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RELEVANCE OF TRYPANOTHIONE REDUCTASE INHIBITORS ON *TRYPANOSOMA CRUZI* INFECTION

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ABSTRACT

Due to the rudimentary antioxidant defenses in trypanosomatids, disruptors of redox balance, especially trypanothione reductase (TR) inhibitors, are promising candidates for new anti-trypanosomal drugs. However, the relevance of these drugs in the treatment of Chagas disease remains poorly explored. From primary and secondary investigations, we analyzed the relevance of TR inhibitors in the treatment of *T. cruzi* infection *in vivo*. In our secondary approach, we used an integrated framework based on systematic review, meta-analyses and molecular modeling. Our findings indicated that the TR inhibitors analyzed, especially clomipramine and thioridazine, presented no beneficial effects on infection-related electrocardiographic abnormalities. However, were effective in reducing parasitemia and mortality in *T. cruzi*-infected mice. The affinity between TR inhibitors and TR was confirmed by molecular docking. However, the molecular affinity score was unable to explain TR inhibition and the anti-parasitic potential of these drugs in vitro and *in vivo*, indicating that the anti-*T. cruzi* effects may not be restricted to TR inhibition. As *in vivo* studies on TR inhibitors are scarce and exhibit methodological limitations, mechanistic and highly controlled studies are required to improve the quality of evidence. From this observations, we used a murine model of Chagas disease to compare the antiparasitic potential of thioridazine (TDZ) and benznidazole (Bz) administered in monotherapy and combined. Female Swiss mice were randomized into six groups: (i) uninfected untreated, (ii) infected untreated and infected and treated with (iii) Bz (100 mg/kg), (iv) TDZ (80 mg/kg), (v) Bz (100 mg/kg) + TDZ (80 mg/kg), (vi) Bz (50 mg/kg) + TDZ (80 mg/kg). Infected animals were inoculated with 2000 T. cruzi trypomastigotes (Y strain) and treated by gavage during 20 days. The animals treated with TDZ presented the highest levels of parasitemia, parasitic load, anti-T. cruzi immunoglobulin G plasma titers, plasma and/or cardiac cytokine levels (IFN-γ, TNF-α, IL-10 and IL-17), as well as cardiac, skeletal muscle and hepatic damage compared to the other groups ($P<0.05$). These parameters were significantly reduced in the group treated with Bz-based monotherapy compared to the other infected groups ($P<0.05$). The combination of TDZ with Bz at the therapeutic dose (100mg/kg) and mainly at half of this dose attenuated the response to treatment, worsening parasitological control, systemic and tissue inflammation, as well as microstructural lesions of all organs investigated compared to the group treated with Bz alone ($P<0.05$). Thus, our results indicated that when administered alone, TDZ potentiated pathological outcomes in T. cruzi-infected animals. Because TDZ attenuated the antiparasitic effect of Bz, impairing parasitological control and potentiating inflammation and pathological remodeling of the heart, skeletal muscle and liver; Bz-based monotherapy remains a better option for the treatment of experimental Chagas disease.

Keywords: Chagas disease. Experimental chemotherapy. Experimental parasitology. Experimental pathology. Human parasite protozoology. Parasitic diseases.

RESUMO

Devido às defesas antioxidantes rudimentares de tripanossomatídeos, os disruptores do equilíbrio redox, especialmente os inibidores da tripanotiona redutase (TR), são candidatos promissores para o desenvolvimento de novos medicamentos anti-tripanossomais. No entanto, a relevância dessas drogas no tratamento da doença de Chagas permanece pouco explorada. A partir de investigações primárias e secundárias, nós analisamos a relevância dos inibidores de TR no tratamento da infecção por T. cruzi in vivo. Em nossa abordagem secundária, utilizamos uma estrutura integrada baseada em revisão sistemática, metanálises e modelagem molecular. Nossos achados indicaram que os inibidores da TR analisados, especialmente a clomipramina e a tioridazina, não apresentaram efeitos benéficos nas anormalidades eletrocardiográficas relacionadas à infecção. No entanto, foram eficazes em reduzir a parasitemia e mortalidade em camundongos infectados com T. cruzi. A afinidade entre os inibidores e a enzima TR foi confirmada por docking molecular. No entanto, o escore de afinidade molecular não foi capaz de explicar a inibição da TR e o potencial antiparasitário desses medicamentos in vitro e in vivo, indicando que o efeito anti-T. cruzi pode não ser restrito à inibição da TR. Como os estudos in vivo sobre inibidores da TR são escassos e apresentam limitações metodológicas, são necessários estudos mecanísticos e altamente controlados para melhorar a qualidade da evidência. A partir dessas observações, utilizamos um modelo murino da doença de Chagas para comparar o potencial antiparasitário da tioridazina (TDZ) e do benznidazol (Bz) administrados em monoterapia e combinados. Nesse estudo primário, camundongos suíços fêmeas foram randomizados em seis grupos: (i) não infectados e não tratados (ii) infectados e não tratados. Infectados e tratados com (iii) Bz (100 mg/kg), (iv) TDZ (80 mg/kg), (v) Bz (100 mg/kg) + TDZ (80 mg/kg), (vi) Bz (50 mg/kg) + TDZ (80 mg/kg). Os animais infectados foram inoculados com 2000 tripomastigotas de T. cruzi (cepa Y) e tratados por gavagem durante 20 dias. Os animais tratados com TDZ apresentaram os maiores níveis de parasitemia e carga parasitária, títulos plasmáticos de imunoglobulina G anti-T. cruzi, elevados níveis plasmáticos e/ou cardíacos de citocinas (IFN-γ, TNF-α, IL-10 e IL-17), além de danos cardíacos, musculares esqueléticos e hepáticos em comparação com os outros grupos ($P<0,05$) Esses parâmetros foram significativamente reduzidos no grupo tratado com monoterapia baseada em Bz em comparação com os outros grupos infectados ($P<0,05$). A combinação de TDZ com Bz na dose terapêutica (100 mg/kg) e principalmente na metade dessa dose atenuou a resposta ao tratamento, piorando o controle parasitológico, a inflamação sistêmica e tecidual, além de promover lesões microestruturais em todos os órgãos investigados em relação ao grupo tratado com Bz em monoterapia ($P<0,05$). Assim, os nossos resultados indicaram que, quando administrados isoladamente, a TDZ potencializou os desfechos patológicos em animais infectados com T. cruzi. Uma vez que a TDZ atenuou o efeito antiparasitário do Bz, prejudicando o controle parasitológico e potencializando a inflamação e a remodelação patológica do coração, músculo esquelético e fígado; a monoterapia baseada em Bz continua sendo uma melhor opção para o tratamento da doença de Chagas experimental.

Palavras-chave: Doença de Chagas. Patologia experimental. Parasitologia experimental. Protozoologia parasitária humana. Quimioterapia experimental.

SUMMARY

CHAPTER 1

RELEVANCE OF TRYPANOTHIONE REDUCTASE INHIBITORS ON *TRYPANOSOMA CRUZI* INFECTION: A SYSTEMATIC REVIEW, META-ANALYSIS AND *IN SILICO* INTEGRATED APPROACH

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ABSTRACT

Due to the rudimentary antioxidant defenses in *Trypanosoma cruzi*, disruptors of redox balance are promising candidates for new anti-trypanosomal drugs. We developed an integrated model based on systematic review, meta-analyses and molecular modeling to evaluate the effect of trypanothione reductase (TR) inhibitors in *T. cruzi* infections. Our findings indicated that the TR inhibitors analyzed were effective in reducing parasitemia and mortality due to *Trypanosoma cruzi* infection in animal models. The most investigated drugs (clomipramine and thioridazine) showed no beneficial effects on the occurrence of infection-related electrocardiographic abnormalities or the affinity and density of cardiac β-adrenergic receptors. The affinity between the tested ligands and the active site of TR was confirmed by molecular docking. However, the molecular affinity score was unable to explain TR inhibition and *T. cruzi* death *in vitro* or the anti-parasitic potential of these drugs when tested in preclinical models of *T. cruzi* infection. The divergence of *in silico*, *in vitro*, and *in vivo* findings indicated that the anti-*T. cruzi* effects of the analyzed drugs were not restricted to TR inhibition. As *in vivo* studies on TR inhibitors are still scarce and exhibit methodological limitations, mechanistic and highly controlled studies are required to improve the quality of evidence.

Keywords: antioxidants agents, antiparasitic drugs, Chagas disease, chemotherapy, parasitic diseases.

1. INTRODUCTION

Chagas disease is a neglected tropical disease caused by the protozoan parasite *Trypanosoma cruzi*. Recent estimates indicate that 6–7 million people are infected by this parasite worldwide, while at least 120 million people are at risk of infection (PEREIRA et al., 2017; WHO, 2018). Chagas disease is associated with poverty and is endemic in Central and South Americas countries. Due to migratory flow of infected people, cases of infection registered in non-endemic areas are on the rise, especially in North America (over 300,167 cases) and Europe (almost 181,181 cases) (PEREIRA et al., 2017; WHO, 2018). While vector-borne transmission, intake of contaminated foods, and congenital transmission are the main forms of *T. cruzi* infection in endemic countries (BERN, 2015; MURCIA et al., 2013), autochthonous iatrogenic cases secondary to blood transfusion, organ transplant from infected donors, and also congenital transmission are the most frequent infection pathways in non-endemic regions (BERN, 2015; MURCIA et al., 2013)..

Chagas disease is a life-threatening illness associated with at least 50,000 deaths/year worldwide, especially due to sudden cardiac death (60%), heart failure (25%), and stroke (15%) (STANAWAY AND ROTH, 2015). Although the course of infection includes neural (autonomic neuropathy) and digestive disorders (mega syndromes), chronic Chagas cardiomyopathy (CCC) is the most severe and incapacitating clinical form of the disease (BERN, 2015; NOVAES et al., 2016). Approximately 30% of the infected patients progress to CCC which manifests as diffuse heart fibrosis and hypertrophy, complex electrical abnormalities and arrhythmias, thromboembolic events, and congestive heart failure (BERN, 2015; BOCCHI, 2013). Chronic Chagas cardiomyopathy is the most common cause of non-ischemic cardiomyopathy in South America (FREITAS et al., 2005) and the third most common cause of heart transplantation in Brazil (ANDRADE et al., 2011). CCC is also associated with a higher mortality hazard ratio (2.48) than non-infectious cardiomyopathies (FREITAS et al., 2005; NUNES et al., 2010).

Due to limited effectiveness of the strategies to control parasite transmission (i.e., vector control, screening of infected pregnant women, blood and organ banks) and infection treatment, Chagas disease incurs US$7.19 billion per year in health-care costs, and more than 10% of that amount comes from non-endemic countries such as the USA and Canada (LEE et al., 2013). Nifurtimox and benznidazole-based chemotherapy (developed more than 40 years

ago) remains the main strategy for the etiological treatment of Chagas disease (LEE et al., 2013; SANTOS et al., 2015). Although these drugs present acceptable effectiveness in acute infections (an approximately 60% cure rate), they are highly toxic and achieve low cure rates (10%–20%) in chronic infections. As Nifurtimox is no longer used in most Central and South American countries due to excessive side effects (i.e., hypersensitivity reactions, polyneuritis, toxic hepatitis, bone marrow depression, immunosuppression, and cancer), benznidazole is often the only drug available (URBINA and DOCAMPO, 2003; URBINA, 2010). As progress in drug development has been very limited in recent decades, new and less toxic anti-trypanosomal treatments are urgently needed (NOVAES et al., 2016; OMAR and KLAN, 2007).

Considering that most neglected tropical diseases are not included in the research and development platforms of pharmaceutical industries (URBINA, 2010; CHIRAC and TORREELE, 2006), the prospect of new drugs for the treatment of Chagas diseases is not promising. Thus, drug repositioning by identifying commercially available products with anti-*T. cruzi* potential (GOUPIL and MCKERROW, 2014) provides a strong rationale and viable screening option (FERREIRA and ANDRICOPULO, 2016). From the characterization of a rudimentary metabolic pathway associated with antioxidant defenses in Trypanosomatids (TURRENS, 2004; KRAUTH-SIEGEL and COMINI, 2008), disruptors of redox balance are proposed as candidates for new anti-trypanosomal drugs (OMAR and KLAN, 2007; TURRENS, 2004; HUNTER et al., 1992). There is evidence that the enzyme trypanothione reductase (TR) plays a pivotal role in maintaining the functional integrity of antioxidant systems in Trypanosomatids. Accordingly, inhibition of this FAD-cystine-oxidoreductase is effective at increasing parasite susceptibility to oxidative stress, which, together with the immune system, integrates hosts major line of defense against parasitic infections (SORCI and FAIVRE, 2009; MACHADO et al., 2012). As TR is not expressed in vertebrate hosts, this enzyme represents a potentially useful molecular target for rational drug design (OMAR and KLAN, 2007; TURRENS, 2004; RIVAROLA and PAGLINI-OLIVA, 2002).

Several anti-inflammatory, antineoplastic, antidepressant, anxiolytic, and antipsychotic drugs are TR inhibitors (OMAR and KLAN, 2007; LO PRESTI et al., 2015); and their specific effects are potentially mediated by interaction of these drugs with active sites on TR, especially the FAD- and NADPH-binding domains (HUNTER et al., 1992). As current

knowledge of enzyme inhibition and anti-trypanosomal effects is based on *in silico* (OMAR and KLAN, 2007; MARTYN et al., 2007) and *in vitro* (BELTRAN-HORTELANO et al., 2017; CHACÓN-VARGAS et al., 2017) systems, it is unknown if and to what extent these effects can be reproduced *in vivo*. In addition to being fragmented, the current evidence is based on few research initiatives, and the specific inhibitors used and their effectiveness at modifying the pathogenesis of *T. cruzi* infection is still obscure.

In the current manuscript, we systematically review the *in vivo* preclinical evidence on the relevance of TR inhibitors in Chagas disease. In addition to exploring the main characteristics of the experimental models, parasite strains and protocols of treatment, meta-analyses were used to calculate the effect sizes and direction of parasitological, biochemical, and electrophysiological outcomes. From an integrated *in silico* approach, we also investigated if and to what extent the effect size obtained for each drug could be explained by variations in chemical structures, molecular interactions and the rate of TR inhibition. The methodological quality of each study identified was also evaluated, and the main sources of bias that undermine the quality of evidence were pointed out.

