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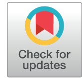
In vitro and *in vivo* drug combination for the treatment of *Trypanosoma cruzi* infection: A multivariate approach



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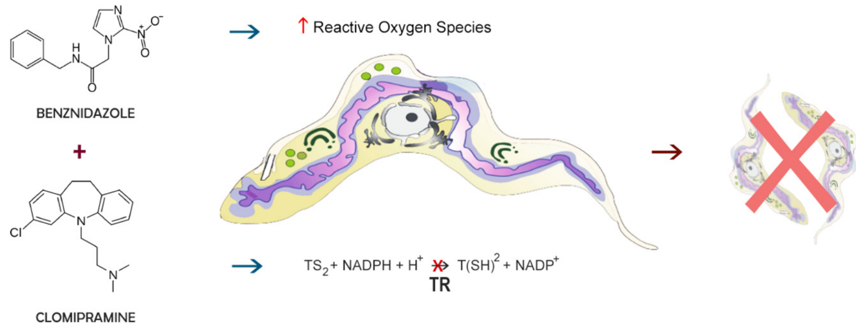
HIGHLIGHTS

- Benznidazole and clomipramine presented an anti-*T. cruzi* synergistic effect *in vitro*.
- Combined drugs are more active against the parasite than toxic to mammalian cells.
- Benznidazole and clomipramine increased the effectiveness of the treatment *in vivo*.

GRAPHICAL ABSTRACT



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ABSTRACT

Combination therapies based on the available drugs have been proposed as promising therapeutic alternatives for many diseases. Clomipramine (CLO) has been found to modify the evolution of the experimental infection. The objective of this study was to evaluate the combined effect of benznidazole (BZ) and clomipramine (CLO) against different life-stages of *Trypanosoma cruzi in vitro* and their efficacy in a murine model. Life-stages of *T. cruzi*, BZ-partially-resistant (Y) strain, were incubated with BZ and CLO and isobolograms and combination index (CI) were obtained. Swiss mice were infected with trypomastigotes and different treatment schedules were performed, each of which consisted of 30 consecutive daily doses. Treatment efficacy was evaluated by comparing parasitemia, qPCR, survival and histological analysis. These results were analyzed using multivariate analysis to determine the combined effect of the drugs *in vivo*. CLO + BZ showed synergistic activity *in vitro* against the clinically relevant life-stages of *T. cruzi*. The most susceptible forms were the intracellular amastigotes (CI: 0.20), followed by trypomastigotes (CI: 0.60), with no toxicity upon mammalian cells. The combination of both drugs CLO (1.25 mg/kg) and BZ (6.25 mg/kg), *in vivo*, significantly diminished the parasitic load in blood and the mortality rate. CLO + BZ presented a similar inflammatory response in cardiac and skeletal muscle (amount of inflammatory cells) to BZ (6.25 mg/kg). Finally, the results from the principal component

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analysis reaffirmed that both drugs administered in combination presented higher activity compared with the individual administration in the acute experimental model.

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1. Introduction

Chagas disease, caused by *Trypanosoma cruzi*, is a complex systemic disease with certain therapeutic limitations. Specific chemotherapy currently based on benznidazole (BZ) and nifurtimox have frequent undesirable side effects and cause biochemical damage to mammalian tissues (Bellera et al., 2015). Some studies highlight the benefit of benznidazole, recognizing however, the need to improve its efficacy and safety (Sperandio da Silva et al., 2017) probably through regimens in monotherapy or in combination (Torrico et al., 2018).

Combination therapies based on the available drugs for Chagas disease treatment and novel uses of old drugs, have been proposed as promising therapeutic alternatives for patients with Chagas disease (Perez-Mazliah et al., 2012; Morillo et al., 2017). Compared with monotherapy, combination therapy presents higher cure rates, reduced treatment times, lower side effects and, additionally delays the appearance of resistant parasites (Barrett and Croft, 2012).

Experimental studies have identified several novel targets for chemotherapy, one of them being the enzyme trypanothione reductase (TR), which is found in trypanosomatid parasites (Manta et al., 2013; Vázquez et al., 2017). The presence of these endogenous antioxidant molecules also provides *T. cruzi* with the ability to counteract the oxidizing environment generated by the cellular immune response of the host and by the action of trypanocidal redox cycling drugs (Thomson et al., 2003). TR is thus a promising target in the development of new chemotherapeutic agents for Chagas disease (Krauth-Siegel et al., 2012; Manta et al., 2013). Clomipramine (CLO), a tricyclic antidepressant, is a competitive inhibitor of TR and it does not inhibit human erythrocyte glutathione reductase (Benson et al., 1992).

Many efforts have been made in order to determine the better way to measure the combinatory effect of drugs. At least 13 different methods for the determination of synergism and antagonism have been categorized (Greco et al., 1995). Chou (2006) proposed a mathematically proofed method based on the mass-action law (Chou, 2006). This equation has been broadly used and expresses the results both numerically, as Combination Index, and graphically, as Isobolograms (Chou, 2006, 2010).

Taking all this into consideration we could hypothesize that the combination of drugs (BZ and CLO) with different mechanism of action, would improve the efficacy and at the same time reduce their doses and consequently diminish adverse reactions.

