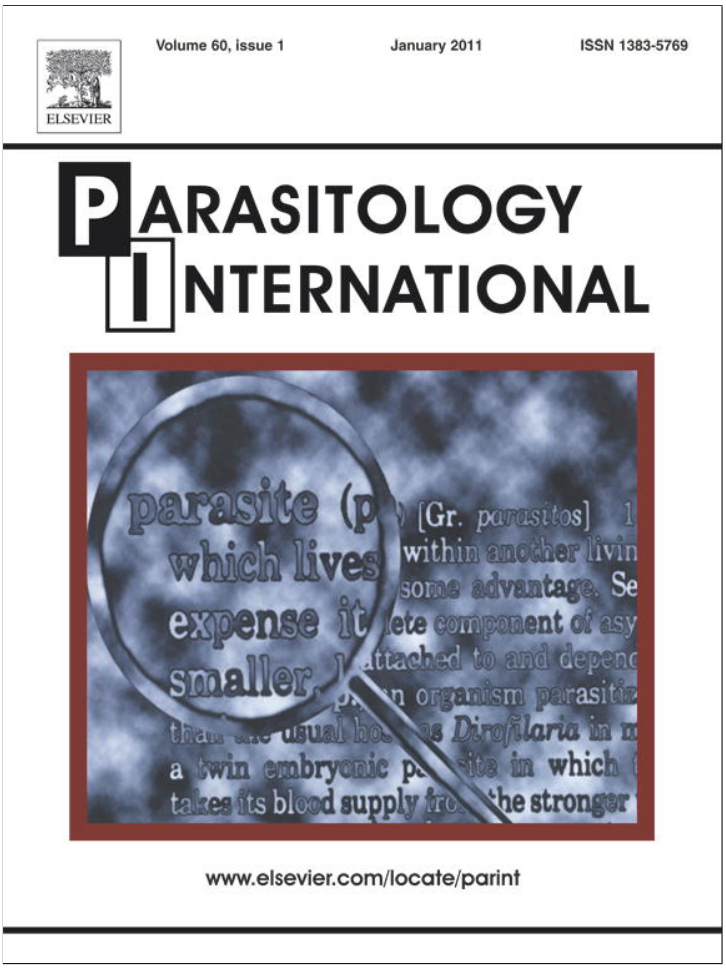

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Clomipramine and benznidazole association for the treatment of acute experimental *Trypanosoma cruzi* infection

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ABSTRACT

Alternative strategies are being designed to identify candidates among drugs already available on the market that could be used in combination to improve the efficacy of Chagas disease treatment. This work evaluates the effect of the association of clomipramine (CLO) with benznidazole (BZN) for the treatment of experimental Chagas disease in the acute stage, in Swiss albino mice infected with *Trypanosoma cruzi* Tulahuen strain. Infected mice were treated with CLO 5 mg/kg/day and BZN 50 and 100 mg/kg/day, each separately or together. Efficacy of the treatment was evaluated through parasitemia, survival, electrocardiography, histopathological studies, serological and PCR assays at 90 days post-infection (dpi). All treatments significantly ($P < 0.05$) reduced mortality and decreased parasitemia. Histopathological analysis of liver and kidneys of mice treated with CLO and the drug combination showed less injury than mice treated only with BZN. The lower dose of BZN (50 mg/kg/day) combined with CLO showed the same efficacy as the habitual dose of BZN (100 mg/kg/day) combined with CLO. The therapeutic results from the combination of BZN with CLO presented lesser side effects than the treatment with BZN.

1. Introduction

Chagas disease, caused by *Trypanosoma cruzi*, is a complex systemic disease that affects over 10 million people in the American continent [1]. There is evidence that Chagas disease is also a serious challenge to public health in European countries, mainly linked to population mobility, especially migration from endemic countries [2]. Specific anti-*T. cruzi* chemotherapy is still unsatisfactory, being based on nitroimidazole or nitrofuran agents such as benznidazole (BZN) (LAFEPE) and nifurtimox (NX) (Lampit®, Bayer), which were developed empirically four decades ago. Currently, posaconazole, a new antifungal agent that has been effective against *T. cruzi* in in vitro and in vivo assays, has moved to clinical trials; however, even if effective, its use may be limited due to its high cost [3,4].