2. METHODOLOGY

2.1. Search strategy

The search strategy was based on two steps according to Pereira et al. (PEREIRA et al., 2017) and based on PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-analyses) statement (MOHER et al., 2009). A direct search was carried out in three comprehensive electronic databases: PubMed/MEDLINE, Web of Science, and Scopus. A secondary search was based on screening of the reference list of all relevant studies identified in the direct search.

Structured search filters were developed for each database (Table S1). The search filters were initially constructed by considering standardized descriptors [Medical Subject Headings (MeSH)] extracted from PubMed thesaurus. All descriptors were combined in a complete three-level search strategy based on (i) *in vivo* preclinical models, (ii) disease (American trypanosomiasis), and (iii) therapeutic intervention (TR inhibitors). Standardized descriptors were defined by the MeSH algorithm, and non-MeSH descriptors were characterized by the [TIAB] algorithm, which was also used to recover recently published but non-indexed studies

(*in process*). A previously published and optimized animal filter was applied in the PubMed search interface (HOOIJMANS et al., 2010). The same search filters used for disease and intervention were adapted for Web of Science and Scopus. An animal filter was created for Web of Science considering the animal models identified in all studies recovered from the PubMed/MEDLINE database. Scopus's own animal filter (Keyword – animals [limit to]) was used in this database. No chronological or language limits were applied in our search strategy. All relevant studies published until September 30, 2017 (updated search date) were recovered and included in the systematic review and meta-analysis.

2.2. Records screening and eligibility

All research records recovered in the database search were analyzed, and duplicates were removed considering the authors, title, journal, and year of publication. After title and abstract screening, all potentially relevant studies were evaluated in full-text for eligibility according to specific inclusion and exclusion criteria. Only studies investigating the relevance of TR inhibitors to *in vivo* preclinical models of Chagas disease were included in the review. The exclusion criteria were (i) no full-text available, (ii) secondary studies (i.e., editorials, commentaries, and letters to the editor), (iii) observational and epidemiological reports, (iv) studies without control groups, and (v) studies exclusively investigating *in vitro* and human systems. Literature reviews were considered only when original data were additionally reported. Eligibility was independently analyzed by the researchers A.A.S.M, R.D.N and R.V.G. Disagreements were resolved by consensus. To enhance the comprehensiveness of the research strategy, the reference lists of all relevant papers identified from database searches were screened for additional studies.

2.3. Data extraction and synthesis

Data extraction was based on basic methodological requirements for preclinical studies, as previously described by Pereira (PEREIRA et al., 2017). Considering a detailed characterization of all relevant studies identified, essential data grouped into four descriptive levels were extracted and summarized as follows: (**i**) publication characteristics: authors, publication year, and countries; (**ii**) experimental design (animal model): species, lineage, sex, age, and weight; (**iii**) experimental design (disease model): parasite species and strain,

inoculum size, route of parasite administration, duration of infection; and (**iv**) main research outcomes (i.e., parasitemia, parasitic load, immunological markers, histopathological findings, and mortality).

Quantitative data on key outcome measures commonly investigated in preclinical *in vivo* parasitological studies on anti-*T. cruzi* chemotherapy were extracted. Mean and standard deviation (mean ± SD) were directly extracted when available in tables or text. When these data were reported in graphics, an image analysis software program (Image-Pro Plus 4.5, Media Cybernetics, MD, USA) calibrated to each image was used to extract these values.

2.4. Molecular docking

Molecular docking analysis was performed in Schrödinger software suite Maestro (Schrödinger, New York, USA) version 10.2.010 using the crystal structure of *Trypanosoma cruzi* TR (PDB code: 1BZL) with two chains. For ligand preparation, the LigPrep program was used with OPLS_3 force field and ionization state for pH 7.0 ± 2.0 (Schrödinger, New York, USA). To the ligands containing metals, the bonds between the metal and the atoms were turned into zero-order bonds, and the formal charges on the atoms were accordingly adjusted to +2 on the manganese atom and to -1 on the oxygen atoms. Protein structure preparation was performed by using the Protein Preparation Wizard program, with hydrogen bonding network optimization at pH 7.0 and minimization performed using the OPLS-3 force field in the Macromodel module (Schrödinger, New York, USA).

For docking analysis, the Induced Fit Docking (IFD) protocol was used, which predicted the protein structure and docking, refined the compounds by using the Prime program, and provided the score by using the Glide program, considering the protein and the ligand to be flexible [32]. The grid box area was defined as 20 × 20 × 20 Å in the active site region. The force field used was OPLS-3. The final ligand protein complexes were visualized using the Maestro interface, and figures were generated using its graphical interface.

2.5. Methodological bias

Reporting bias was analyzed according to Pereira et al. (PEREIRA et al., 2017) and based on methodological requirements described in the Animal Research: Reporting of *In Vivo* Experiments guidelines (KILKENNY et al., 2010). This strategy requires the complete

screening of all manuscript sections (abstract to acknowledgements and funding) to evaluate the completeness of scientific reports on animal studies. The screening strategy was based on short descriptions of essential characteristics such as baseline measurements, sample size, animal allocation, randomization, experimental concealment, statistical methods, ethical statement, and generalizability. A table summarizing all relevant and applicable aspects was constructed considering the specificity and aims of the systematic review. The individual adherence to bias criteria and the overall mean adherence were expressed as absolute and relative values (PEREIRA et al., 2017).

2.6. Statistical analysis

Taking into account potential heterogeneity in the studies identified, we used a statistical model based on random effects weighted mean difference meta-analysis, in which some heterogeneity and sampling error is admitted to calculate a summary estimate of effect size and its 95% confidence intervals (DERSIMONIAN and LAIR, 1986). For this model, standard error (SE) was converted to standard deviation using the formula $SD = SE x \sqrt{n}$, where n is the number of animals used in each experimental group. The variability of each outcome assessed was presented as the heterogeneity statistic (I^2) (HIGGINS et al., 2003). For dichotomous (survival/mortality and electrocardiographic normality/abnormality) and continuous variables, the risk ratio (RR) and standardized mean difference (SMD) were used as estimates of effect, respectively. Where outcomes were repeatedly measured, we established two time points of infection (acute phase and chronic phase) at which the mean result was calculated for each phase. From the relative similarity among the experimental models, subgroup analyses based on variables such as mouse lineage, weight and parasite strain were not applicable to exploring heterogeneity. The sex of animals was not admitted, because there is no evidence of a sex-dependent pharmacological response for the drugs investigated or any anti-*T. cruzi* drug. Furthermore, age-based heterogeneity was not taken into consideration as this variable was underreported. When appropriate, subgroup analysis was based on methodological (specific drugs and phase of infection) rather than biological characteristics to try to explain possible causes of heterogeneity. A subgroup was defined as a group containing a minimum of two studies (HOOIJMANS et al., 2012).

3. RESULTS

3.1. Publication characteristics

From our search strategy, 15 relevant studies (11 identified in electronic databases and 4 recovered in the secondary search) were included in the systematic review (Fig. 1). Most of these studies originated in South America countries, especially Argentina (80%, n = 12), followed by European countries (20%, n = 3).

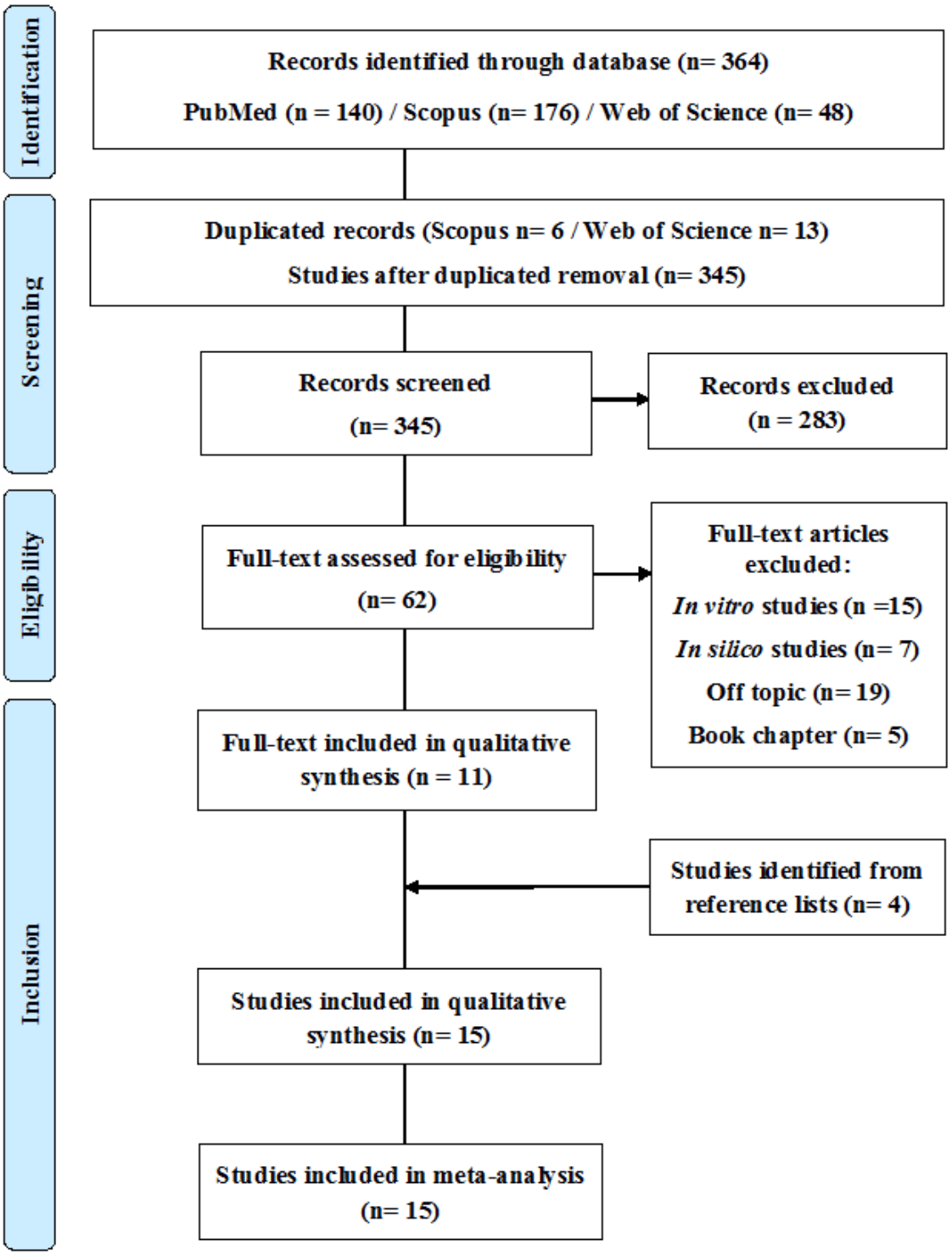

Fig. 1. Flow diagram with the search results obtained in the systematic review. Based on PRISMA statement "Preferred Reporting Items for Systematic Reviews and Meta-Analyses".

3.2. Reporting bias

Detailed results of bias analysis are shown in Table S2 and Fig. 2. The original studies adhered to a mean of 52.3 ± 5.6 bias items (Fig. 2). No study reported experimental blinding, baseline procedures and measures, housing of experimental animals (type of facility or housing), sample size calculation, welfare-related assessments and interventions, order in which the groups were treated and assessed, adequacy of the statistical approach, animal or data exclusion, and study limitations (sources of intrinsic and extrinsic bias). The rationale for the choice of administration route and details of animal allocation (7.9%, n = 1), as well as the rational basis for dosage definition and age of the animals, were also underreported (14.2%, n = 2). Fifty-seven percent of studies presented clear objectives and ethical approval. Information on parasitemia, mortality, parasite strain, and mouse weight was cited in 11 studies (78.6%). Sex of the animals, experimental conditions, number of animals, relevance to human biology, statistical methods (85.7%, n = 12), and route of administration (92.8%, n = 13) were consistently reported.

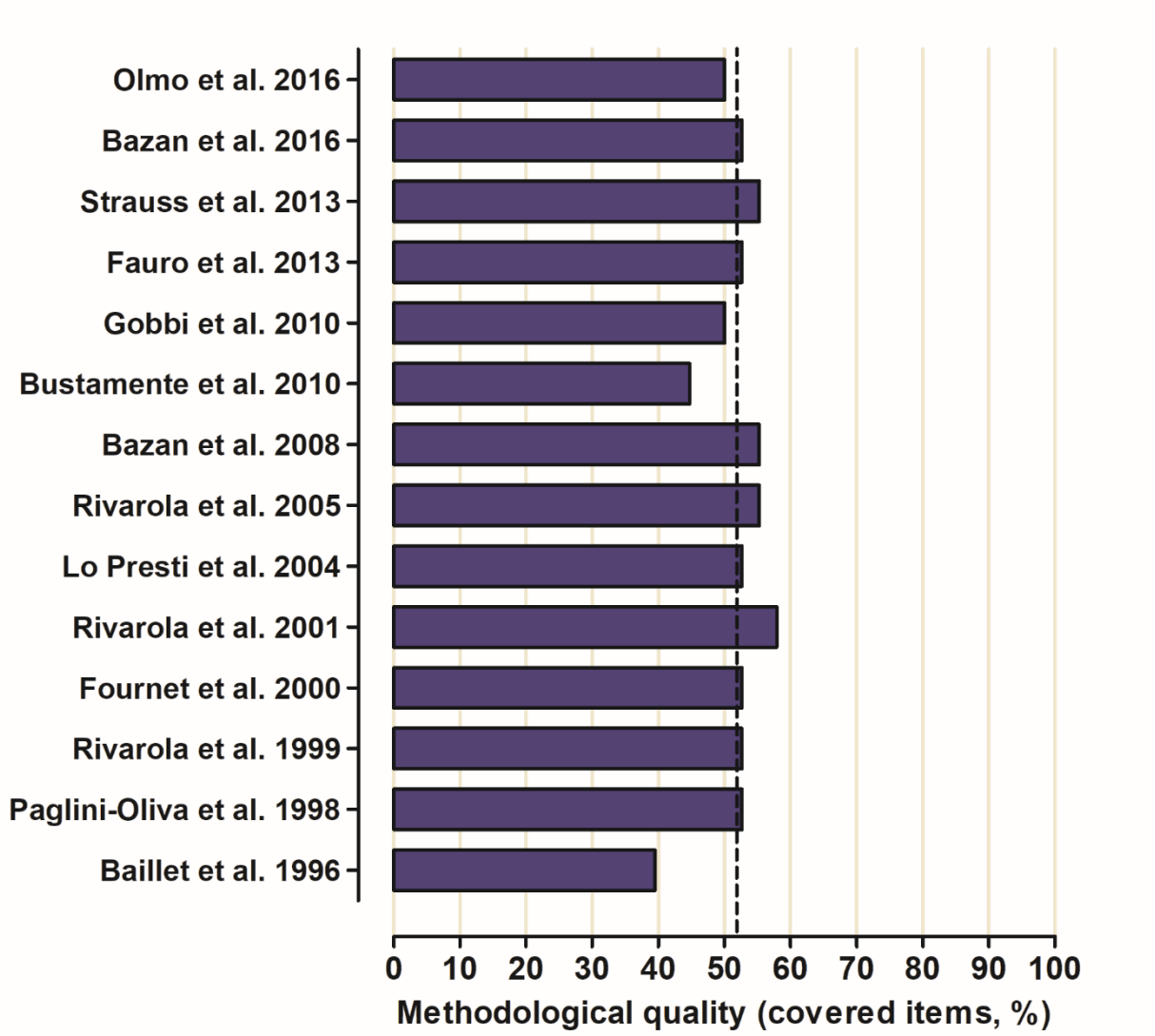

Fig. 2. Analysis of methodological bias (reporting quality) for each study included in the review. The dotted line indicates the mean quality score (%). Drugs investigated: Olmo et al. 2016 a, b and c (Compound 2, Compound 3 and Compound 5); Lo Presti et al. 2015, Bustamante et al. 2010**,** Lo Presti et al. 2004, Rivarola et al. 1999, Paglini-Oliva et al. 1998 (Thioridazine); Fournet el at. 2000 a and b (Daphnoline and Cepharanthine); Baillet et al. 1996 2-Amino Diphenylsulfide; Bazan et al. 2016, Strauss et al. 2013, Fauro et al. 2013, Gobbi et al. 2010, Bazan et al.2008, Rivarola et al. 2005, Rivarola et al. 2001 (Clomipramine). Detailed bias analysis stratified by domains and items evaluated is presented in Table S7.