Previous studies have tested the activity of BZ and CLO in mice infected with the Tulahuen strain (Strauss et al., 2013; García et al., 2016), a BZ-susceptible *T. cruzi* strain (Cencig et al., 2012). Since parasite strain susceptibility could be a factor determining the effectiveness of the treatment, in the present work we evaluated the effect of BZ in combination with CLO *in vitro* and in mice acutely infected with a BZ-partially-resistant (Y) strain of *T. cruzi* and we used a multivariate approach (a principal component analysis, PCA) to better elucidate the effect of this combination.

2. Materials and methods

2.1. In vitro assay

2.1.1. Chemicals

Clomipramine hydrochloride was purchased from Sigma-Aldrich (St. Louis, MI, USA). Benznidazole tablets (100 mg - Abarax®, ELEA, Argentina) were ground in a mortar. The powder was transferred into a glass container and dispersed with 200 ml of distilled ethanol under heating (40–45 °C) and constant stirring for 30 min. The remaining solid was filtered and BZ was obtain from the dripped liquid by recrystallization, using temperature and solvent change, through the addition of 200 ml of cold distilled water. The BZ in solid state was extracted by filtration under vacuum and dried in oven at 45 °C until constant weight. The crystalline material (melting temperature 190.2–191.2 °C) had a purity of 99.2 ± 0.5% and yield of 87 ± 2% determined spectrofotometrically and was stored at room temperature in tightly-closed light resistant glass containers.

2.1.2. Cells and parasites

The *in vitro* experiments were performed with *T. cruzi* Y strain (TcII) (Zingales et al., 2009). Epimastigote forms were axenically cultivated at 28 °C in Liver Infusion Tryptose (LIT) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco Invitrogen, Grand Island, NY, USA), and maintained by weekly transfers (Camargo, 1994). The trypomastigotes were daily collected from the supernatant of infected LLCMK2 cells (Macaca mulatta epithelial kidney cells), and harvested by centrifugation. The mammalian cells, infected or not, were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco Invitrogen), pH 7.4, supplemented with 2 mM L-glutamine, 10% FBS, and 50 mg/L gentamicin at 37 °C in a humidified 5% CO2 atmosphere (Andrews and Colli, 1982).

2.1.3. Anti-proliferative activity against epimastigote forms

Epimastigotes (1×10^6 cells/mL) in the exponential phase of growth (96 h) were harvested and incubated in the presence of LIT supplemented with 10% FBS. Increasing concentrations of CLO and BZ combined were added. Parasites were then incubated at 28 °C in 24-well flat-bottom plates, and counted in a Neubauer hemocytometer after 96 h.

2.1.4. Activity against trypomastigote forms

Trypomastigotes (1×10^7 parasites/mL), obtained from the supernatant of infected $LLCMK_2$ cells, were inoculated in DMEM in 96-well plates in the presence or absence of increasing concentrations of CLO and BZ. Parasites were incubated for 24 h at 37 °C in a 5% CO_2 atmosphere, and then their viability was examined by mobility under a light microscope (Olympus CX31) using the Brener method (Brener, 1962).

2.1.5. Activity against intracellular amastigotes

$LLCMK_2$ cells were harvested and resuspended in DMEM plus 10% FBS and plated (2.5×10^5 cells/mL) in 24-well plates containing

round glass coverslips. When confluent growth was achieved, the cells were infected with trypomastigotes obtained from pre-infected cultures at a ratio of 10 parasites per 1 mammalian cell. After 24 h, the medium was removed and new DMEM with or without increasing concentrations of CLO and BZ was added. After 96 h at 37 °C in a 5% CO_2 atmosphere, the glass coverslips were subjected to fixation with methanol and Giemsa staining and permanently prepared with Entellan (Merck, Germany). The number of amastigotes was determined under a light microscope by counting randomly 200 cells in duplicate cultures.

2.1.6. Cytotoxicity assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay was applied. $LLCMK_2$ cells were collected from confluent cultures, plated in 96-well plates, and incubated at 37 °C in a humid 5% CO_2 atmosphere. After 24 h, the medium was replaced with new DMEM containing increasing concentrations of CLO and BZ. Following 96 h incubation, the cells were washed in PBS, and 50 μL of MTT (2 mg/mL) was added to each well. The formazan crystals were solubilized in DMSO and absorbance was read at 570 nm in a microplate reader (BioTek—Power Wave XS). The concentration that reduced 50% of the absorbance value observed in the control represented the CC_{50} (cytotoxic concentration for 50% of the cells).

2.1.7. In vitro data analysis

In order to measure the effect of the combination of CLO and BZ we applied the Combination Index method proposed by Chou and Talalay (1984) and reviewed by Zhao (2010). The experimental design consisted of combinations of at least five concentrations of each drug arranged in a checkerboard at a 1:2 concentration ratio. The concentrations of drugs that expressed 50% of inhibition (IC50) against the cells were determined for each drug by regression analysis. A selectivity index (SI) (SI = 50% cytotoxic concentration/50% effective concentration) was calculated. The combinatory effect was mathematically expressed as the Combination Index (CI = [IC50BZ combined/IC50BZ alone] + [IC50CLO combined/IC50CLO alone]; where the numerators are the concentrations of each drug that in combination are active against 50% of the cells, and the denominators are the concentrations that have this same effect for each drug alone). The interpretation of the CI was based on the broadly used specifications established by Chou and Talalay in which CI values less than, equal to, and more than 1 indicate synergism, additive, and antagonism, respectively (Choy and Talalay, 1984). The data were also graphically expressed as isobolograms.