Based on literature data, around 40% of different *T. cruzi* strains, from various sources and geographical origins, show partial sensitivity or resistance to BZN and/or NX [5,6]. The occurrence of *T. cruzi* strain drug resistance at least partially explains why, although BZN treatment is effective when administered during the acute phase of infection, it may not be able to eliminate all parasites from the vertebrate host [6]. Additionally, specific treatments have frequent undesirable side effects and produce biochemical damage in mammalian tissues [7,8]. Identifying metabolic pathways and finding new molecular targets in the parasite are helpful to develop more effective trypanocidal drugs. These targets must be parasite-specific, and their interaction with the trypanocidal drug should not produce any metabolic alteration in the mammalian host. Trypanothione reductase (TR) and hypoxanthine-guanine ribosyltransferase (HGRT) are specific enzymes of *T. cruzi*, both of which play vital functions in the parasite [9]. Clomipramine is a tricyclic drug used in psychiatric treatment that has been found to disable TR [10]. Taking into account the possible existence of resistant parasite strains and the coexistence of more than one strain in the same patient, it is possible that the simultaneous administration of two or more drugs with different mechanisms of action, different targets, and with fewer adverse side effects could optimize the treatment [11–13].

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The combination of drugs can be a valuable way to improve the efficacy of treatment in a variety of diseases. This has been demonstrated by several authors in the treatment of tuberculosis [14], and in Chagas disease with the combination of benznidazole and ketoconazole [15], clomipramine and allopurinol [9], among others. The present work evaluated the effect of the association of clomipramine and benznidazole (at different doses) for the treatment of experimental Chagas disease in the acute stage, in Swiss albino mice infected with *T. cruzi* Tulahuen strain (Tc VI) [16].

2. Materials and methods

2.1. Animals and experimental design

One hundred and twenty Swiss Albino female and male mice weighing 30 ± 1 g were intraperitoneally inoculated with 50 trypomastigotes forms of *T. cruzi*, Tulahuen strain [17,11]. Another twenty mice left uninfected were used as controls.

Mice were divided into the following groups: *uninfected* (NI), treated daily intraperitoneally with isotonic saline solution (n = 20); *infected untreated* (NT), infected with *T. cruzi* (n = 20); infected with *T. cruzi* and treated with benznidazole 100 mg/kg per day (n = 20), (BZN); infected with *T. cruzi* and treated with benznidazole 50 mg/kg per day (n = 20), (BZN/2); infected with *T. cruzi* and treated with clomipramine 5 mg/kg/day (n = 20), (CLO); infected with *T. cruzi* and treated with benznidazole 100 mg/kg/day and clomipramine 5 mg/kg/day (n = 20), (BZN + CLO); infected with *T. cruzi* and treated with benznidazole 50 mg/kg/day and clomipramine 5 mg/kg/day (BZN/2 + CLO), (n = 20, all groups consisted of 10 male and 10 female mice). All the experiments were performed with the complete animal group.

2.2. Drugs

The drugs used were clomipramine (Sigma Chemical, St. Louis, MI, USA) and benznidazole (LAFEPE, Brazil). For all treatment schedules, clomipramine was administered intraperitoneally and benznidazole orally for 30 days, with the first dose 24 h after infection.

The experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (revised 2011) [18].

2.3. Parasitemia and mortality parameters

Parasites were determined in a Neubauer hemocytometer using tail vein blood samples obtained once a week, beginning 7 days post-infection (dpi). The survival of each group was monitored every day.

2.4. Electrocardiographic studies (ECG)

Electrocardiograms were obtained with an electrocardiograph (Model FD-16 Fukuda Denshi) under ketamine hydrochloride (Parke Davis) anesthesia, 10 mg/kg, at 90 dpi. ECG tracings were obtained with six standard leads (dipolar leads DI, DII, DIII, and unipolar leads aVR, aVL, and aVF), recording at 50 mm/s with amplitude set to give 1 mV/10 mm.

In order to follow the progress of the cardiopathy, ECG parameters evaluated were: (i) heart rate (beats per minute), (ii) modifications in atrioventricular conduction (prolonged PR interval) and ventricular conduction (prolonged QT interval) in milliseconds.

2.5. Serological assay

Microplates sensitized with 6 recombinant antigens (SAPA, 1, 2, 30, 13 and 36) were used; 1/20 serum dilutions were conducted. The specifications of the Wiener Recombinant 3.0 ELISA kit were followed. The cut-off was set at 0.22 [10,19].