3.3. Characteristics of the animal models

All studies used mice as the animal model. Swiss mice were the main lineage used (73.3%, n = 11), and only two studies (13.33%) used BALB/c mice. Forty percent of studies (n = 6) used male animals, 13.3% (n = 2) used female animals, and 26.6% (n = 4) used animals of both sexes. Three studies (20%) did not specify the sex of the animals. The animals' age was neglected in most studies (86.6%, n = 13), and the animals' weight ranged from 20 to 30 g. Tulahuen (53.3%, n = 8), SGO-Z12 (13.33%, n = 2), or both (13.33%, n = 2) *T. cruzi* strains were used to induce infection. Parasite strain was not mentioned by three studies (20%) (Table S3).

3.4. Characteristics of the treatments

As shown in Table S3, clomipramine (46.6%, n = 7), thioridazine (33.3%, n = 5), tetra-amine-based compounds (6.6%, n = 1), bisbenzylisoquinoline alkaloids (6.6%, n = 1), and metallodrugs (6.6%, n = 1) were the drugs tested against *T. cruzi* infection. The doses administered ranged from 4 mg/kg to 80 mg/kg. The administration routes were intraperitoneal (46.6%, n = 7), oral (40%, n = 6), or both (6.6%, n = 1). The treatment period ranged from 1 hour to 90 days.

3.5. Parasitological outcomes and mortality

The results of parasitemia and mortality for each study are detailed in Table S4. In general, TR inhibitors were effective in controlling parasitemia and mortality. When the studies were grouped by drug and by dose investigated, no heterogeneity ($I^2 = 0$) or significant differences were detected among subgroups (P = 0.41). Thus, the overall effect of treatment on parasitemia was analyzed for the set of studies reporting these data. Eight of nine studies showed a significant reduction in parasitemia, indicating a consistent effect in favor of treated animals compared with untreated infected mice (SMD: −1.11 [95% CI: −1.73, −0.49], P = 0.0004) (Fig. 3).

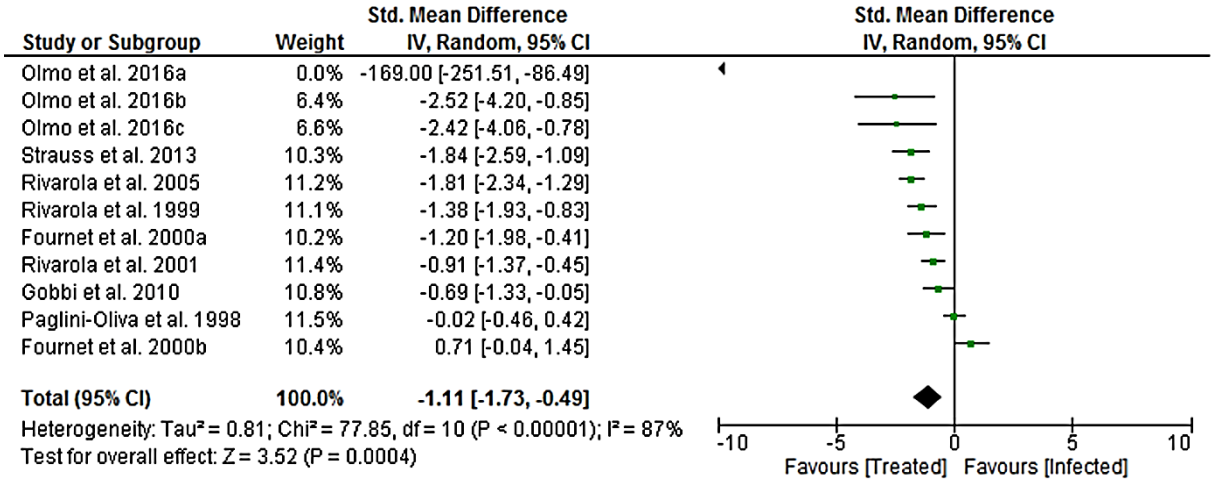

Fig. 3. Forest plot obtained from meta-analysis comparing the mean difference of parasitemia in infected mice treated with trypanothione reductase inhibitors and those untreated (control). Drugs investigated: Olmo et al. 2016a, b and c (Compound 2, Compound 3 and Compound 5); Fournet el at. 2000a and b (Daphnoline and Cepharanthine); Rivarola et al. 1999, Paglini-Oliva et al. 1998 (Thioridazine); Rivarola et al. 2005, Strauss et al. 2013, Rivarola et al. 2000, Gobbi et al. 2010 (Clomipramine).

As shown in Table S5, only one study (20%, n = 1) objectively reported parasitic load, indicating reduced heart parasitism in animals treated with clomipramine (5 mg/kg). Parasitological cure was investigated in four studies (80%, n = 4). The cure rate (CR) was similar in all of them, except for those using cepharanthine and daphnoline (CR = 51% and 84% respectively, *vs.* infected untreated mice CR = 17%) and two tetraamine-based metallodrugs (CR = 33.33% and 50%, *vs.* infected untreated mice CR = 0%).

Overall mortality (Fig. 4) was significantly reduced in animals treated with TR inhibitors (RR: 0.54 [95% CI: 0.35, 0.81], P = 0.003). A favorable effect of treatment on mortality (P < 0.05) was observed in the acute phase of infection (RR: 0.36 [95% CI: 0.22, 0.61]). Conversely, these drugs had no impact (P > 0.05) on mortality in chronic infections (RR: 0.76 [95% CI: 0.44, 1.32]).

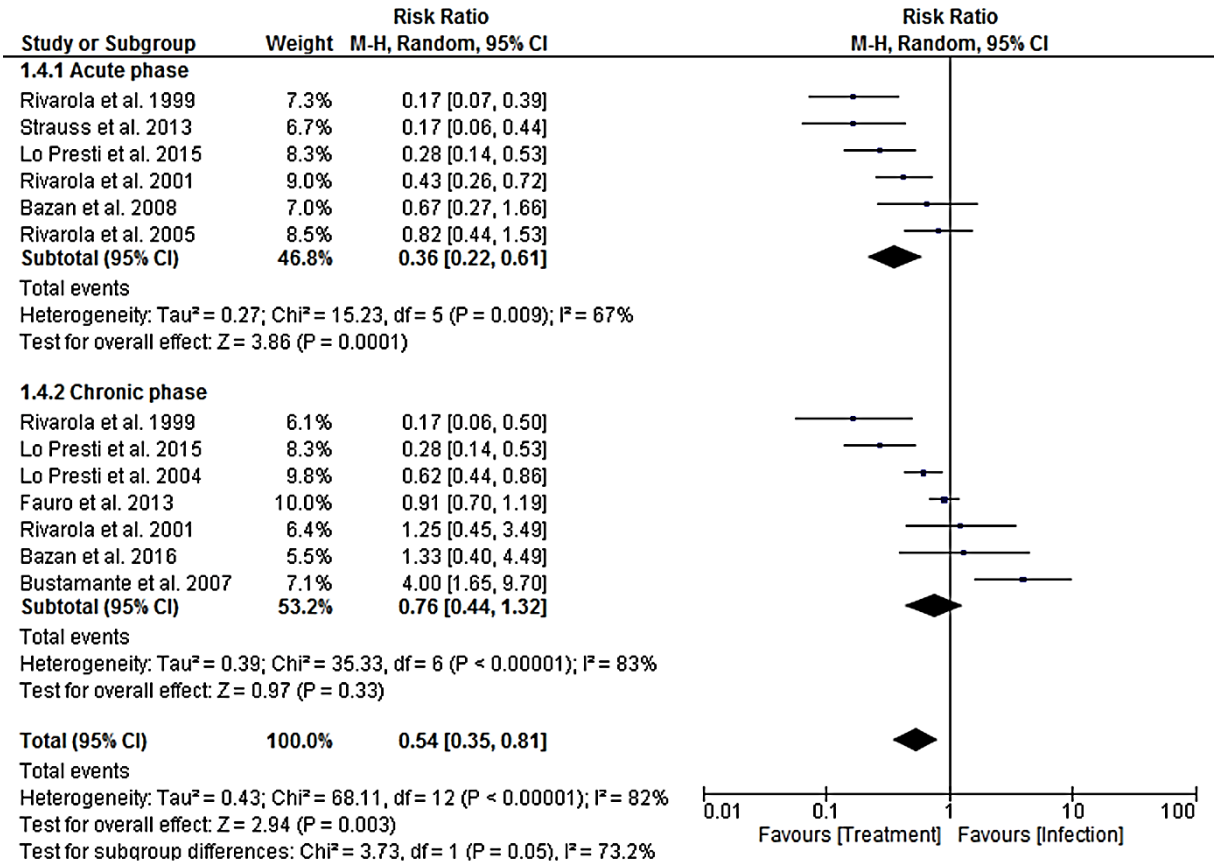

Fig. 4. Forest plot obtained from meta-analysis comparing the risk ratio for mortality in infected mice treated with trypanothione reductase inhibitors.

3.6. Electrocardiographic changes and β-adrenergic receptors

Electrocardiographic findings are detailed in Tables S6 and S7. Electrocardiographic and cardiac receptor data were reported in seven studies (46.6%, n = 7) investigating clomipramine or thioridazine. The overall effect showed no difference in electrocardiographic abnormalities (i.e., arrhythmias and intra-ventricular block) comparing treated and untreated infected mice (RR: 0.66 [95% CI: 0.35, 1.26], P = 0.21). In subgroup analysis, treatment with thioridazine reduced the occurrence of electrocardiographic abnormalities in chronic infection compared with untreated mice (RR: 0.42 [95% CI: 0.21, 0.83). Conversely, the risk of electrocardiographic disturbances in both phases of infection was similar (P > 0.05) in clomipramine-treated and untreated animals (acute phase RR: 1.09 [95% CI: 0.70–1.70] and chronic phase RR: 1.05 [95% CI: 0.10, 11.09]) (Fig. 5).

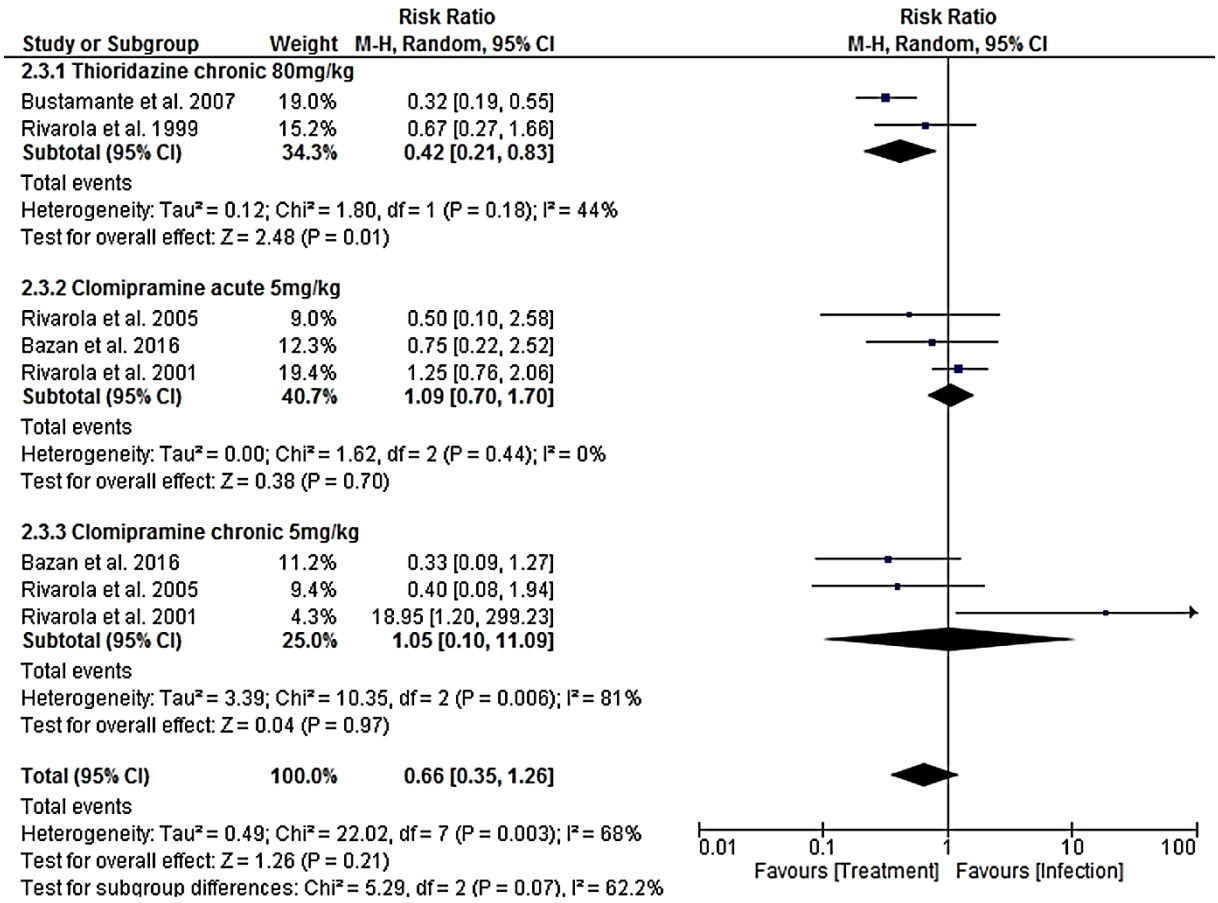

Fig. 5. Forest plot obtained from meta-analysis comparing the risk ratio for electrocardiographic abnormalities in infected mice treated with trypanothione reductase inhibitors and those untreated (control). Abnormalities were determined by evidence of arrhythmias and intra-ventricular block.

