Chagas disease in the chronic cardiac form of the infection since it diminished cardiac damage and improved the survival of the infected individuals.

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