2.6. PCR assay

PCRs were performed in all groups using blood samples obtained on day 90 post infection. The detection of parasites in each sample was determined by PCR amplification of a fragment of 220 bp, corresponding to the gene family E13 [20]. The sequence of the oligonucleotide O1 is 5′-TGG CTT GGA GGA GTT ATT GT-3′; the sequence of the oligonucleotide O2 is 5′-AGG AGT GAC GGT TGA TGA TCA GT-3′. PCR amplifications were performed in a final volume of 50 μl. The reaction mixtures contained 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM of each of the 4 deoxynucleotide triphosphates, 1 μM of each primer and 1.25 U of Taq DNA polymerase (Promega). Prior to the amplification reaction, the DNA was denatured for 10 min at 94 °C. The reaction mixtures were subjected to 30 cycles of amplification in a programmable thermal cycler. During each cycle, the samples were incubated at 94 °C for 1 min, cooled to 55 °C for 1 min and heated to 63 °C for 2 min. For visualization of the results, 10 μl of each sample were subjected to electrophoretic fractionation on 1.6% agarose gels [21]. The quality of the DNA samples was checked by PCR amplification of a 289 bp constitutive gene from the host (β-actin), using the corresponding primers: β-act-F (5′CGG AAC CGC TCA TTG CC 3′) and β-act-R (5′AAC CAC ACT GTG CCC ATC TA 3′).

2.7. Histopathological analysis

After treatment, at 31 dpi, two mice were randomly selected from each group and the liver, kidney and intestine were extracted, in order to evaluate structural alterations due to possible toxic drug effects. Samples were classified as follows: *without alterations*; *mild*: with only inflammatory infiltrates; *moderate*: inflammatory infiltrates, vascular congestion and dilatation; and *severe*: inflammatory infiltrates, vascular congestion and dilatation, foci of necrosis and/or amastigote nests. A total of 126 slices were analyzed, 18 from each group. All mice groups were euthanized at 90 dpi by ketamine anesthesia and fixed in 10% buffered (pH 7.0) formaldehyde, and embedded in paraffin. Each heart was cut horizontally into 5 μm sections from the apex to the auricles. The cardiac and skeletal muscle sections were stained with hematoxylin–eosin, and with Masson's technique for evaluation of inflammation and fibrosis, respectively, by optical microscopy. A section of each sample was taken as the total area; the area occupied by inflammatory infiltrates was calculated using a 4× objective. A total of 210 slices were analyzed, 30 from each group. The percentage of inflammatory infiltrates and fibrosis in each section was quantified using AxioVision 4.8. Program (Carl Zeiss).

2.8. Statistical analysis

Statistical analyses were performed with Sigma Plot 11.0 and InfoStat Software/Pversion 2011. Data were compared by ANOVA and multiple comparisons by the Fisher Test (percentage of inflammatory infiltrates and fibrosis, serological and ECG parameter data). Parasitemia data were analyzed by multivariate analysis MANOVA (Hotelling's test). Survival data were analyzed by the Kaplan–Meier survival test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Parasitemias, Serology and PCR

The parasitemia level was significantly higher in infected and untreated mice than in the other groups ($P < 0.05$) (Fig. 1). In the group treated with CLO, it was significantly higher ($P < 0.05$) than in other treatments, while the curves of the groups treated with BZN, BZN/2, BZN + CLO and BZN/2 + CLO presented no significant differences between them, demonstrating that treatment reduces parasitemia levels. The drug combinations (BZN + CLO and BZN/2 + CLO) notably

reduced parasitemia, and no parasites were detected with BZN + CLO treatment during the infection.

Parasite DNA or anti-*T. cruzi* antibodies were detected in all groups at 90 dpi. Anti-*T.cruzi* antibody was detectable in both infected and infected-treated mice, and no significant difference in antibodies levels were observed ($P > 0.05$) between groups. Positive PCR results were obtained in the blood samples from all infected and infected-treated groups. Furthermore, the group treated with BZN + CLO that showed negative parasitemia was also positive. There was a correlation between serology and PCR in all groups — infected, treated and untreated.

3.2. Survival

Fig. 2 shows the survival of the infected and infected-treated groups until 90 dpi. It can be seen that 45% of the infected and untreated mice survived, while survival in the groups treated with BZN, BZN/2, BZN + CLO and BZN/2 + CLO was 90–100%. The group treated with CLO presented a significantly lower survival rate (65%) compared with the other treated groups.