Beta-adrenergic receptor affinity and density are detailed in Table S7. Four studies (57.1%, n = 4) analyzed β-adrenergic receptors, of which three (42.8%, n= 3) using clomipramine were included in the meta-analysis. Considering both phases of infection, β-adrenergic affinity presented a similar overall effect when clomipramine-treated and untreated animals were compared (SMD: −1.21 [95% CI: −2.57, 0.14], P = 0.08) (Fig. 6). Whereas clomipramine-treated animals showed reduced β-adrenergic affinity in chronic infection compared with control animals (SMD: −2.95 [95% CI: −5.45, −0.46], P < 0.00001), no significant effect was observed in acute infection (SMD: 0.60 [95% CI: −0.57, 1.78], P = 0.31).

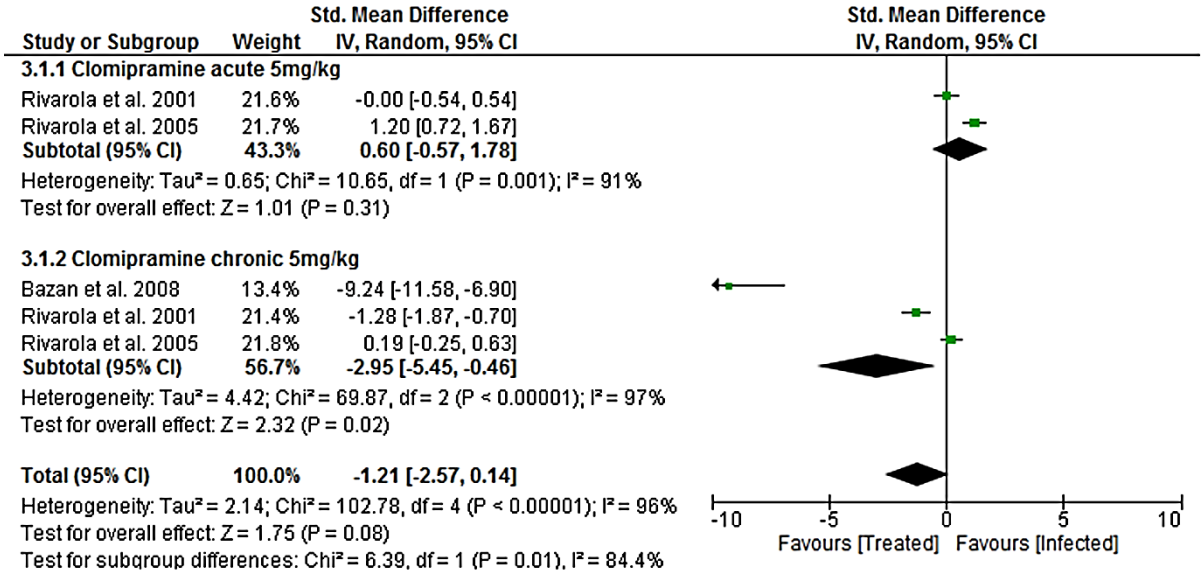

Fig. 6. Forest plot obtained from meta-analysis comparing the mean difference of β-adrenergic receptors affinity in infected mice treated with trypanothione reductase inhibitors and those untreated (control).

Considering β-adrenergic receptor density, the overall analysis indicated a positive effect in favor of infected untreated compared with clomipramine-treated mice (SMD: 1.15 [95% CI: 0.81, 1.15], P < 0.00001) (Fig. 7). In subgroup analysis, while untreated animals showed increased β-adrenergic density in chronic infection compared with clomipramine-treated animals (SMD: 1.86 [95% CI: 1.43−2.29], P < 0.0001), no significant effect was observed in acute infection (SMD: –0.04 [95% CI: −0.61, 0.52], P = 0.88).

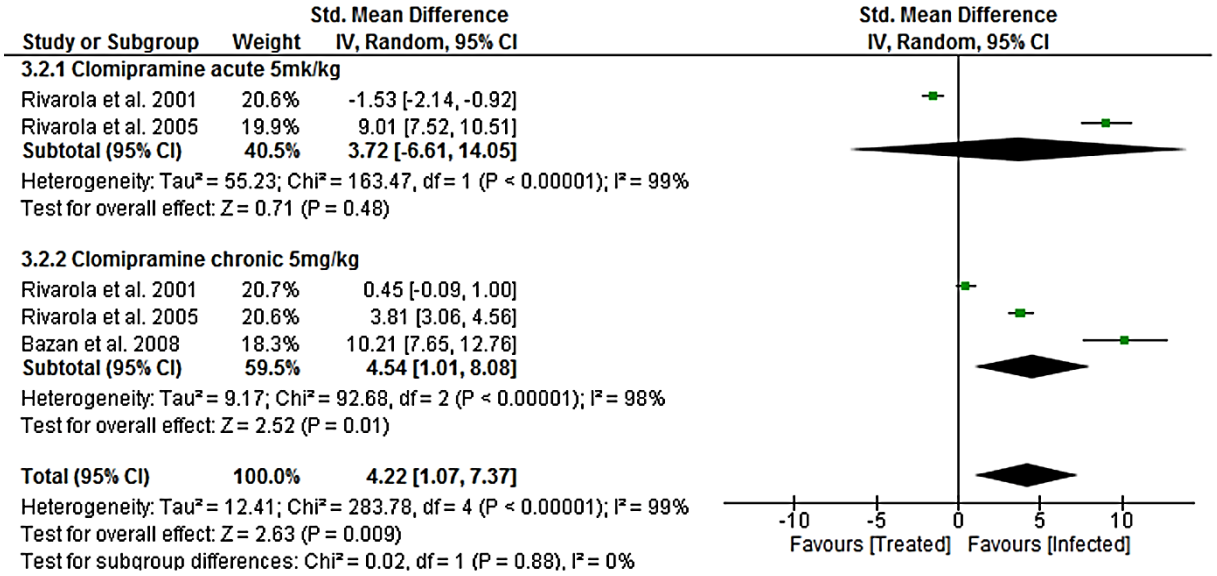

Fig. 7. Forest plot obtained from meta-analysis comparing the mean difference of β-adrenergic receptors density in infected mice treated with trypanothione reductase inhibitors and those untreated (control).

3.7. Immunological, histopathological and biochemical findings

Table S8 shows additional morphological, immunological, and biochemical findings. Histopathological data of the heart were reported in 11 studies (73.3%, n= 11). In general, TR inhibitors, especially thioridazine and clomipramine, reduced heart inflammation, tissue necrosis, and fibrosis compared with untreated infected mice. Immunoglobulin levels were investigated in 8 studies (53.3%), which generally reported reduced anti-*T. cruzi* antibody levels in animals treated with TR inhibitors. In four studies (26.6%, n= 4), antibody levels were similar (LO PRESTI et al., 2004; STRAUSS et al., 2013), while in two studies (13.3%, n= 2), they were greater in treated *vs.* untreated control groups (RIVAROLA et al., 1999; GOBBI et al., 2010). Only one study (6.6%, n= 1) investigated biochemical parameters of systemic toxicity. In this study (OLMO et al., 2016), while CK-MB, uric acid, and urea serum levels were increased, LDH was reduced (30% and 40%) in metallodrugs-treated compared with untreated animals.

3.8. In silico molecular interaction

Molecular docking of a group of nine different bioactive molecules (ligands) and trypanothione disulfide (substrate) was performed with TR (Fig. 8). Values of the Glide Score (GScore), the number of interactions by hydrogen bonds (*Hbond*), van der Waals (*good vdW*), π-π stacking and cation π between the ligands and TR are shown in Table 1. Figures 9 and 10 represent the results of molecular docking in 2D format. Eleven compounds with amino acid residues in the active site of TR showed the types of interactions relevant to these studies.

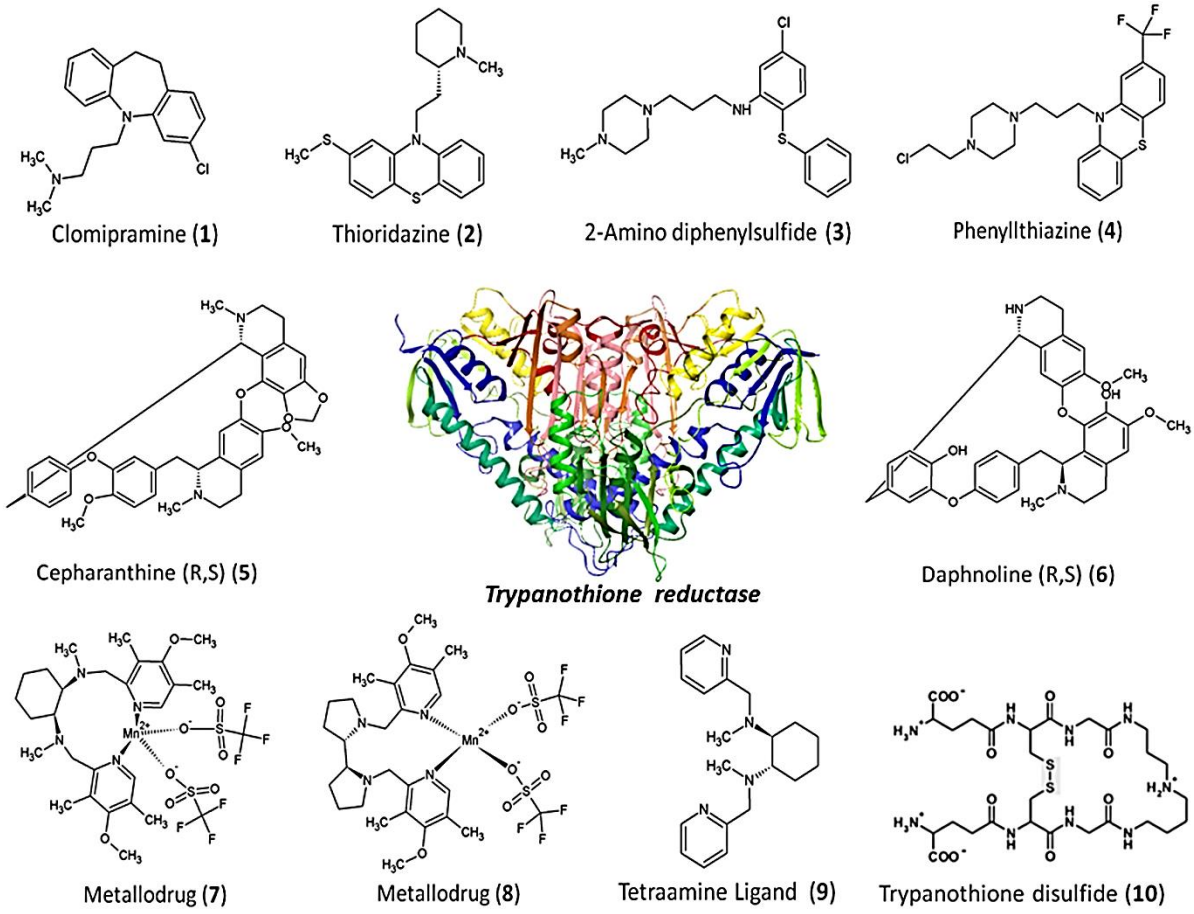

Fig. 8. Molecular structures of trypanothione reductase inhibitors (ligands) used in docking studies.

Table 1. Values of Glide Score (*GScore*), the number of interactions by hydrogen bonds (*Hbond*), Van der Waals (*good vdW*), π-π stacking and cation π between the ligands (1-10), the substrate trypanothione disulfide and trypanothione reductase.

Ligand	GScore *(kcal.mol^{-1})*	H Bond	Amino acids that perform H Bond	Good vdW	π-π stacking	cation π
Clomipramine (**1**)	-3.902	1	GLU467	143	0	1
Thioridazine (**2**)	-6.099	1	GLU466	236	0	0
2-Amino diphenylsulfide (**3**)	-4.871	1	GLU19	280	1	0
Phenylthiazine (**4**)	-5.511	1	TYR111	269	0	0
Cepharanthine (R,S) (**5**)	-3.107	0	-	257	2	2
Daphnoline (R,S) (**6**)	-6.247	2	THR397, GLU467	338	0	1
Metallodrug (**7**)	-4.280	0	-	127	0	0
Metallodrug (**8**)	-3.760	0	-	236	1	1
Tetraamine Ligand (**9**)	-5.400	3	TYR111, HIS461	280	1	0
Trypanothione disulfide (substrate) (**10**)	-8.768	9	GLU19, TYR111, THR335, LEU399, GLY459, THR463, GLU466, GLU467	329	0	2

Figures 11 to 13 show a superimposition of the molecular docking configurations of the compounds evaluated. The images were divided according to structural similarity. Our results suggested an interaction that could lead to an inhibitory activity of all the compounds towards TR. However, the strength of molecular affinity was not always aligned with the experimental results *in vivo* reported in this work.