2.2. In vivo assay

2.2.1. Animals and experimental design

Adult male and female albino Swiss mice, weighing 25±3 g, were inoculated, by intraperitoneal injection, with 50 trypomastigotes, Y strain; this amount of parasites is enough to reproduce the disease in this model (Bustamante et al., 2003; Lo Presti et al., 2014). The parasites used have been maintained in the laboratory by successive infections of new mice every 15 days. Mice were kept in controlled housing conditions (12 h' light period, 23 ± 3 °C, with food and water ad libitum) and the experimental procedures were carried out according to the Guide for the Care and Use of Laboratory Animals (Tolosa de Talamoni et al., 2010).

The infected animals were divided into: infected and non-treated (NT); infected and treated with benznidazole: 100 mg/kg (BZ100) and 6.25 mg/kg (BZ6.25); infected and treated with clomipramine: 5 mg/kg (CLO5) and 1.25 mg/kg (CLO1.25); infected and treated with the co-administration of BZ6.25 + CLO1.25; n = 10 in each group. BZ100 and CLO5 were selected according to previous studies (Khare et al., 2015; Rivarola et al., 2005, respectively). BZ6.25 and CLO1.25 correspond to the minimum concentrations that were able to kill the parasites, according to previous experiments using several concentrations, evaluating the results through parasitemia, qPCR and survival (data not shown).

In all treated groups the drugs were administered orally for 30 days daily, using as criteria for initiation of treatment the appearance of parasites in blood.

2.2.2. Parasitemia and survival

Parasitemias in all groups were determined in a Neubauer hemocytometer using blood samples obtained from the tail of the mice. Parasitemia was evaluated once a week until day 35 p. i., as previously described for similar models (Bustamante et al., 2003; Lo Presti et al., 2014). Survival of the different groups was monitored daily until 35 days p. i.

2.2.3. qPCR assay

Real-time Polymerase Chain Reaction (qPCR) was performed using blood, cardiac and skeletal samples from three mice of each group at day 35 p. i. DNA was extracted from the samples using the method described in Lachaud et al. (2001).

T. cruzi detection in each sample was performed by the amplification of a 188 bp nuclear fragment of the parasite DNA using two specific primers: TCZ-1 (5′- CGA GCT CTT GCC CAC ACG GGT GCT -3′) and TCZ-2 (5′- CCT CCA AGC AGC GGA TAG TTC AGG -3′) (Virreira et al., 2003). The quality of the DNA samples was verified by the 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′). Real-time PCR was performed by using KAPA SYBR Fast qPCR Master Mix (2X) (Kapa Biosystems) with 10 ng of total genomic DNA (gDNA). The samples were amplified with a Rotor-Gene Q (Quiagen) thermal cycler under the following conditions: a first step for 3 min at 94 °C and 40 cycles of 15 s at 94 °C, 30 s at 58 °C, 30 s at 72 °C, followed by a dissociation step. The reactions were performed by duplicate and a template control was included in each run as the real-time PCR negative control.

The results were based on a standard curve constructed with DNA from culture samples of *T. cruzi* epimastigotes, Y strain. Standard curve was generated from nine serial dilutions in water (1:10) of DNA extracted from culture containing 1×10^7 parasites and were performed by triplicate. The coefficient of determination (R^2) was calculated and considered as suitable when not lower than 0.99. The slope of the standard curves was used for the determination of PCR efficiency ($E = 10[\text{-1/slope}]^{-1}$). The efficiency was considered satisfactory when it is not lower than 90% and not above 110% (Wong et al., 2005).

2.2.4. Histopathological studies

After the treatment, three mice per group were selected and the cardiac and skeletal muscles were extracted. Tissues were fixed in buffered 10% formaldehyde (pH 7.0), embedded in paraffin, and 5 μm sections were stained with hematoxylin and eosin. A total of 9 slices from each group for each tissue was analyzed and the number of inflammatory infiltrates was determined with a 400X magnification for each slide. The number of inflammatory infiltrates per area in each section was quantified using the software ImageJ.

All mice were sacrificed by decapitation, using Ketamine ClH

(Ketalar®, Parke Davis, Warner Lambert Co, USA) anesthesia (10 mg/kg).

2.2.5. Statistical analysis

Statistical analyses were performed using SigmaPlot 12.0 and InfoStat software. The number of inflammatory infiltrates and parasites were compared using analysis of variance and multiple comparisons by the Fisher Test. Parasitemia data were analyzed by multivariate analysis MANOVA (Hotelling's test). Survival data were analyzed by Kaplan-Meier survival test. $P < 0.05$ was considered statistically significant.

Principal component analysis (PCA) was performed to improve the interpretability of the results of the drugs combinations *in vivo*. Four variables were used for the analysis: number of parasites/mL, number of inflammatory infiltrates in skeletal and cardiac muscles and percentage of survival. Logarithmic transformation for database normalization was performed in: number of parasites/mL, inflammatory infiltrates in skeletal and cardiac muscles. A biplot was generated based on the standardized data.