3.3. Electrocardiograms

Table 1 shows the ECG results from all groups (NI, NT, BZN, BZN/2, CLO, BZN + CLO and BZN/2 + CLO). At 90 dpi, the ECG of untreated infected mice showed significantly longer PR and QT intervals than uninfected mice (42.4; 57.4 versus 19.3; 36.2 ms, respectively). PR intervals greater than 40 ms suggest slower transmission of electrical impulses and atrioventricular block (AVB), which is characteristic of acute *T. cruzi* infection [22]. Mice treated with CLO presented a significantly prolonged PR interval, indicating that this drug may not prevent atrioventricular block. Similarly, treatment with BZN/2 presented a significantly prolonged QT interval, suggesting that the lower dose of BZN was not effective at preventing intraventricular blockades (IVB). Bradycardia was also observed more frequently in infected untreated mice than in the control group. None of the combined-treated groups presented ECG abnormalities, which shows that the drug combination was effective in preventing significant alterations of the cardiac electric conduction system during acute experimental *T. cruzi* infection.

3.4. Histopathological analysis

3.4.1. Liver, kidney and intestine

Table 2 shows the characteristics of intestine, liver and kidney at 31 dpi, after completion of treatment. The kidneys of infected mice

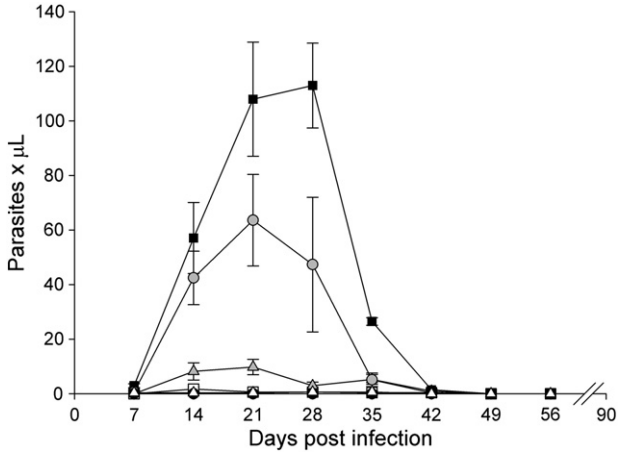


Fig. 1. Evolution of parasitemia in Albino Swiss mice inoculated with *Trypanosoma cruzi*, Tulahuen strain, untreated NT (black filled squares) and treated with BZN (open squares), BZN/2 (grey filled triangle), CLO (grey filled circles), BZN + CLO (black filled circles), BZN/2 + CLO (open triangle). For all the groups n = 20 (10 male and 10 female).

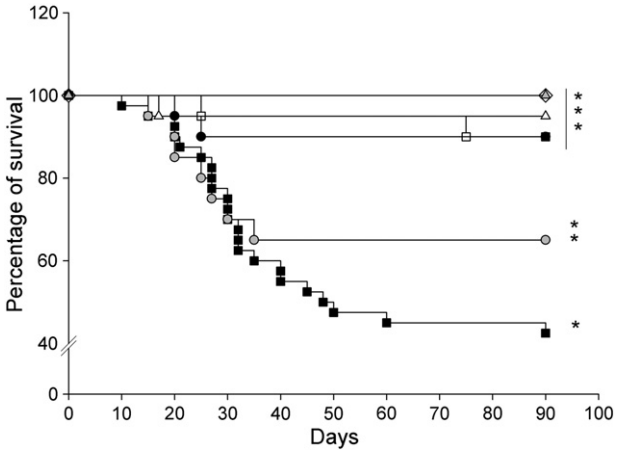


Fig. 2. Survival curve of the groups uninfected NI (open rhombus), infected with *Trypanosoma cruzi*, Tulahuen strain, untreated NT (black filled squares) and infected and treated with BZN (open squares), BZN/2 (grey filled triangle), CLO (grey filled circles), BZN + CLO (black filled circles) and BZN/2 + CLO (open triangle). This figure was performed using Kaplan Meier Survival test (Y axis shows percentages). Asterisks indicate a significant difference between the groups NT, BZN, BZN/2, BZN + CLO, BZN/2 + CLO; CLO and NT. (Log-rank test–Chi square, $P < 0.05$.) All groups consisted of female and male mice.

treated with BZN/2 presented amastigote nests and inflammatory infiltrates. The livers of infected mice treated with BZN and BZN/2 showed abnormalities such as foci of necrosis, perivascular inflammatory infiltrates, etc. No necroses were observed in the groups infected and treated with CLO, BZN + CLO and BZN/2 + CLO. Clomipramine seems to have a protective effect on hepatocytes.

3.4.2. Myocardium

Fig. 3A and B shows myocardial sections obtained at 90 dpi from uninfected mice. Fig. 3C and D shows intense inflammatory infiltrates and fibrosis, respectively, from infected untreated mice. The percentage of inflammatory infiltrates and myocardial fibrosis was significantly higher in the NT group than in the treated groups (Fig. 5).