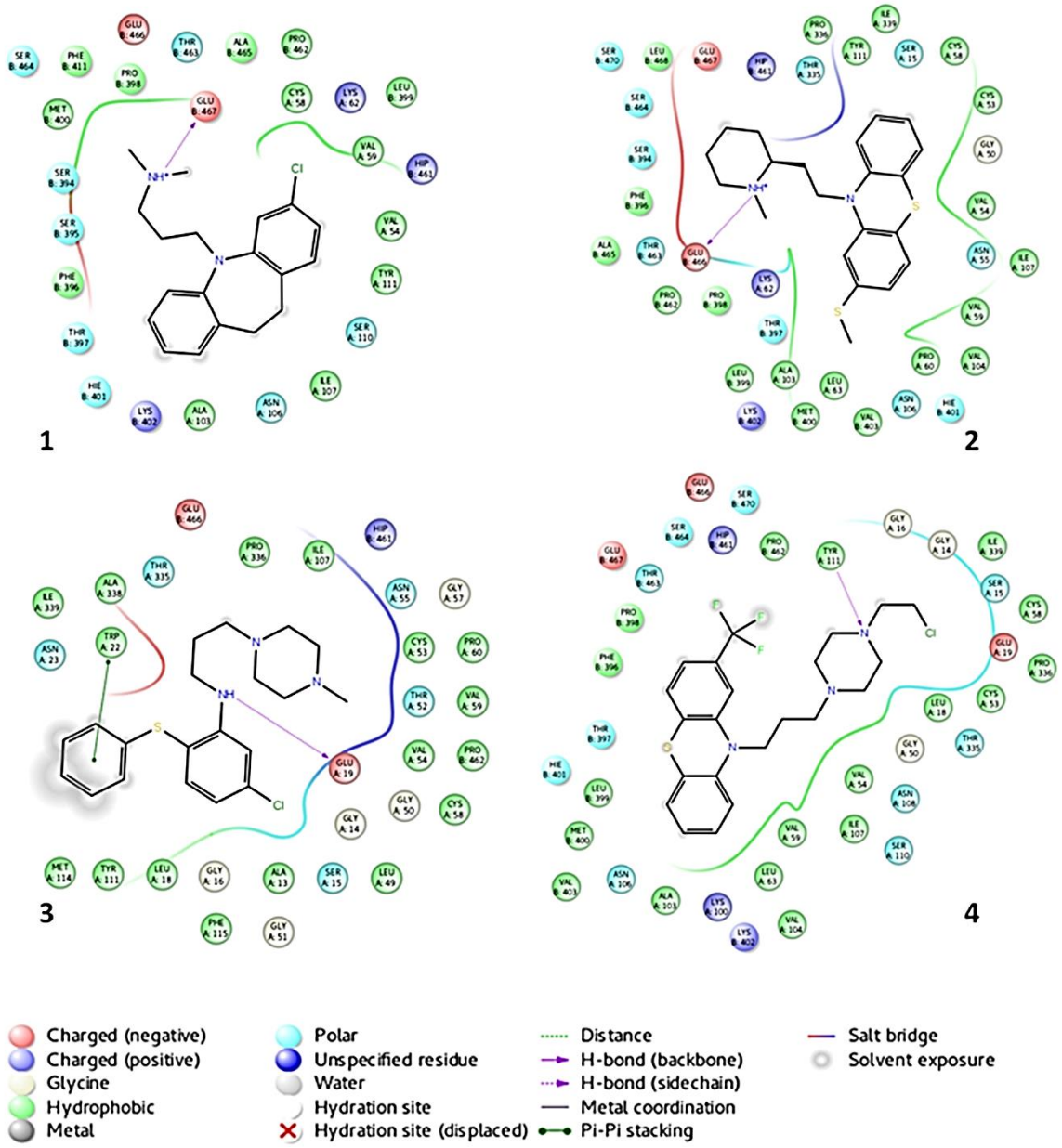

Fig. 9. Interactions between amino acids of trypanothione reductase active site and clomipramine (1), thioridazine (2), 2-Amino diphenylsulfide (3) and phenylthiazine (4).

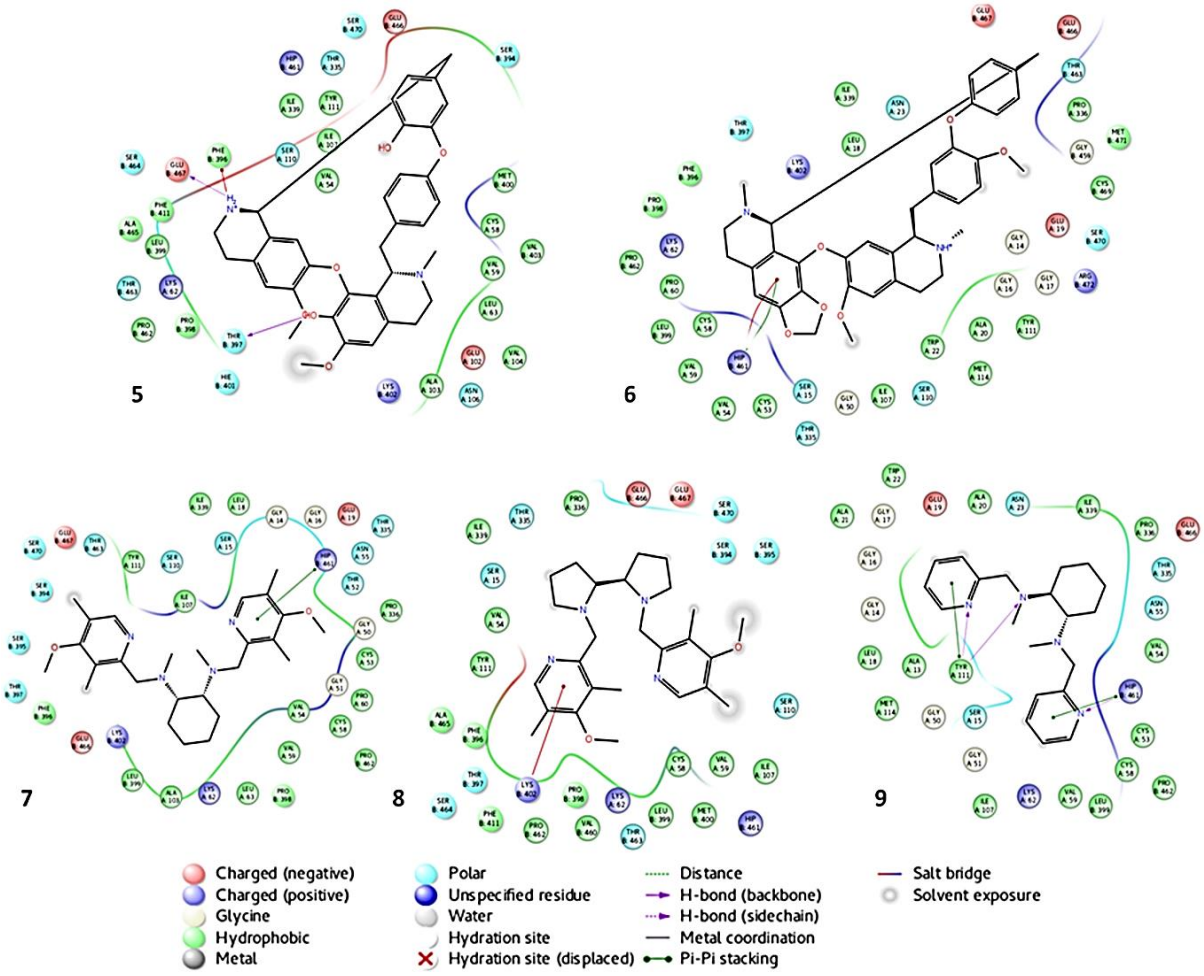

Fig. 10. Interactions between amino acids of trypanothione reductase active site and cepharanthine (R,S) (5), daphnoline (R,S) (6), metallodrug (7), metallodrug (8) and tetraamine Ligand (9).

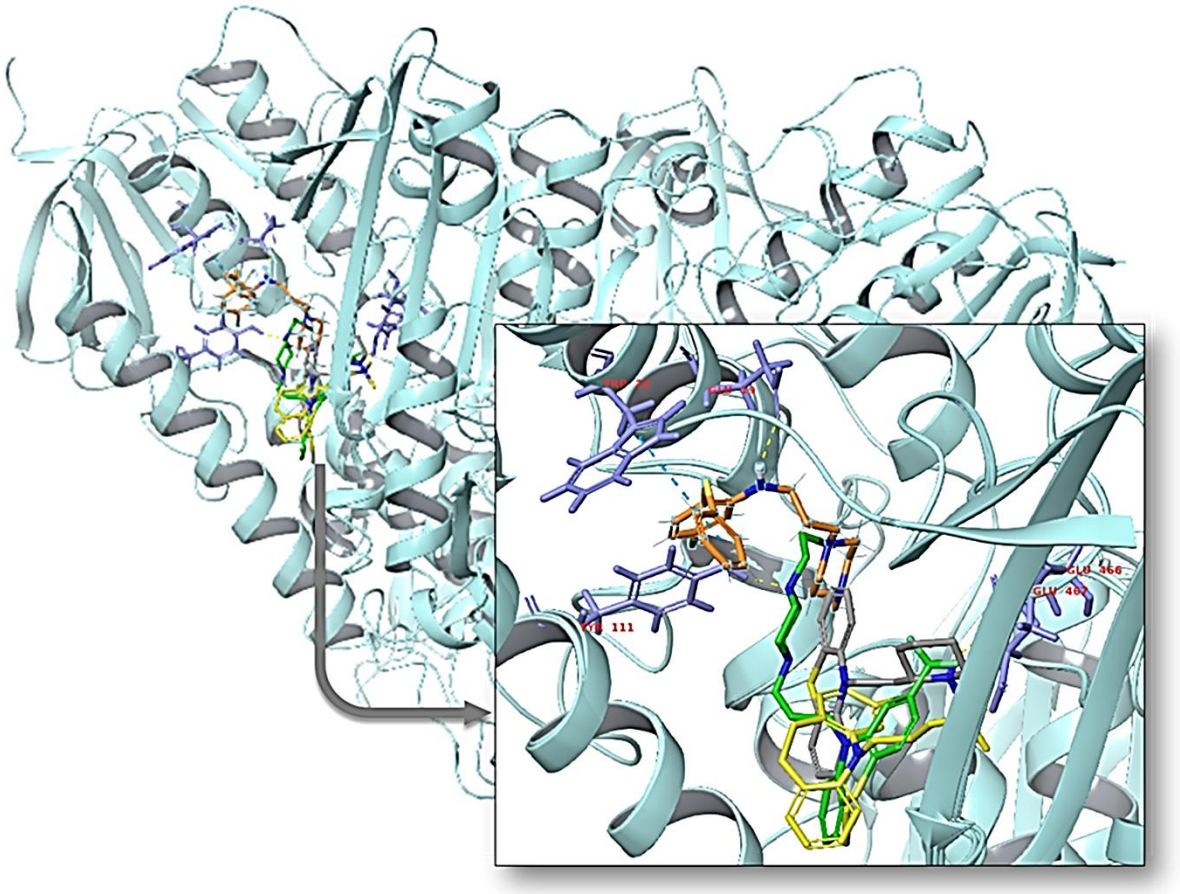

Fig. 11. Representation of molecular docking superimposition of clomipramine (1) (yellow carbon), thioridazine (2) (gray carbon), 2-Amino diphenylsulfide (3) (orange carbon) and phenylthiazine (4) (green carbon) with trypanothione reductase active site. The highlighted image shows the interaction of the investigated drugs with the binding site of trypanothione reductase.

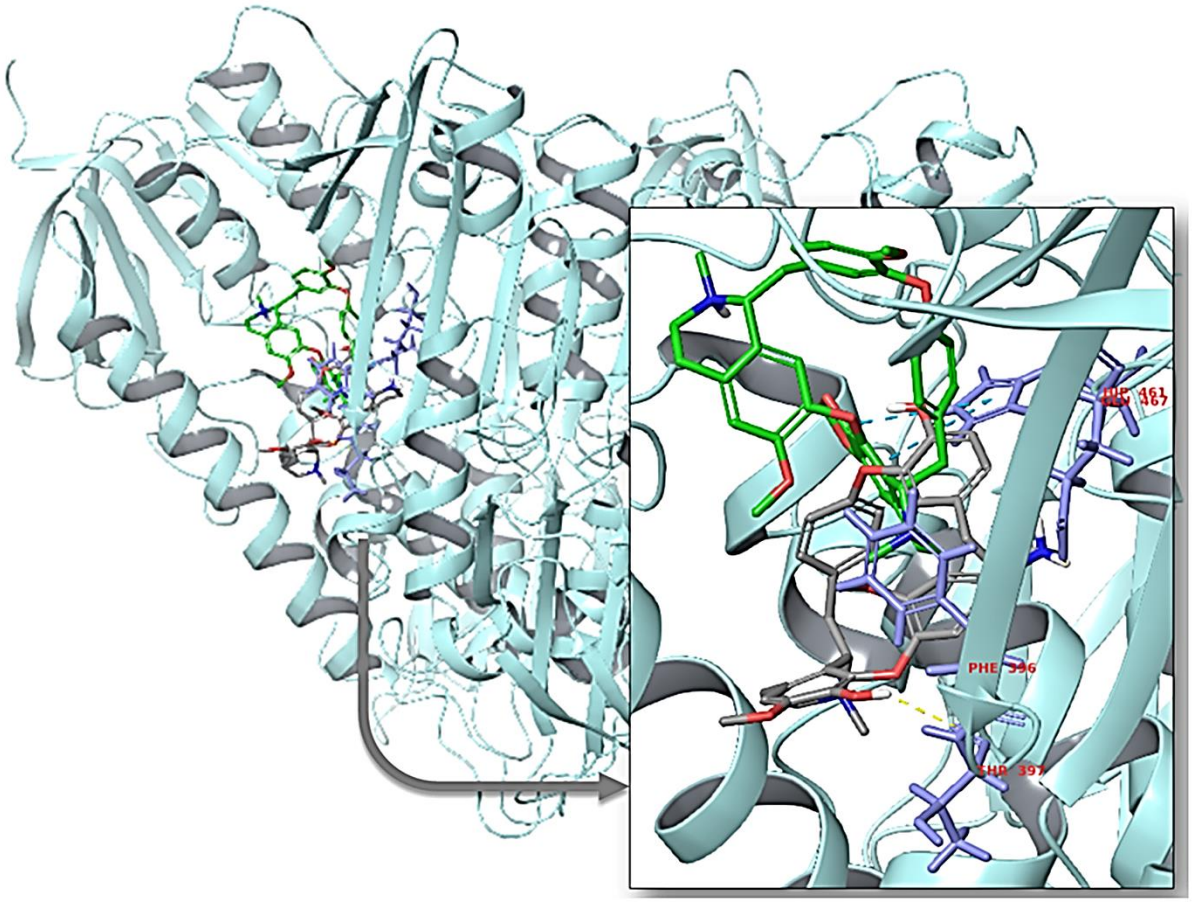

Fig. 12. Representation of molecular docking superimposition of cepharanthine (R,S) (5) (green carbon) and daphnoline (R,S) (6) (gray Carbon) with trypanothione reductase active site. The highlighted image shows the interaction of the investigated drugs with the binding site of trypanothione reductase.