3. Results

3.1. In vitro activity assays

CLO presented selective activity *in vitro* against all the parasitic forms of *T. cruzi*. The tricyclic compound was able to inhibit epimastigotes (IC_{50}: 30.84 μM) in a time and dose-dependent manner. We also observed a clear reduction on the viability of trypomastigotes (IC_{50}: 9.34 μM) after 24 h treatment with CLO. Interestingly, the most susceptible forms were intracellular amastigotes (IC_{50}: 3.73 μM). Additionally, the anti-*T. cruzi* activity of CLO was improved when applied in combination with BZ *in vitro*. The isobolographs (Fig. 1H–K) indicate the synergistic interaction between BZ and CLO against *T. cruzi*, this effect is evidenced by the numerous points observed below the additivity. When tested against epimastigotes forms CLO in association with BZ showed a Combination Index (CI) of 0.96 and against trypomastigotes forms a CI of 0.60. The synergistic effect can be clearly noticed by comparing the activity against the intracellular parasites (CI: 0.20), while CLO

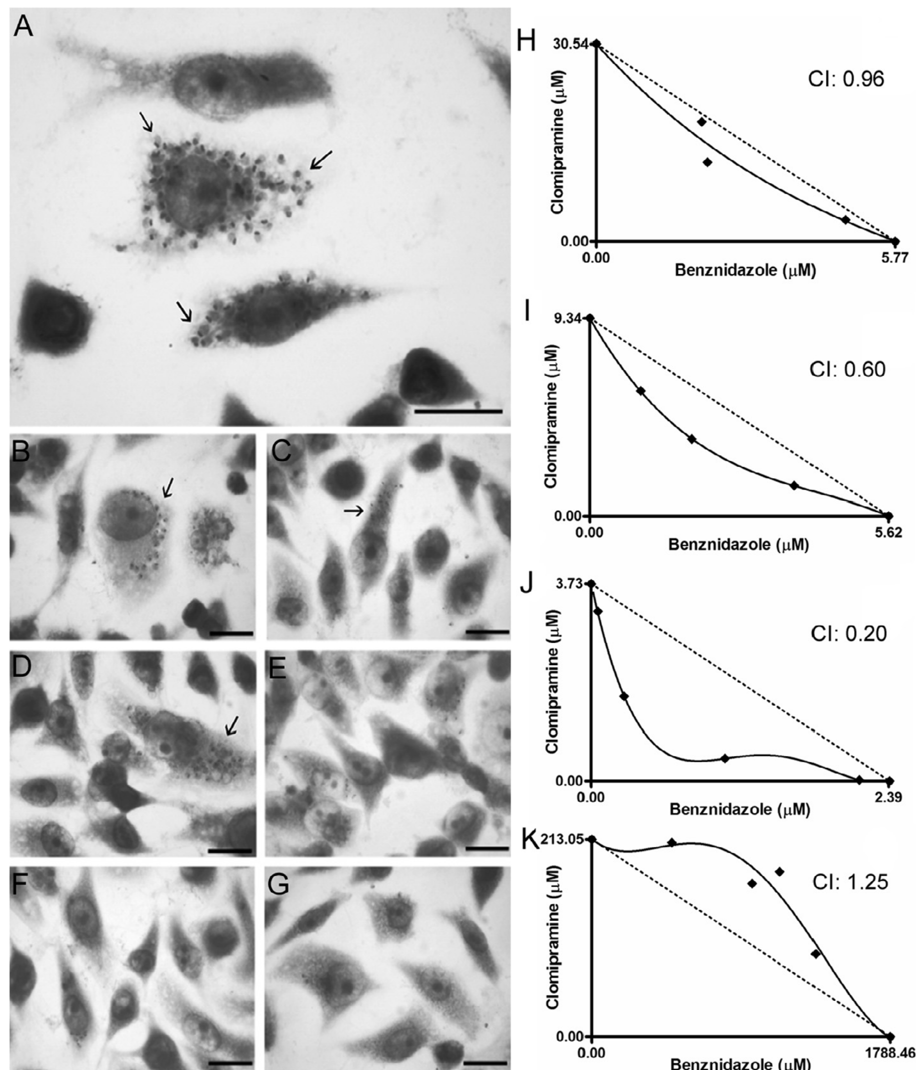

Fig. 1. A-G: Activity of clomipramine and benznidazole against intracellular amastigotes of *T. cruzi*. Control untreated cells (A), CLO 1.6 μM (B), CLO 3.2 μM (C), BZ 1.1 μM (D), BZ 2.2 μM (E), BZ 1.1 μM plus CLO 1.6 μM (F), BZ 2.2 μM plus CLO 3.2 μM (G). Representative images of three independent experiments. Arrow indicates intracellular amastigotes. Bars: 20 μm. **H-K**: Combination effect of clomipramine and benznidazole against *T. cruzi* and mammalian cells. Isobolographs describe the effect of the combination of clomipramine and benznidazole against epimastigotes (H), trypomastigotes (I), amastigotes (J) and LLCMK2 cells (K). The dotted lines correspond to the additivity effect. Points below the line indicate a synergistic effect. Points above the line indicate an antagonistic effect. The experiment was repeated three times. Dots show median values. Combination Indexes (CI) are also shown.