Hearts from mice treated with BZN + CLO and BZN/2 + CLO (Fig. 4G, H and I, J) presented a few cardiac alterations including isolated inflammatory infiltrates and fibrosis. Mice treated with BZN + CLO showed no myocardial fibrosis. There was no statistically significant difference between these treatments ($P < 0.05$) (Fig. 5). While treatments with CLO showed a similar percentage of fibrosis to treatment with BZN/2, the fibrosis area of BZN-treated mice was significantly lower than in mice treated with BZN/2 and CLO (Fig. 5). However, treatment

Table 1
Electrocardiograph parameters of uninfected mice (NI, n = 15) and infected with *T. cruzi* and untreated (NT, n = 9) and treated with BZN (n = 15), BZN/2 (n = 15), CLO (n = 13), BZN + CLO (n = 15), BZN/2 + CLO (n = 15).

Groups	Electrocardiographic characteristics (mean ± SE)[a]		
	PR(ms)	QT(ms)	Heart rate (bpm)
NI	19.3 ± 1	36.2 ± 1.1	556.81 ± 8.86
NT	42.4 ± 2	57.4 ± 2.1	491.25 ± 2.1
BZN	23.03 ± 1.6	41.7 ± 2.5	524.37 ± 18.76
BZN/2	24.1 ± 1.1	53.4 ± 2.9[b]	511.84 ± 19.43
CLO	34.7 ± 2.1[b]	47.0 ± 1.9	500.5 ± 27.14
BZN + CLO	29.1 ± 1.7	41.3 ± 1.1	525.6 ± 18.39
BZN/2 + CLO	28.1 ± 1.6	42.3 ± 1.8	522 ± 23.9

[a] Values show mean ± standard error. ECG parameters were evaluated at 90 dpi using the following standard criteria: the variation of the PR and QT intervals, all measured in milliseconds, and heart rate (monitored by beats/min).

[b] Significant differences ($P < 0.05$) between the values for mice treated with CLO and BZN/2 relative to the NI.

Table 2
Histopathological analysis of intestine, liver and kidney of the groups uninfected (NI) and infected with *T. cruzi* Tulhauen strain and treated with BZN, BZN/2, CLO, BZN + CLO, BZN/2 + CLO and NT (not treated) at 31 dpi (n = 14, 2 for each treatment).

Groups	NI			BZN			BZN/2			CLO			BZN + CLO			BZN/2 + CLO			NT		
Tissues	I	L	K	I	L	K	I	L	K	I	L	K	I	L	K	I	L	K	I	L	K
	−	−	−	−	+++	+++	−	+++	++	−	+	+	−	+	++	−	+	++	−	+	+

I: intestine, L: liver, K: kidney. Symbols means tissue injure levels: without alterations: −; mild: +; moderate: ++; severe: +++.

with BZN/2 presented a lower percentage of inflammatory infiltrate than BZN and CLO treatment. These results can be seen in Fig. 4 A–F.

3.4.3. Skeletal muscle

Skeletal muscle from mice treated with BZN + CLO and BZN/2 + CLO presented significantly lower inflammatory infiltrates than mice treated with CLO, BZN and BZN/2 (Fig. 5). The decrease in the percentage of inflammatory infiltrates in skeletal muscle in mice treated with the different drugs matched the decrease in blood parasitemia.

4. Discussion

This study shows that treating *T. cruzi* Tulahuen strain-infected mice with BZN, BZN/2, CLO, BZN + CLO, BZN/2 + CLO reduces the severity of infection and tissue lesions, leading to a significant decrease in mortality. Benznidazole has shown efficacy as a trypanocidal agent, but it has serious side effects.

Treatment of human cases of Chagas disease is still limited: even when is clinically used in the acute (children and adults) and chronic phases (adults) of the disease, treatment drop-out rate in the chronic phases due to the appearance of adverse reactions is important and generally higher for adults than for children [23–29]; additionally, several authors have shown the resistance of some *T. cruzi* strains to BZN [30].

Another study demonstrated that, in mice infected with Y strain (BZN moderately resistant), treatment in the acute phase with BZN 50 mg/kg/day did not cure the infection, whereas BZN at 100 mg/kg/day cured 30.8% of the animals [15]. In our work, with Tulahuen strain-infected mice treated with BZN 100 and 50 mg/kg/day we obtained a survival of 90 and 100% respectively; however, positive serological test and PCR results were obtained not only in the BZN treatment but also in the combination with CLO.