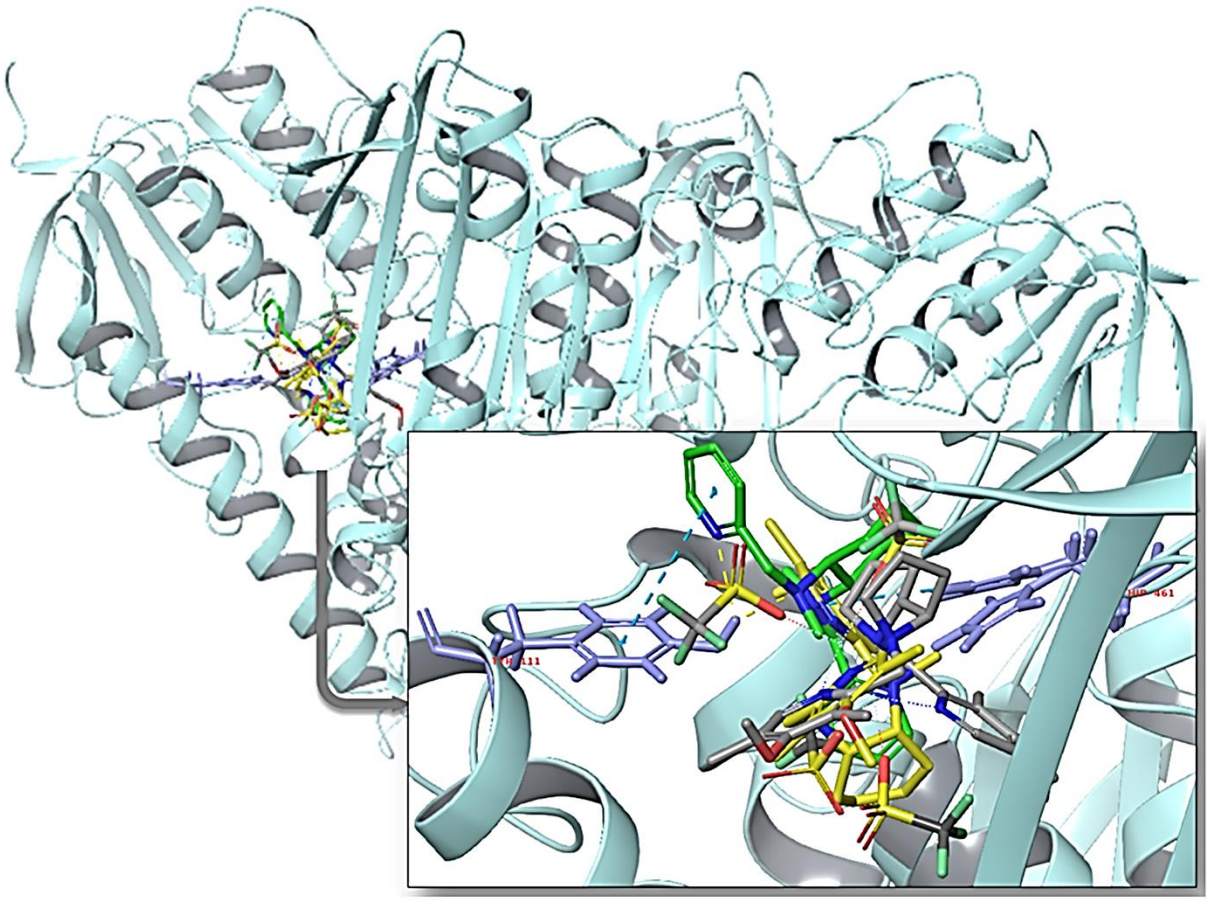

Fig. 13. Representation of molecular docking superimposition of metallodrug (7) (gray carbon), metallodrug (8) (yellow carbon) and tetraamine ligand (9) (green carbon) with trypanothione reductase active site. The highlighted image shows the interaction of the investigated drugs with the binding site of trypanothione reductase.

4. DISCUSSION

The knowledge of *T. cruzi* redox metabolism and the role of oxidative stress in the pathophysiology of Chagas disease has indicated potential targets for antiparasitic chemotherapy (OMAR and KLAN, 2007; TURRENS, 2004, RIVAROLA and PAGLINI-OLIVA, 2002). Due to the rudimentary antioxidant defenses, *T. cruzi* is highly susceptible to oxidative and nitrosative events activated during infection of multiples organs, such as heart (RIVAROLA and PAGLINI-OLIVA, 2002; LO PRESTI et al., 2015; NOVAES et al., 2016; NOVAES et al., 2017a), skeletal muscles (NOVAES et al., 2017b), liver (NOVAES et al.,

2015) and placenta (TRIQUELL et al., 2018). There is evidence that inflammatory, oxidative and nitrosative events are coupled processes potentially mediated by pro-inflammatory cytokines such as TNF-α, IFN-γ and IL-1 and IL-6, which stimulates the intense production of reactive species such as oxygen peroxide, superoxide anion, hydroxyl radicals, nitric oxide and peroxynitrite (NOVAES et al., 2017a; GUPTA et al., 2009; RODRIGUES et al., 2017). In general, decoupling of electron transport chain in host cells (GUPTA et al., 2009), upregulation of endothelial and inducible nitric oxide synthase expression (TRIQUELL et al., 2018; GUPTA et al., 2009), and the respiratory burst in leucocytes recruited in infected tissues (NOVAES et al., 2017a; NOVAES et al., 2015) are the primary sources of these highly reactive molecules. Reactive oxygen (ROS) and nitrogen (RNS) species are relevant defense molecules against *T. cruzi* infection, and its inhibition (especially NO) has been associated with intense parasite load and severe tissue damage (TRIQUELL et al., 2018; RODRIGUES et al., 2017). The blockade of the parasite's antioxidant defenses has been proposed as a relevant strategy to increase host resistance to *T. cruzi* infection (RIVAROLA and PAGLINI-OLIVA, 2002; LO PRESTI et al., 2015; BELTRAN-HORTELANO et al., 2017). However, due to its interspecific action, host cells are also an important target of ROS and RNS cytotoxicity (NOVAES et al., 2017a; NOVAES et al., 2015; GUPTA et al., 2009). Thus, to identify specific molecules involved in the control of redox metabolism in *T. cruzi* may represent a rational and useful strategy to develop antiparasitic drugs with little or no impact on the host's antioxidant defenses. In this sense, TR inhibitors are currently the most promising drugs with direct impact on *T. cruzi* antioxidant defenses.

Most studies investigating TR inhibitors were performed in developing countries, indicating that research efforts are coherently concentrated in South American countries where Chagas disease is endemic (MURCIA et al., 2013). A limited general score of methodological quality was identified for the set of studies. As the analysis of reporting bias was structured from basic requirements to the rational acquisition and interpretation of the results, a limited quality of evidence could be attributed to studies with low methodological scores (ZOLTOWSKI et al., 2014). Accordingly, it is important a rigorous analysis and interpretation of the available evidence, taking into account all critical elements that could hinder construct validity (the degree to which the analytical tool measures what it purports to), internal validity (cause-effect relationships), and external validity (generalizability) of each

study, as well as the individual weight associated with meta-analysis effect size (SILVA FILHO et al., 2005).

Although individual bias scores were variable, they did not present a temporal influence (year of publication). This finding indicates that reporting bias was systematically reproduced through the research process, independent of notorious advances in the analytical and statistical methods as well as the increasing availability of guidelines and regulatory strategies adopted to stimulate the completeness of the scientific reports in preclinical studies (KILKENNY et al., 2010). In all studies included, simple constructs such as experimental blindness, animal allocation and age, sample size calculation, and rational choice of administration route were the main source of bias. Although these elements are important sources of intrinsic bias (CURTIS et al., 2015; MCGRATH and LILLEY, 2015), they also are easily adjustable, and the construction of more rigorous experimental designs aligned with acceptable construct validity can be achieved in future studies.

Despite these methodological limitations, similar elements of experimental design were identified, contributing to the reliability and reproducibility of the results. Parasitemia, mortality, parasite strain, animal number, sex and weight, route of administration, relevance to human biology, and statistical methods were consistently reported. Furthermore, Swiss mice infected by the Tulahuen strain were used as the main animal model. A proper selection of animal species and genetic background is crucial in preclinical investigations of parasitic diseases as these factors are directly related to host resistance and susceptibility to the pathogen (ANDRADE, et al., 2002; LÉON, et al., 2017). Swiss mice are highly applicable, especially considering that they are highly susceptible to *T. cruzi* infection, and their genotype and phenotype variability resembles the one of the human condition (ANDRADE, et al., 2002; LÉON, et al., 2017). Swiss mice also recapitulate many features of human disease, especially the immunological profile and histopathological manifestations (JELICKS and TANOWITS, 2011). From an operational perspective, mice are also good animal models due to ease of handling and housing and the low cost of maintenance. Conversely, the applicability of larger animals as models of Chagas disease, especially dogs and non-human primates, is limited by availability, cost, and ethical considerations (PEREIRA et al., 2017).

Similar strains were also applied to induce *T. cruzi* infection. Careful selection of a parasite strain is essential in preclinical studies, especially considering the variable profiles of

infectivity, pathogenicity, and virulence (ANDRADE, et al., 2002; LÉON, et al., 2017; MANOEL-CAETANO and SILVA, 2007). These elements require good alignment with the outcome measures, which are closely associated with the phase of infection under analysis. Thus, highly pathogenic and virulent parasite strains are realistically applicable only in acute models as the animals often die before developing a chronic infection (CHATELAIN and KONAR, 2015). Conversely, as parasitological cure and Chagas cardiomyopathy attenuation or reversion are the primary focus of anti-*T. cruzi* chemotherapy, models using pathogenic strains with low virulence are a more rational strategy (CHATELAIN and KONAR, 2015; SILVA et al., 2013). Based on these latter models, the chances of instilling morphological, electrical and mechanical changes typical of Chagas' cardiomyopathy are enhanced (SILVA et al., 2013; GUERREIRO et al., 2015). Because the parasite strains used were aligned with the phases of interest in Chagas disease, the analyzed studies exhibited an important element of methodological consistence, with a positive reflection on construct validity.

Due to the similarity in experimental designs, analytical methods, and outcome measures, meta-analysis estimates were calculated to evaluate the relevance of TR inhibitors in preclinical treatment of *T. cruzi*. All common and relevant outcomes, such as parasitemia, mortality, electrocardiographic abnormalities, β-adrenergic receptor density, and affinity reported in two or more studies were combined. Taken together, the results of our meta-analyses indicated that these inhibitors are highly effective at attenuating multiple pathological events of experimental Chagas disease. Parasitemia was reduced by TR inhibitors, and no subgroup differences (drugs and doses) were identified. This finding indicated that parasitemia measures were highly consistent and not affected by bias. It is recognized that heterogeneity in meta-analytical studies can be affected by multiple elements of bias, especially those from methodological (experimental design and scientific reporting) or biological sources (genotypic and phenotypic variability) (DOHOO et al., 2007; LIN et al., 2017). Thus, controlling all potential sources of bias is essential to ensure reliable effect sizes in a meta-analysis, with a direct reflection on the quality of evidence (DOHOO et al., 2007; LIN et al., 2017).

The meta-analysis also indicated that TR inhibitors were effective at reducing overall mortality. However, this effect was determined by benefits limited to acute infections. This finding was coherent with the results of parasitemia and phase of infection. Control of

parasitemia is a pivotal objective of anti-parasitic chemotherapy, with a direct impact on mortality rates (RIVAROLA et al. 2005; STRAUSS et al., 2013; BAZÁN et al., 2008). Parasitemia is integrated in the reproductive cycle of *T. cruzi* and can reflect the parasite's ability to overcome host defenses and propagate the infection (MIYAZAKI et al., 2010; SANCHES et al., 2014). Strategies that control parasite replication are essential to attenuate tissue damage and secondary mortality in Chagas disease (FAURO et al., 2013; OLMO et al., 2016). As supported by current evidence, parasitological control is more effective in acute infections with positive repercussions in the chronic phases (i.e., reduction of heart damage) (LO PRESTI et al., 2015; GOBBI et al., 2010; BAZÁN et al., 2016). However, after the parasite spreads and establishes stable reservoirs (amastigote nests) in multiple organs during the chronic infection, anti-parasitic treatments exhibit limited effects (VÁZQUEZ et al., 2017). The mechanisms associated with this limited efficacy are poorly understood. It is possible that in the late stages of infection, parasites that survive the host's defenses are more resistant to anti-parasitic drugs (ALVIANO et al., 2012; KELLY and WILKINSON, 2013). Low detection limits of circulating parasites by conventional microscopic methods can also be related to the inefficacy of TR inhibitors in chronic infection. As parasitemia decreases to very low or undetectable levels in the chronic phase, the relevance of this parameter to indicate infection is controversial, especially considering that disease progression and development of severe manifestations (i.e., chronic cardiomyopathy) frequently occur in the absence of circulating parasites (SANTOS et al., 2016; ROJO et al., 2017). Therefore, in chronic infections, parasitic load and specific abnormalities associated with Chagas cardiomyopathy are better parameters to evaluate the efficacy of anti-*T. cruzi* chemotherapy than parasitemia (SANTOS et al., 2016)..