and BZ used alone were able to inhibit 50% of amastigotes proliferation at 3.73 μM and 2.39 μM, respectively; when combined, the same effect was observed at remarkably lower concentrations for both drugs, 0.58 μM and 0.87 μM. After 96 h incubation in the presence of both drugs, the number of infected cells and the amount of amastigotes inside each cell were reduced compared with the untreated control (Fig. 1A–G). Regarding the *in vitro* cytotoxicity, after 96 h incubation, the cytotoxic concentration 50 (CC_{50}) of CLO was 213.05 μM. An antagonistic effect on the cytotoxicity (CI: 1.25) was observed for the combination of both drugs. By comparing the CC_{50} with the concentration of the drug that inhibited 50% of the parasites, the drug was found to exhibit promising values of Selectivity Index (SI) for all the clinically relevant forms of *T. cruzi* (SI_{trypo}: 22.8; SI_{ama}: 57.1), indicating that clomipramine is more active against the parasite than toxic to mammalian cells *in vitro*.

3.2. Parasitaemia and survival

The evolution of parasitaemia levels can be observed in Fig. 2 A. Parasite levels from mice treated with BZ (6.25 and 100 mg/kg/day) were significantly lower than the NT group (Hotelling's test: $P < 0.05$). Parasite levels from mice treated with CLO (5 and 1.25 mg/kg/day) showed statistical differences on day 14 and 28 p. i. with the NT group (Fisher test: $P < 0.05$). The combined treatment, BZ6.25 + CLO1.25, showed statistical differences with CLO5 and CLO1.25 groups (Hotelling's test: $P < 0.05$). There was no statistical difference between BZ6.25 and BZ6.25 + CLO1.25, but the group treated with the combination presented an earlier decrease in the parasitic load than the BZ6.25.

By day 35 p. i., BZ6.25 + CLO1.25 group showed a significant reduction in blood parasite number shown by qPCR compared with either monotherapy (Fig. 2 C), (Fisher test: $P < 0.05$). The group of mice treated with BZ100 showed the lowest parasite load in blood and skeletal muscle (Fig. 2 C, E; Fisher test: $P < 0.05$). In cardiac muscle the parasitic load was lower compared to blood and skeletal muscle. Significant differences were found between the NT group and the treated groups, however no differences were found among the treated groups (Fig. 2 D Fisher test: $P < 0.05$). For the quantification of the parasites, a standard curve was plotted based on Ct values against the log of the estimated DNA copy number in the sample. The coefficient of determination (R^2) was 0.9981 (Fig. 2 C).

The survival of the groups BZ6.25 (9/10), BZ100 (10/10) and BZ6.25 + CLO1.25 (10/10) was significantly higher than the NT group (4/10), (Kaplan Meier: $P < 0.05$). The groups CLO5 (5/10) and CLO1.25 (5/10) showed similar survival rate to the NT group (Fig. 2 B).

3.3. Histology

Inflammatory infiltrates were observed in histological sections of cardiac and skeletal muscles from all groups (Fig. 3). In cardiac muscle, no statistical differences were found among all the studied groups regarding the number of inflammatory infiltrates (Fig. 4). In the skeletal muscle, statistical differences were found between the group treated with BZ100 and the rest of the groups (ANOVA among all groups Fisher test: $P < 0.05$); however when an ANOVA was performed among only the treated groups (without NT), BZ6.25 + CLO1.25 did significantly decrease the number of inflammatory infiltrates compared to CLO1.25, CLO5 and BZ6.25 (Fisher test: $P < 0.05$).

3.4. Analysis of the interaction with PCA

PCA computes new variables called principal components (PC) which are obtained as linear combinations of the original variables. The first PC (PC 1) is defined as the linear combination with maximal sample variance among all linear combinations of the variables. The next (PC 2) represents the linear combination that explains the maximal sample variance that remains unexplained by PC 1, with the additional condition that it is orthogonal to the first (Rencher, 1998). These two PCs were used to construct the biplot