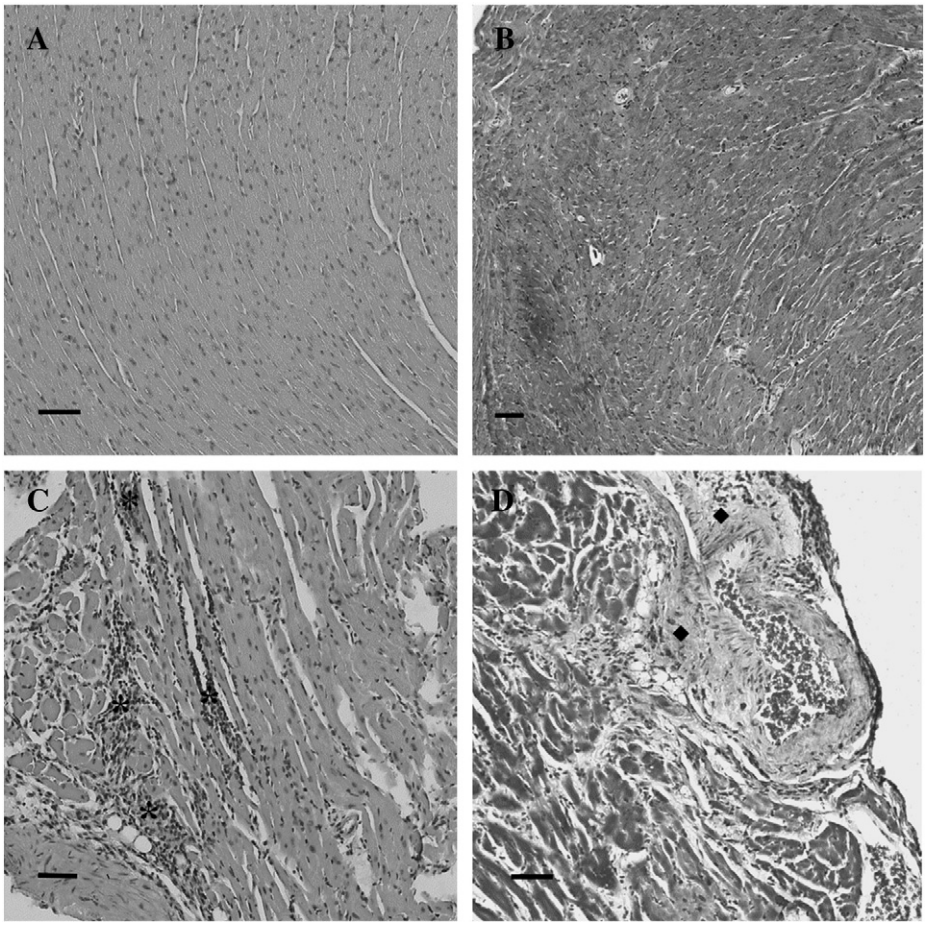
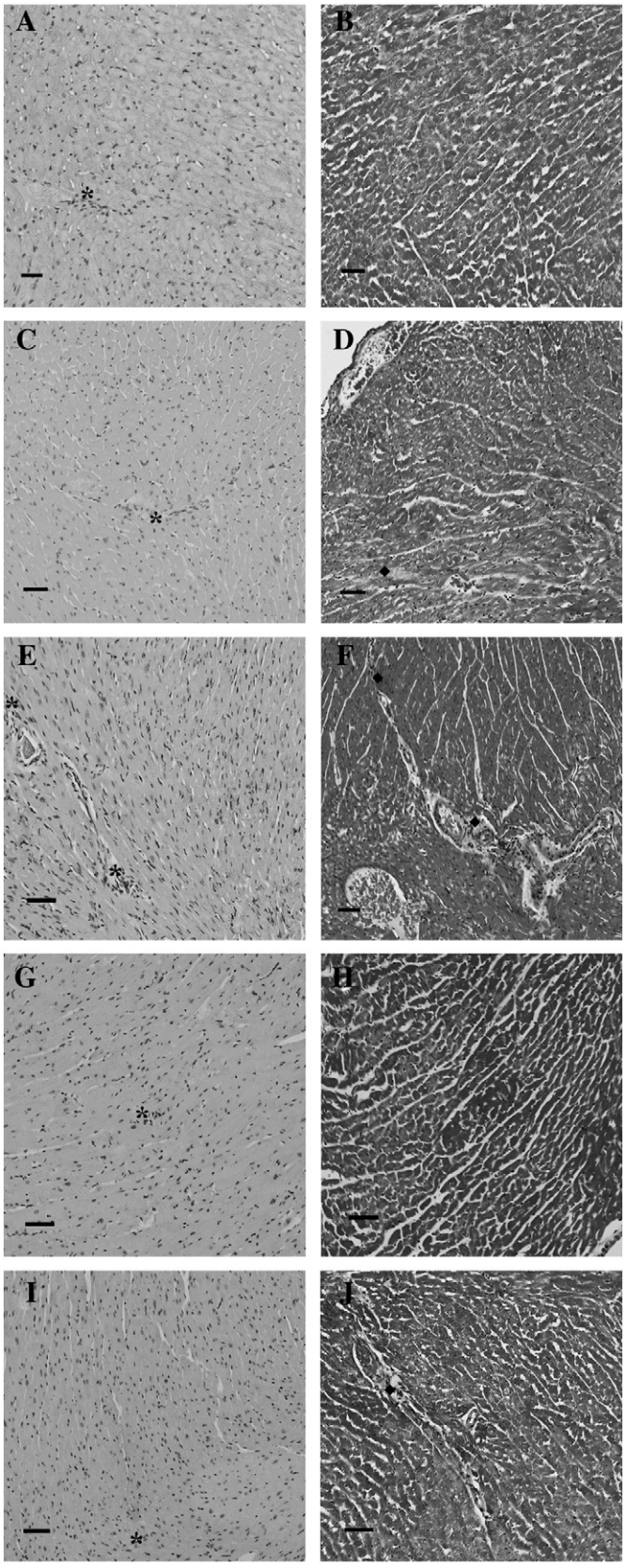