As cardiac function outcomes were analyzed only in studies testing clomipramine (1) and thioridazine (2), these drugs were combined in a meta-analysis considering subgroup differences (phases of disease and doses) and heterogeneity. All parameters of cardiac function (electrocardiographic abnormalities, β-adrenergic receptor density and affinity) were not influenced by clomipramine and thioridazine treatment. Conversely, an overall effect in favor of *T. cruzi* infection was identified for β-adrenergic density. There is no doubt that heart is highly parasitized and damaged by *T. cruzi* (ROMANO et al., 2017; TRAINA et al., 2017). Structural and electromechanical cardiac damage is the most serious manifestations of Chagas

disease and is closely associated with morbidity and mortality, especially in the chronic phase of infection (RASSI JR et al., 2010; PRADO et al., 2012). In fact, in all studies included in this review that investigated cardiac function, the main electrocardiographic changes identified in animals infected with *T. cruzi* were atrial and ventricular conduction defects (prolonged PQ and QRS segments) and arrhythmias (PAGINI-OLIVA et al., 2012). These changes were possibly associated with molecular abnormalities in β-adrenergic receptors, which are identified as central elements in the pathogenesis of Chagas cardiomyopathy and partially responsible for disturbances in the autonomic regulation of cardiac function (JAHNS et al., 2006; LABOVSKY et al., 2007). Interestingly, the results of our meta-analysis and the findings reported in the original studies (RIVAROLA et al., 2005; RIVAROLA et al., 1999; RIVAROLA et al., 2001) indicated an opposite profile of β-adrenergic affinity and density. While receptor affinity was altered by treatment, especially with clomipramine, density was altered by infection. This profile indicates the typical balance between molecular properties of these receptors. There is evidence that damage to β-adrenergic receptors is mediated by autoantibodies produced against *T. cruzi* antigens that exhibit molecular mimicry to heart molecules (STERIN-BORDA and BORDA, 2000; NUSSINOVITCH and SHOENFELD, 2013). It is natural that reduction in receptor density from immunological attack is accompanied by a compensatory increase in the affinity of the remaining receptors (RIVAROLA and PAGLINI-OLIVA, 2002; GARCIA et al., 2016). This behavior represents an important adaptive and counter-regulatory mechanism in the attempt to adjust the cardiac function and to resist organ parasitism (RIVAROLA et al., 2005). Considering that *T. cruzi* infection is not restricted to the heart, mortality rates are determined by the accumulation of lesions and failure in multiple tissues and organs, including nervous structures (i.e., spinal cord and brain) and digestive organs (i.e., liver, esophagus and colon) (HIGUCHI et al., 2003; CAMPOS et al., 2016). As evidenced in our meta-analysis, the cardiac electrocardiographic parameters cannot explain the positive effects of TR inhibitors on mortality reduction.

In general, TR inhibitors attenuated microstructural damage (i.e., necrosis and fibrosis), tissue inflammation and immunological markers commonly increased in *T. cruzi* infection (i.e., cytokines and specific antibody titers). Taken together, these are important effects potentially related to the reduction of mortality rates in animals treated with TR inhibitors. Attenuation of immunological effectors, especially cytokines and anti-*T. cruzi*

antibody levels in animals treated with TR inhibitors (i.e., thioridazine, clomipramine, and tetraamine-based compounds) cannot be interpreted as a disadvantage of chemotherapy that could enhance host susceptibility to infection. On the contrary, reduction in these markers indicated a direct anti-parasitic effect of the drugs. Considering parasitological control reflected by low parasitemia levels, attenuation of the immunological response in the face of reduced antigenic load would be expected (FAURO et al., 2013; RIVAROLA et al., 2001). This proposition was reinforced by the marked attenuation of heart damage (i.e., myocarditis, necrosis, and fibrosis) in animals treated with TR inhibitors, which indicated that drugs such thioridazine and clomipramine increased host resistance to *T. cruzi* infection. It is broadly recognized that Chagas disease is associated with intense immunological responses that modulates the host-pathogen interaction (CUERVO et al., 2011). At the same time that innate (i.e., macrophages and dendritic and NK cells) and acquired (i.e., T and B lymphocytes) effector agents are essential to reducing parasite survival and replication, exacerbated cellular and humoral responses are detrimental to host cells (MACHADO et al., 2012; CARDOSO et al., 2016). Thus, excessive immunological downregulation or upregulation can increase host mortality, in the first case by insufficient defenses to attenuate parasitism and direct organ damage and in the second by inducing tissue lesions in response to massive inflammatory processes (MCAULEY et al., 2015; CARDOSO et al., 2016). From this perspective, drugs with combined anti-parasitic and immunomodulatory properties, including those analyzed in this review, present a great potential for their evaluation in Chagas disease.

Few studies analyzed parasitic load and parasitological cure (OLMO et al., 2016; BAZÁN et al., 2016; FOURNET et al., 2000), parameters essential to estimating the efficacy of experimental chemotherapy with greater reliability (BAZÁN et al., 2016; GUEDES et al., 2011). As parasites are rarely found in tissues examined by routine histopathological techniques in chronic infections, highly sensitive molecular screening based on PCR methods are strongly recommended (VAGO et al., 2000; CALDAS et al., 2012). An important disadvantage of PCR measures is the controversial relation between molecular marker levels and real parasitic load (PIRON et al., 2007). However, this method is currently the gold standard since it was proven that parasite DNA detected by PCR is derived from parasite persistence in host tissues and not from DNA persistence over long periods of time (MARCON et al., 2011). Although PCR methods are limited to estimating parasitic load, this

approach is highly sensitive and reliable as criteria for parasitological cure (CALDAS et al., 2012; DUFFY et al., 2009).

Considering the inhibitory potential of TR by all drugs investigated, the molecular affinity of the enzyme for its ligands was evaluated *in silico*. As the large size of the active site of TR complicates docking studies of the quantitative structure-activity relationship (QSAR), the identification and theoretical development of selective inhibitors from bioinformatics methods is challenging. Our results showed variable molecular affinity between the ligands investigated and TR. In our docking studies, the dibenzazepine clomipramine (1) presented a Glide Score of −3.902 kcal.mol^{-1}, which can be correlated with its affinity, although it was lower than that of the substrate trypanothione disulfide (GScore of −8.768 kcal.mol^{-1}). Our findings also indicated that the amino acid residue Glu467 of TR is a direct site of interaction with clomipramine by hydrogen bonding. In addition, the phenothiazine thioridazine (2) exhibited in a Gscore higher than that of clomipramine (−6.099 kcal.mol^{-1}) and presented hydrogen bond interaction with Glu466.

Clomipramine and thioridazine were the most tested TR inhibitors against *T. cruzi* infection *in vivo*. There is evidence that clomipramine (BENSON et al., 1992) and thioridazine (LO PRESTI et al., 2015; FAIRLAMB, 1999) inhibit 100% TR activity at low concentrations (Ki = 6.5 µM and 10 µM, respectively). It is worth mentioning that these drugs were primarily developed for the treatment of psychiatric disorders (GUTIERREZ-CORREA et al., 2001). The dosage and routes of administration of these drugs were based on human equivalent dosages aligned with pharmacological indications for psychiatric disorders (CONTRERA et al., 2004; NAIR and JACOB, 2016). Clomipramine and thioridazine do not induce high toxicity (BALANT-GORGIA et al., 1991; THANACOODY, 2007) and are easily absorbed orally and rapidly distributed through the body (DANIEL et al., 2000; KOBUCHI et al., 2011). Among their biological properties, they exhibit powerful anti-inflammatory, antitumor, antifungal, antibacterial, anthelmintic, and anti-protozoal activities (FAURO et al., 2013; AMARAL et al., 2001).

The inhibitory activity of tricyclic antidepressants on TR is not completely understood. It has been postulated that these drugs bind to TR with the ring system lodged against the hydrophobic wall formed by Trp21 and Met113 and the aminopropyl side chain extending toward Glu466´ and Glu467´ (KRAUTH-SIEGEL and INHOFF, 2003). More recently,

molecular modeling techniques showed that tricyclic drugs, in particular phenothiazine's, contained some of the best-fitting probes at the active site of TR and could indeed inhibit the parasite's flavoenzymes (PAULINO et al., 2005). Although there is evidence of parasitological cure after clomipramine and thioridazine administration in *T. cruzi*-infected mice (LO PRESTI et al., 2015; GARCIA et al., 2016), it is still debatable whether this effect is exclusively mediated by TR inhibition and induction of redox imbalance, or other alternative mechanisms. There is evidence that clomipramine and thioridazine also interact with membranes and their components, intracellular proteins, and dopaminergic receptors; inhibit Mg^{2+}-dependent ATPase activity; present a strong anti-calmodulin activity; induce condensation of cytoplasm organoids; and disrupt mitochondria as well as kinetoplasts (RIVAROLA and PAGLINI-OLIVA, 2002; PAGLINI-OLIVA and RIVAROLA, 2003). Possibly mediated by the combination of different effects, the lethality induced by clomipramine and thioridazine in trypomastigote and epimastigote forms of *T. cruzi* has been associated with the beneficial effects of chemotherapy in controlling tissue damage and mortality in acute and chronic infections (LO PRESTI et al., 2015; RIVAROLA et al., 2005; BAZÁN et al., 2016).

The psychotropic and other side effects of clomipramine and thioridazine preclude their ample use in the treatment of trypanosome infections (O´SULLIVAN et al., 2015). However, employing these drugs as molecular scaffolds to construct more active structures by molecular synthesis could be a reasonable strategy in the development of new anti-trypanosomal drugs. As clomipramine and thioridazine have high biodistribution and can cross the blood-brain barrier (LO PRESTI et al., 2015; THANACOODY, 2007), reservoirs of *T. cruzi* in the central nervous tissues that are not accessible to several anti-parasitic drugs could also be treated. This is a notorious pharmacological advantage potentially applicable to overcoming low cure rates, especially in the chronic phases of infection, when *T. cruzi* reservoirs are well established and quiescent (PITELLA et al., 2009; SILVA et al., 2010). In fact, divergent profiles in the biodistribution of reference drugs (i.e., benznidazole and nifurtimox) have been associated with limited parasite clearance in multiple organs (ESPERANDIM et al., 2010; BAHIA et al., 2014). Moreover, the limited pharmacological efficacy of the reference drugs appears to be associated more significantly with their low bioavailability, a feature that

increases the relevance of clomipramine and thioridazine, which exhibit high bioavailability following oral administration (THANACOODY, 2007; LAINESSE et al., 2006).

Chemical synthesis was effective in developing two TR inhibitors from phenothiazine's (2-aminodiphenylsulfide [3] and phenothiazine derivative [4]) without significant psychotropic activity (FERNANDEZ-GOMEZ et al., 1995). In our molecular docking studies, the GScores of 2-aminodiphenylsulfide (-4.871 kcal.mol^{-1}) and the phenothiazine derivative (-5,511 kcal.mol^{-1}) indicated a small difference in molecular affinity, which was lower than that of trypanothione disulfide. Although the interaction is based on hydrogen bonds, 2-aminodiphenylsulfide and the phenothiazine derivative interact with different amino acids residues in the TR active site, specifically Glu19 and Tyr111, respectively. This interesting finding reinforces the specificity of these molecules in inhibiting TR as molecular interactions occur with amino acids residues that are absent in the human glutathione reductase, the antioxidant enzyme with the highest degree of molecular similarity to TR (IRIBARNE et al., 2009). Although these molecules induce inhibitory activity against TR (Ki 10 to 25 µM) and *in vitro* cytotoxicity against *T. cruzi* trypomastigotes, *in vivo* toxicity was also identified in a murine model of Chagas disease (FERNANDEZ-GOMEZ et al., 1995). However, the low methodological score of this study prompts caution in the interpretation of *in vivo* toxicity results. The main limitations were due to incomplete information on the experimental design of the *in vivo* assays and the absence of key outcome measures (i.e., parasitemia, parasitic load, and markers of toxicity). Therefore, more controlled and comprehensive studies are needed to evaluate the *in vivo* anti-*T. cruzi* potential of phenothiazine derivatives.

An additional strategy in chemical synthesis to potentiate the anti-parasitic activity of promising substances is the insertion of metallic components in the primary structure of target molecules (NAVARRO et al., 2010; BARRY and SADLER, 2013). In this sense, two metallodrugs with inhibitory activity against TR were developed and tested *in vivo* by Olmo (OLMO et al., 2016). These drugs (7 and 8) are manganese coordination complexes containing polyamine ligands, which are also capable of generating highly oxidizing species in *T. cruzi* (OLMO et al., 2016; COMPANY et al., 2011; CUSSÓ ET AL., 2013). From our docking model, tetraamine ligand (9) exhibited the best affinity value (GScore −5.400 kcal.mol^{-1}), followed by metallodrugs (7) (GScore −4.280 kcal.mol^{-1}) and (8) (−3,760 kcal.mol^{-1}). However, the affinity indexes were not able to explain the anti-parasitic activity

identified by Olmo (OLMO et al., 2016) *in vitro* or *in vivo*. While metallodrugs (7 and 8) and tetraamine ligand (9) exhibited IC_{50} values of 1.4, 4.4, and 13.6 µM for trypomastigotes *in vitro*, respectively, these drugs reduced parasitemia in 59%, 35%, and 27% in mice infected with *T. cruzi*, respectively. These findings indicate that the anti-parasitic effect of these drugs seem to be independent of TR inhibition. This proposition is reinforced by the fact that more than 90% TR inhibition maybe required to kill the parasites (FARILAMB, 1999), and these metallodrugs determined only moderate or low TR inhibition *in vitro* (compound 9, 72% *vs.* compound 8, 23%) at 100 µM. As identified by Olmo (OLMO et al., 2016), iron superoxide dismutase (Fe-SOD) inhibition and mitochondrial and kinetoplast damage are the potential mechanisms related to *T. cruzi* cytotoxicity. Despite variable anti-parasitic effects, both metallodrugs showed promising anti-*T. cruzi* potential, especially considering that they prevented mortality in all treated groups and induced important parasitological cure rates in infected mice (compounds 7, 55% and 8, 33%) (OLMO et al., 2016).

Beyond synthetic drugs, natural plant products, such as the bisbenzylisoquinoline alkaloids cepharanthine and daphnoline, also showed TR inhibition and were tested in a murine model of *T. cruzi* infection (FOURNET et al., 2000). Docking studies showed higher affinity values for daphnoline (GScore of -6.247 $kcal.mol^{-1}$) than for cepharanthine (GScore of -3.107 $kcal.mol^{-1}$), which was potentially related to two hydrogen bond interactions with the amino acid residues Thr397 and Glu467 in the TR active site. Based on *in vitro* data reported by Fournet (FOURNET et al., 1998), TR inhibition and parasite death could not be explained by molecular affinity, since daphnoline and cepharanthine achieved IC_{50} at 50 and 15 µM for TR, and 10 and 30 µM for *T. cruzi* trypomastigotes, respectively. In addition, despite excellent TR inhibition, cepharanthine was ineffective at reducing parasitemia or preventing mortality, which occurred in 60% of treated animals, in *T. cruzi*-infected mice. Conversely, daphnoline induced a marked reduction in parasitemia and a high rate of serological cure (60%) compared with the reference drug benznidazole (31.0% cure) (FOURNET et al., 1998). Although a positive relationship between TR inhibition and *in vivo* effects has indicated a promising potential for daphnoline in anti-*T. cruzi* therapy, the trypanocidal mechanisms are possibly not restricted to TR inhibition. Blockage of calcium channels and modulation of the host immune response are also suggested as potential antitrypanosomal mechanisms for this molecule (FOURNET et al., 1998), but the evidence is

mainly based on *in vitro* models not directly related to *T. cruzi* infection (KONDO et al., 1993; SEOW et al., 1993). Further studies are required to evaluate the role of daphnoline in the treatment of Chagas disease.