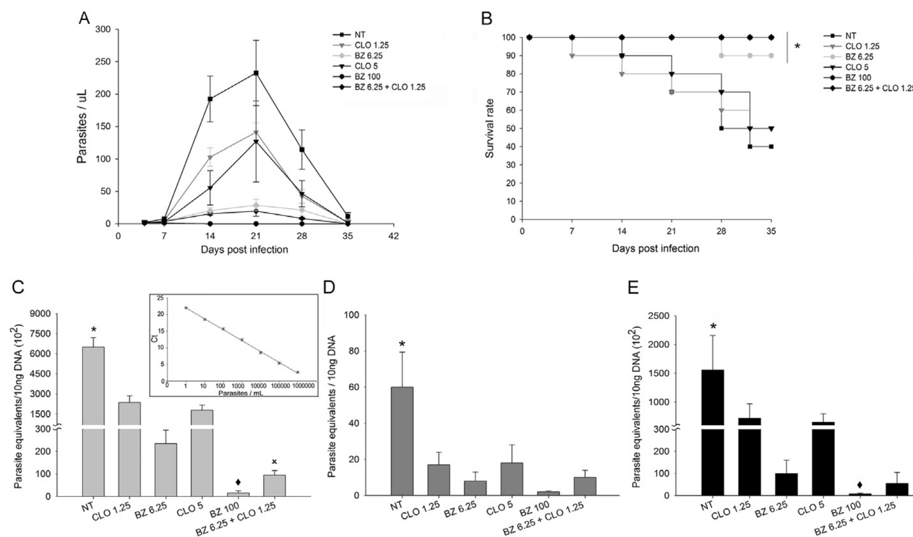

Fig. 2. A: Evolution of parasitemia in Albino Swiss mice inoculated with *T. cruzi*, Y strain, (n = 10, each group) and treated with BZ, CLO and BZ + CLO. **B**: Kaplan-Meier survival curve of infected and treated mice. Asterisks indicate a significant difference between the groups BZ100, BZ6.25, BZ6.25 + CLO1.25 and NT, CLO5, CLO1.25 (Log-rank test–Chi square, $P < 0.05$.) **C, D** and **E:** Blood, cardiac and skeletal muscle parasite load, respectively, by day 35 p. i. in Swiss mice infected with *T. cruzi* (Y strain) and under the different treatment schedules. Parasite equivalents/10 ng of DNA. Bars indicate standard error, (n = 3, each group). (*)The parasite load of the tissues from NT group was significantly higher compared with all the groups. (◆) Parasite load of BZ100 group in blood and skeletal muscle were significantly lower compared with all the groups. (✗) The blood parasite load was significantly lower in the BZ6.25 + CLO1.25 group among the BZ6.25 and CLO1.25 groups (Fisher test, $P < 0.05$). The insert in figure C is the dynamic range of the real-time PCR. DNA from epimastigotes (Y strain) was amplified in triplicates; 10-fold serial dilutions were used. The limit of detection was 1 parasite. Regression coefficient (R^2): 0.9981. Slope: -3.1964. Efficiency: 108%.

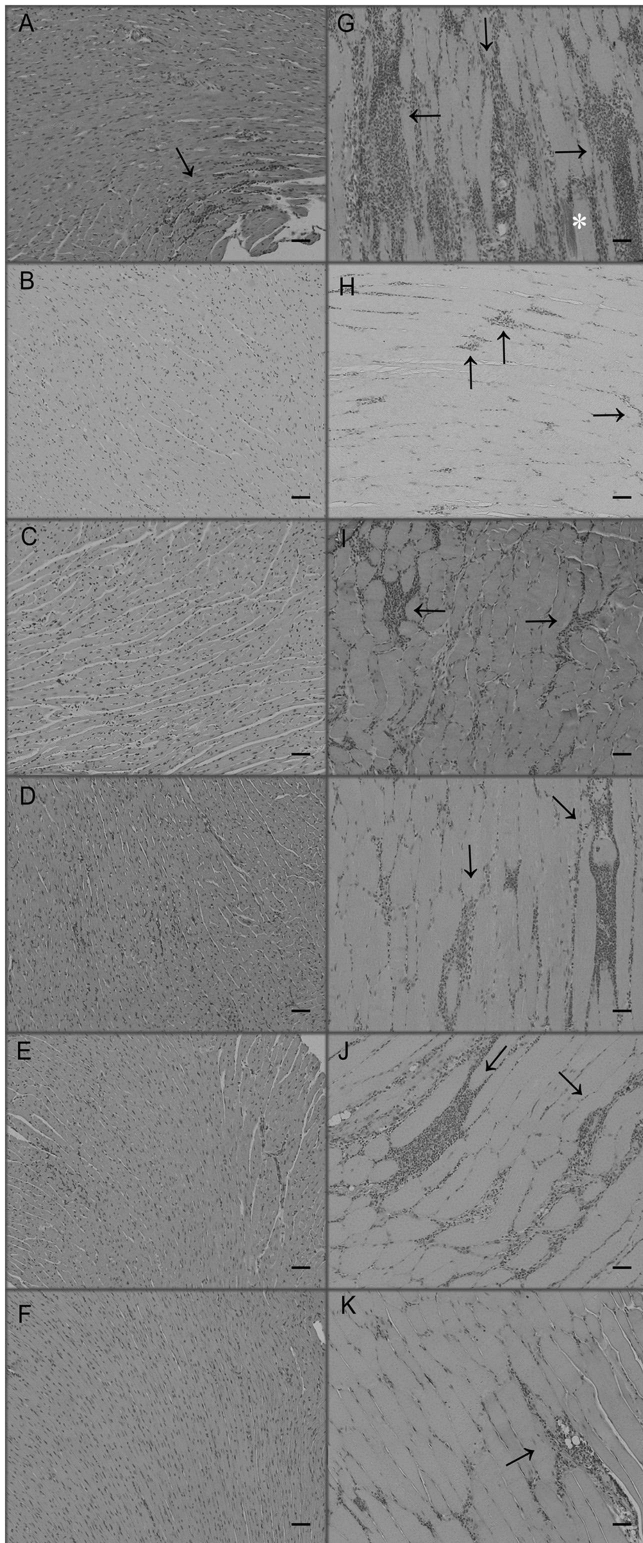

Fig. 3. Histological section from *T. cruzi* infected mice stained with hematoxylin-eosin, by day 35 p. i. 200X. A-F) Cardiac histological section from NT, BZ100, BZ6.25, CLO5, CLO1.25 and BZ6.25 + CLO1.25 respectively. G-L) Skeletal muscle histological section from NT, BZ100, BZ6.25, CLO5, CLO1.25 and BZ6.25 + CLO1.25 respectively. Amastigote nest (*) can be observed. Arrows indicate intense lympho-monocitary infiltrates. The bars correspond to 50 μm.