Fig. 3. A) Cardiac histological section from uninfected mice stained with hematoxylin–eosin, 90 dpi. 200×; B) Cardiac histological section from uninfected mice stained with Masson's technique, 90 dpi. 200×. No alterations were observed in either section (panels A and B). C) Cardiac histological section from *T. cruzi* (Tulahuen strain) infected mice, 90 dpi. Intense lympho-monocitary infiltrates (*) can be observed. 200×. D) Cardiac histological section from *T. cruzi* (Tulahuen strain) infected mice, 90 dpi. Fibrosis (♦) can be observed. 200×. The bars correspond to 50 μm.

A longer period of time is probably necessary to reverse the serology response, as has been demonstrated by other authors finding that parasite antigens persist for long periods in the infected host, even when parasitemia has been negativized by the treatment [31]. Several therapeutic studies confirm the usefulness of PCR to evaluate treatment outcome in either acute or chronic cases of Chagas disease [32–35]. PCR is a helpful tool for the early detection of treatment failure. Treatment with BZN and BZN/2 produced a decrease in parasitic load but the parasite was still detected by conventional techniques, and therefore the positive PCR results were expected.



Previous studies in our laboratory have reported that CLO is an important anti-parasitic agent, without apparent side effects [36,37]. Interestingly, our results show that CLO alone was less effective than BZN and BZN/2 to modify the values studied. It did not completely eliminate the presence of *T. cruzi* from the host, showing the highest parasitemia load in relation to the other treatments (BZN, BZN/2, BZN + CLO, BZN/2 + CLO) and lower survival (65% at 90 dpi). However, the kidneys and liver analyzed at the end of treatment with CLO showed no apparent injuries, confirming Bazán's observation [37].

Treatment with BZN + CLO produced a decrease in the parasitic load to levels undetectable by conventional techniques throughout the experience. It would therefore be advantageous to evaluate parasite load by quantitative real-time PCR, which can more precisely measure the impact of trypanocidal therapy on the course of the disease.

At 31 dpi mild abnormalities were found in the liver and kidneys of mice treated with BZN (50 and 100 mg/kg/day) plus CLO, in contrast to BZN and BZN/2 treatment, which showed more profound alterations such as necrosis, vascular congestion and dilatation, among others. In these cases, CLO seems to have a protective effect on these organs.

The most severe form of Chagas disease leads to cardiac arrhythmia, thromboembolism and congestive heart failure. Histologically, there is often severe chronic myocarditis caused mainly by mononuclear cells, with great hypertrophy of cardiomyocytes [38].

All treatments performed in this study, (BZN, BZN/2, CLO, BZN + CLO, BZN/2 + CLO) showed a significantly lower percentage of fibrosis in the myocardium compared to the infected and untreated group, at 90 dpi ($P < 0.05$). The cardiac disorders caused by inflammatory infiltrates and fibrosis that involve the conduction system can be detected through ECG studies, which provide important information about heart function and are a useful tool to measure the severity of chagasic cardiomyopathy [39]. In the chronic stage, a major complication is heart failure due to fibrosis and arrhythmias [40]. The parameters measured in ECG studies conducted in this study at 90 dpi (PR and QT) showed mild alterations in all groups, although combined-treated and BZN groups presented no significant differences with the uninfected group ($P < 0.05$). The treatments did not completely prevent electrocardiographic changes, but prevented them from becoming more serious, as also was found in Garcia et al. (2005) [41].

5. Conclusion

In conclusion, all treatments modified the values of most of the variables studied, with respect to untreated infected mice (NT). Benznidazole showed a higher efficacy in the acute phase than clomipramine, but necrosis foci were observed in the liver. Meanwhile, the combination of BZN + CLO and BZN/2 + CLO had results

Fig. 4. A–J Cardiac histological section from *T. cruzi* (Tulahuen strain) infected mice, 90 dpi. 200×. Left, cardiac histological section stained with hematoxylin–eosin and, right, stained with Masson's technique. Asterisk indicates inflammatory infiltrate and diamond indicates fibrosis. A) Mild inflammatory infiltrate can be observed in mice treated with BZN. B) No fibrosis can be observed in mice treated with BZN. C) Mild inflammatory infiltrate can be observed in mice treated with BZN/2. D) Mild fibrosis can be observed in mice treated with BZN/2. E) Moderate inflammatory infiltrate can be observed in mice treated with CLO. F) Mild fibrosis can be observed in mice treated with CLO. G) Mild inflammatory infiltrate can be observed in mice treated with BZN + CLO. H) No fibrosis can be observed in mice treated with BZN + CLO. I) Mild inflammatory infiltrate and can be observed in mice treated with BZN/2 + CLO. J) Mild fibrosis can be observed in mice treated with BZN/2 + CLO. The bars correspond to 50 μm.

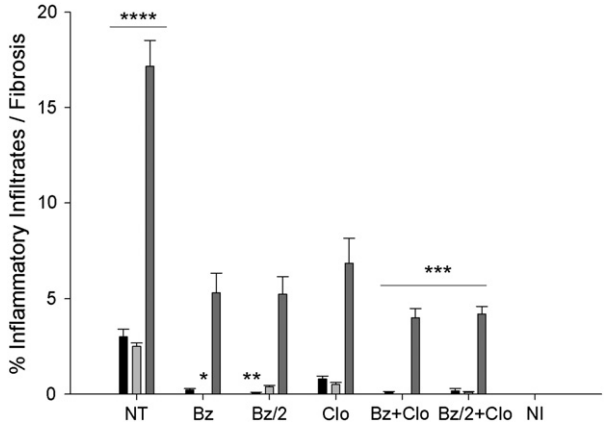


Fig. 5. Myocardium inflammatory infiltrates (black bars) and fibrosis (light gray bars) percentage; and skeletal muscle inflammatory infiltrates (dark gray bars) percentage from uninfected (NI) (n = 15), Tulahuen strain infected untreated (NT) (n = 9) and treated with BZN (n = 15), BZN/2(n = 15), CLO (n = 13), BZN + CLO (n = 15) and BZN/2 + CLO (n = 15) at, 90 dpi. Bars show standard error. *Mice heart, treated with BZN, did not present fibrosis and presented no significant difference between BZN + CLO and BZN/2 + CLO treatments, although there were significant differences with BZN/2 and CLO treatments and with the infected not treated group (Fisher test, $P < 0.05$). **Mice treated with BZN/2 significantly decreased the percentage of myocardium inflammatory infiltrates compared to BZN and CLO treatments, and with the infected not treated group (Fisher test, $P < 0.05$). But there was no significant difference with BZN + CLO and BZN/2 + CLO treatments. ***Myocardium inflammatory infiltrates and fibrosis; there were no significant differences in the percentage of skeletal muscle inflammatory infiltrates in mice treated with BZN + CLO and BZN/2 + CLO. ****The percentage of myocardium inflammatory infiltrates and fibrosis and skeletal muscle inflammatory infiltrates was significantly higher in the NT group than in the treated groups (Fisher test, $P < 0.05$).

comparable to those of benznidazole (100 mg/kg/day), but no necrosis foci were observed in the liver with these combinations. Further experimental investigations are needed to explain the protective effect of CLO on the tissues studied.

These results suggest the need to continue testing new protocols with different combinations and doses of new and safer anti-parasitic drugs that may be a hope for thousands of chagasic patients.

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