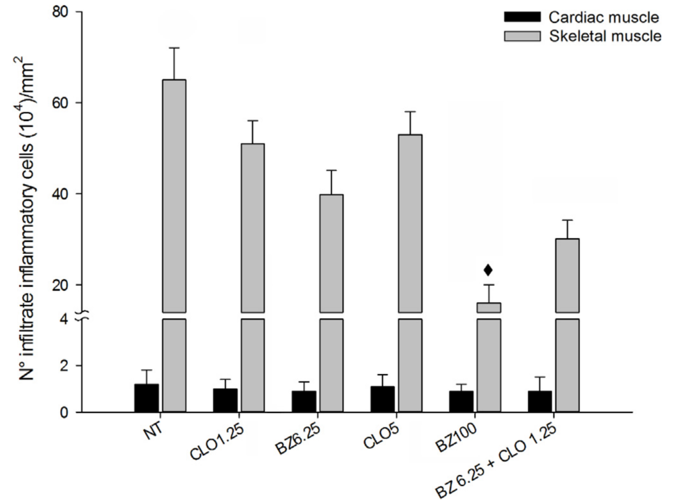

Fig. 4. Number of inflammatory infiltrates in cardiac and skeletal muscle from mice infected non-treated (NT) or under the different treatment schedules. Bars indicate standard error. (◆) The number of muscle inflammatory infiltrates was significantly lower in the BZ100 group among the non-treated and treated groups (Fisher test, $P < 0.05$).

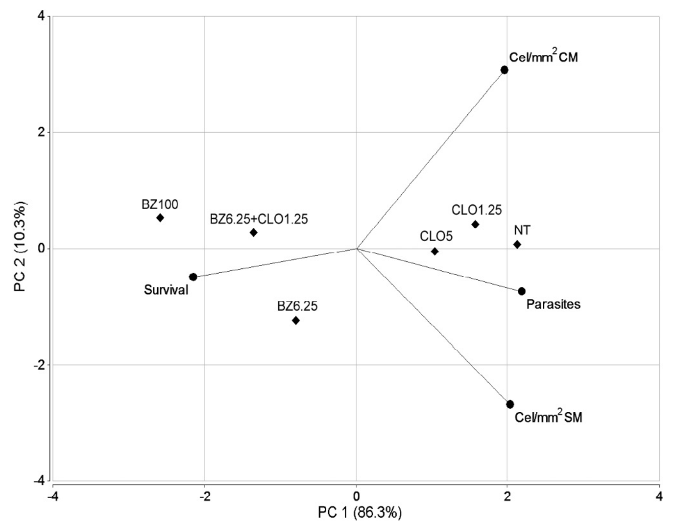

Fig. 5. Biplot of a principal component analysis (PCA) performed on the interaction between treatments and the variables analyzed. The axes represent the first two principal components (PC) and the percentage of the total variability explained by each PC is shown between parenthesis. As can be observed, the first two components explain more than 96% of the variance. Black circles indicate the variables included in the analyses: N° of inflammatory infiltrates in cardiac (Cel/mm2 CM) and skeletal muscle (Cel/mm2 SM), n° of parasites and percentage of survival. Black diamonds indicate the different treatments.

shown in Fig. 5. PC 1, shown on the x-axis (which explains more than 86% of the variation), contrasts survival with the rest of the variables (parasitemia and number of inflammatory cells in cardiac and skeletal muscles), since they are plotted on opposite positions in this axis (lines with end black circle). From this, PC 1 would represent characteristics that are inversely correlated with the survival of the mice after the treatments (that is, a higher parasitemia and a higher inflammation in the tissues would result in a lower survival). PC 2 is shown on the y-axis and only explains 10.3%

of the variation; therefore, the grouping of the variables according to this axis is somehow negligible.

As can be observed in the figure, PC 1 also indicates variation which distinguishes among the treatments. Firstly, BZ100 contrasts with the NT group; the distribution of these groups in relation to the variables analyzed indicates that BZ100 showed higher survival (since it is plotted closer to this parameter) and lower number of parasites and inflammatory infiltration than NT group. The group treated with the combination of BZ6.25 + CLO1.25 is plotted between BZ100 and BZ6.25 indicating that the use of both drugs (CLO and BZ) in combination increases the effectiveness of the treatment, when a lower dose of BZ is used.

4. Discussion

Several studies have been carried out using combination of drugs for the treatment of *T. cruzi* infection. For instance, promising results have been found in different stages of the disease using BZ + posaconazole (Diniz et al., 2013), BZ + pentoxifylline (Vilar-Pereira et al., 2016), BZ + E1224 (Torrico et al., 2018), among others.

On the present study, we found a synergistic effect of CLO in combination with BZ, *in vitro*, against clinically relevant forms of a BZ-partially-resistant strain (Y), evidenced by isobolograms and a CI < 1 ($CI_{trypo} = 0.60$; $CI_{ama} = 0.20$). Similar results were found with Tulahuen strain trypomastigotes (CI = 0.375) (García et al., 2016), a BZ-susceptible *T. cruzi* strain (Cencig et al., 2012). In addition, an antagonistic effect upon mammalian cells (CI: 1.25) was observed, which allow us to infer that the combination was not cytotoxic.

The synergistic combination between BZ and CLO can be understood as a result of a cooperative interaction between the mechanisms of action of both drugs. Knowing that BZ is a prodrug able to generate electrophilic metabolites and induce oxidative stress in the parasite (Hall and Wilkinson, 2012) and considering that CLO acts through the inhibition of trypanothione reductase (Vázquez et al., 2017), they would act cooperatively against the parasite: BZ would deplete the antioxidant defenses of the parasite while CLO would avoid the generation of new reduced molecules (Maya et al., 2007). Thus, this combination of effects would be able to kill trypanosomes more efficiently, since they are particularly more susceptible to oxidative stress than mammalian cells.

Conclusions drawn from *in vivo* studies to determine synergism or antagonism are not as evident as those from *in vitro* studies. Therefore, in the present study, principal component analysis was performed to better elucidate the combination effect of BZ6.25 + CLO1.25 upon the murine model. This multivariate analysis is widely used in different areas with the purpose to improve the interpretation of the results when multiple variables contribute to a certain event/outcome (de Haan et al., 2007; ur Rehman et al., 2015).

In the present work, we used 6.25 mg/kg/day of BZ and 1.25 mg/kg/day of CLO; these concentrations were previously found to be the minimum effective doses assessed through parasitemia, qPCR and survival (data not shown). The combination of these doses in the murine model, demonstrated an improvement in most of the parameters analyzed here: parasite load (in blood and cardiac and skeletal muscles), histological analysis and survival. Taking all these variables into consideration, the multivariate analysis plotted the BZ6.25 + CLO1.25 group between BZ100 and BZ6.25 being closer to BZ6.25, which would indicate that the therapeutic effect would be mostly given by this concentration of BZ. This analysis showed that the combination of both drugs (CLO and BZ) increased the effectiveness of the treatment in mice infected with a BZ-partially-resistant strain of *T. cruz*i (Y strain) (Cencig et al., 2012).

In previous studies we showed, in Swiss mice infected with the Tulahuen strain (a BZ-susceptible *T. cruzi* strain), that the combination of CLO (5 mg/kg/day) and BZ (50 mg/kg/day) in the acute phase resulted in no electrocardiographic abnormalities when studied by day 90 p. i. (Strauss et al., 2013). This result was associated with the data obtained in BALB/c mice also infected with the Tulahuen strain and treated with CLO (7.5 mg/kg/day) and BZ (25 mg/kg/day), which showed less cardiac damage in the chronic phase (90 d. p.i.) than mice treated only with BZ (100 mg/kg/day) (García et al., 2016). In these studies, however, BZ doses were high enough to have these effects on their own and, therefore, the effect of the combination is difficult to define. For instance, Cevey et al. (2016), found that treatment with 25 mg/kg/day was as effective as 100 mg/kg/day, when used in the acute phase in BALB/c mice infected with the RA strain, a BZ-susceptible *T. cruzi* strain. Here, by using the minimum effective doses of both drugs and by analyzing the results using a multivariate approach, we could evidence an increased effectiveness of the treatment, although the therapeutic effect would be still mostly given by BZ as shown by the results using BZ6.25. Additionally, to better assess the effectiveness of the combination, a follow-up study would be necessary, including electro and echocardiographic parameters, to determine the therapeutic effect upon clinical evolution of the infection.

Even though treatment with BZ100 showed better results improving all the parameters analyzed, this treatment has been shown to have several limitations, including a variety of adverse effects and limited efficacy in the later chronic phase of the infection (Soeiro et al., 2009; Machado et al., 2010; Morillo et al., 2015). Clinical efficacy of BZ in chronic patients is still under debate: the BENEFIT clinical trial, in individuals with established heart disease associated with Chagas disease, demonstrated that benznidazole treatment decreased the number of patients with *T. cruzi* DNA detected by PCR but did not change the clinical outcome over 5 years of follow-up (Morillo et al., 2015). Additionally, some studies point to a high proportion of treatment suspension due to BZ adverse effects (Viotti et al., 2009; Sales Junior et al., 2017). Consequently, there is an urgent need for the development of alternative treatments. Combined therapies like the present, are a valid approach, since they may enhance efficacy by acting upon different cellular targets, may reduce toxicity due to the lower dose levels and minimize the risk of drug resistance (Diniz et al., 2013; Vilar-Pereira et al., 2016).

5. Conclusions

The *in vitro* experiments performed in this work showed a synergistic interaction between BZ and CLO upon different cellular forms of *T. cruzi*; the *in vivo* experiments additionally showed a positive interaction between the drugs when administered in the acute phase of the infection with a BZ-partially-resistant strain of *T. cruzi.* In conclusion, all these results reaffirm the possibility of the use of combined therapies for the treatment of Chagas disease and provide useful information that could be used in new combination schemes using other experimental models.

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Competing interests

All authors: none to declare.

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