In this review, we developed an integrated model based on systematic review, meta-analysis and molecular modeling to gather and analyze the preclinical evidence on the effect of TR inhibitors in *T. cruzi* infections. Our findings indicated that the main methodological limitations of the identified studies were based on recurrent underreporting of the experimental designs and outcome measures, but developing more comprehensive and controlled studies seems to be a feasible task. Our meta-analysis showed a beneficial overall effect in reducing parasitemia and mortality in *T. cruzi*-infected animals. Clomipramine and thioridazine were the most studied drugs, and they did not show protective effects against the occurrence of electrocardiographic abnormalities and affinity and density of cardiac β-adrenergic receptors. Although variable enzyme-ligand affinity has been confirmed for all drugs in molecular docking studies, the molecular affinity score was unable to explain TR inhibition and *T. cruzi* death *in vitro* as well as the anti-parasitic potential of these drugs when tested in preclinical models of *T. cruzi* infection. The divergence of *in silico*, *in vitro,* and *in vivo* findings indicated that the anti-*T. cruzi* preclinical effect of the drugs is not be restricted to TR inhibition. Controlled studies are required to determine whether and to what extent additional mechanisms contribute to *T. cruzi* cytotoxicity and trypanocidal effects induced by the TR inhibitors.

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APENDIX - SUPPLEMENTARY MATERIALS

Table S1. Complete search strategy with search filters and number of studies recovered in databases PubMed-Medline, Scopus and Web of Sciences.

PubMed-MEDLINE – Search filters	*Retrieved records*
#1 Disease: (“Chagas disease”[TIAB] OR “American trypanosomiasis”[TIAB] OR “Trypanosoma cruzi”[TIAB] OR “Trypanosoma cruzi”[TIAB])	17.002
#2 Therapeutic target / intervention: (“trypanothione reductase”[TIAB] OR “trypanothione reductase inhibitors”[TIAB])	378
#3 First animal filter: ("animal experimentation"[MeSH Terms] OR "models, animal"[MeSH Terms] OR "invertebrates"[MeSH Terms] OR "Animals"[Mesh:noexp] OR "animal population groups"[MeSH Terms] OR "chordata"[MeSH Terms:noexp] OR "chordata, nonvertebrate"[MeSH Terms] OR "vertebrates"[MeSH Terms:noexp] OR "amphibians"[MeSH Terms] OR "birds"[MeSH Terms] OR "fishes"[MeSH Terms] OR "reptiles"[MeSH Terms] OR "mammals"[MeSH Terms:noexp] OR "primates"[MeSH Terms:noexp] OR "artiodactyla"[MeSH Terms] OR "carnivora"[MeSH Terms] OR "cetacea"[MeSH Terms] OR "chiroptera"[MeSH Terms] OR "elephants"[MeSH Terms] OR "hyraxes"[MeSH Terms] OR "insectivora"[MeSH Terms] OR "lagomorpha"[MeSH Terms] OR "marsupialia"[MeSH Terms] OR "monotremata"[MeSH Terms] OR "perissodactyla"[MeSH Terms] OR "rodentia"[MeSH Terms] OR "scandentia"[MeSH Terms] OR "sirenia"[MeSH Terms] OR "xenarthra"[MeSH Terms] OR "haplorhini"[MeSH Terms:noexp] OR "strepsirhini"[MeSH Terms] OR "platyrrhini"[MeSH Terms] OR "tarsii"[MeSH Terms] OR "catarrhini"[MeSH Terms:noexp] OR "cercopithecidae"[MeSH Terms] OR "hylobatidae"[MeSH	5.990.987

Terms] OR "hominidae"[MeSH Terms:noexp] OR "gorilla gorilla"[MeSH Terms] OR "pan paniscus"[MeSH Terms] OR "pan troglodytes"[MeSH Terms] OR "pongo pygmaeus"[MeSH Terms])	
#4 Second animal filter: ((animals[tiab] OR animal[tiab] OR mice[Tiab] OR mus[Tiab] OR mouse[Tiab] OR murine[Tiab] OR woodmouse[tiab] OR rats[Tiab] OR rat[Tiab] OR murinae[Tiab] OR muridae[Tiab] OR cottonrat[tiab] OR cottonrats[tiab] OR hamster[tiab] OR hamsters[tiab] OR cricetinae[tiab] OR rodentia[Tiab] OR rodent[Tiab] OR rodents[Tiab] OR pigs[Tiab] OR pig[Tiab] OR swine[tiab] OR swines[tiab] OR piglets[tiab] OR piglet[tiab] OR boar[tiab] OR boars[tiab] OR "sus scrofa"[tiab] OR ferrets[tiab] OR ferret[tiab] OR polecat[tiab] OR polecats[tiab] OR "mustela putorius"[tiab] OR "guinea pigs"[Tiab] OR "guinea pig"[Tiab] OR cavia[Tiab] OR callithrix[Tiab] OR marmoset[Tiab] OR marmosets[Tiab] OR cebuella[Tiab] OR hapale[Tiab] OR octodon[Tiab] OR chinchilla[Tiab] OR chinchillas[Tiab] OR gerbillinae[Tiab] OR gerbil[Tiab] OR gerbils[Tiab] OR jird[Tiab] OR jirds[Tiab] OR merione[Tiab] OR meriones[Tiab] OR rabbits[Tiab] OR rabbit[Tiab] OR hares[Tiab] OR hare[Tiab] OR diptera[Tiab] OR flies[Tiab] OR fly[Tiab] OR dipteral[Tiab] OR drosphila[Tiab] OR drosophilidae[Tiab] OR cats[Tiab] OR cat[Tiab] OR carus[Tiab] OR felis[Tiab] OR nematoda[Tiab] OR nematode[Tiab] OR nematoda[Tiab] OR nematode[Tiab] OR nematodes[Tiab] OR sipunculida[Tiab] OR dogs[Tiab] OR dog[Tiab] OR canine[Tiab] OR canines[Tiab] OR canis[Tiab] OR sheep[Tiab] OR sheeps[Tiab] OR mouflon[Tiab] OR mouflons[Tiab] OR ovis[Tiab] OR goats[Tiab] OR goat[Tiab] OR capra[Tiab] OR capras[Tiab] OR rupicapra[Tiab] OR chamois[Tiab] OR haplorhini[Tiab] OR monkey[Tiab] OR monkeys[Tiab] OR anthropoidea[Tiab] OR anthropoids[Tiab] OR saguinus[Tiab] OR tamarin[Tiab] OR tamarins[Tiab] OR leontopithecus[Tiab] OR hominidae[Tiab] OR ape[Tiab] OR apes[Tiab] OR pan[Tiab] OR paniscus[Tiab] OR "pan paniscus"[Tiab] OR bonobo[Tiab] OR bonobos[Tiab] OR troglodytes[Tiab] OR "pan troglodytes"[Tiab] OR gibbon[Tiab] OR gibbons[Tiab] OR siamang[Tiab] OR	288.098

siamangs[Tiab] OR nomascus[Tiab] OR symphalangus[Tiab] OR chimpanzee[Tiab] OR chimpanzees[Tiab] OR prosimians[Tiab] OR "bush baby"[Tiab] OR prosimian[Tiab] OR bush babies[Tiab] OR galagos[Tiab] OR galago[Tiab] OR pongidae[Tiab] OR gorilla[Tiab] OR gorillas[Tiab] OR pongo[Tiab] OR pygmaeus[Tiab] OR "pongo pygmaeus"[Tiab] OR orangutans[Tiab] OR pygmaeus[Tiab] OR lemur[Tiab] OR lemurs[Tiab] OR lemuridae[Tiab] OR horse[Tiab] OR horses[Tiab] OR pongo[Tiab] OR equus[Tiab] OR cow[Tiab] OR calf[Tiab] OR bull[Tiab] OR chicken[Tiab] OR chickens[Tiab] OR gallus[Tiab] OR quail[Tiab] OR bird[Tiab] OR birds[Tiab] OR quails[Tiab] OR poultry[Tiab] OR poultries[Tiab] OR fowl[Tiab] OR fowls[Tiab] OR reptile[Tiab] OR reptilia[Tiab] OR reptiles[Tiab] OR snakes[Tiab] OR snake[Tiab] OR lizard[Tiab] OR lizards[Tiab] OR alligator[Tiab] OR alligators[Tiab] OR crocodile[Tiab] OR crocodiles[Tiab] OR turtle[Tiab] OR turtles[Tiab] OR amphibian[Tiab] OR amphibians[Tiab] OR amphibia[Tiab] OR frog[Tiab] OR frogs[Tiab] OR bombina[Tiab] OR salientia[Tiab] OR toad[Tiab] OR toads[Tiab] OR "epidalea calamita"[Tiab] OR salamander[Tiab] OR salamanders[Tiab] OR eel[Tiab] OR eels[Tiab] OR fish[Tiab] OR fishes[Tiab] OR pisces[Tiab] OR catfish[Tiab] OR catfishes[Tiab] OR siluriformes[Tiab] OR arius[Tiab] OR heteropneustes[Tiab] OR sheatfish[Tiab] OR perch[Tiab] OR perches[Tiab] OR percidae[Tiab] OR perca[Tiab] OR trout[Tiab] OR trouts[Tiab] OR char[Tiab] OR chars[Tiab] OR salvelinus[Tiab] OR "fathead minnow"[Tiab] OR minnow[Tiab] OR cyprinidae[Tiab] OR carps[Tiab] OR carp[Tiab] OR zebrafish[Tiab] OR zebrafishes[Tiab] OR goldfish[Tiab] OR goldfishes[Tiab] OR guppy[Tiab] OR guppies[Tiab] OR chub[Tiab] OR chubs[Tiab] OR tinca[Tiab] OR barbels[Tiab] OR barbus[Tiab] OR pimephales[Tiab] OR promelas[Tiab] OR "poecilia reticulata"[Tiab] OR mullet[Tiab] OR mullets[Tiab] OR seahorse[Tiab] OR seahorses[Tiab] OR mugil curema[Tiab] OR atlantic cod[Tiab] OR shark[Tiab] OR sharks[Tiab] OR catshark[Tiab] OR anguilla[Tiab] OR salmonid[Tiab] OR salmonids[Tiab] OR whitefish[Tiab] OR whitefishes[Tiab] OR salmon[Tiab] OR salmons[Tiab] OR sole[Tiab] OR solea[Tiab] OR "sea lamprey"[Tiab] OR lamprey[Tiab] OR lampreys[Tiab] OR pumpkinseed[Tiab] OR

sunfish[Tiab] OR sunfishes[Tiab] OR tilapia[Tiab] OR tilapias[Tiab] OR turbot[Tiab] OR turbots[Tiab] OR flatfish[Tiab] OR flatfishes[Tiab] OR sciuridae[Tiab] OR squirrel[Tiab] OR squirrels[Tiab] OR chipmunk[Tiab] OR chipmunks[Tiab] OR suslik[Tiab] OR susliks[Tiab] OR vole[Tiab] OR voles[Tiab] OR lemming[Tiab] OR lemmings[Tiab] OR muskrat[Tiab] OR muskrats[Tiab] OR lemmus[Tiab] OR otter[Tiab] OR otters[Tiab] OR marten[Tiab] OR martens[Tiab] OR martes[Tiab] OR weasel[Tiab] OR badger[Tiab] OR badgers[Tiab] OR ermine[Tiab] OR mink[Tiab] OR minks[Tiab] OR sable[Tiab] OR sables[Tiab] OR gulo[Tiab] OR gulos[Tiab] OR wolverine[Tiab] OR wolverines[Tiab] OR minks[Tiab] OR mustela[Tiab] OR llama[Tiab] OR llamas[Tiab] OR alpaca[Tiab] OR alpacas[Tiab] OR camelid[Tiab] OR camelids[Tiab] OR guanaco[Tiab] OR guanacos[Tiab] OR chiroptera[Tiab] OR chiropteras[Tiab] OR bat[Tiab] OR bats[Tiab] OR fox[Tiab] OR foxes[Tiab] OR iguana[Tiab] OR iguanas[Tiab] OR xenopus laevis[Tiab] OR parakeet[Tiab] OR parakeets[Tiab] OR parrot[Tiab] OR parrots[Tiab] OR donkey[Tiab] OR donkeys[Tiab] OR mule[Tiab] OR mules[Tiab] OR zebra[Tiab] OR zebras[Tiab] OR shrew[Tiab] OR shrews[Tiab] OR bison[Tiab] OR bisons[Tiab] OR buffalo[Tiab] OR buffaloes[Tiab] OR deer[Tiab] OR deers[Tiab] OR bear[Tiab] OR bears[Tiab] OR panda[Tiab] OR pandas[Tiab] OR "wild hog"[Tiab] OR "wild boar"[Tiab] OR fitchew[Tiab] OR fitch[Tiab] OR beaver[Tiab] OR beavers[Tiab] OR jerboa[Tiab] OR jerboas[Tiab] OR capybara[Tiab] OR capybaras[Tiab]) NOT medline[subset])	
Combined search: (#1 AND #2) AND (#3 OR #4)	140

SCOPUS – *Search filters*	***Retrieved records***
#1 Disease: ((TITLE-ABS-KEY("Chagas disease") OR TITLE-ABS-KEY("American trypanosomiasis") OR TITLE-ABS-KEY("Trypanosoma cruzi") OR TITLE-ABS-KEY("Trypanosoma cruzus")))	*23.654*
#2 Therapeutic target / Intervention: ((TITLE-ABS-KEY("trypanothione reductase") OR TITLE-ABS-	*495*

KEY("trypanothione reductase inhibitors")))

Combined search: #1 AND #2	*247*
Search limits: *in vitro, review, conference paper,* book chapter *[exclude]; animals [limit to]*	*175*

WEB OF SCIENCE – ***Search filters***	***Retrieved records***
#1 Disease: TS=Chagas disease OR TS=American trypanosomiasis OR TS=Trypanosoma cruzi OR TS=Trypanosoma cruzus	*21.596*
#2 Therapeutic target / Intervention: TS= trypanothione reductase OR TS= trypanothione reductase inhibitors	*866*
#3 Animal filter: TS=mice OR TS=mouse OR TS=rat OR TS=rats OR TS=dog OR TS=dogs OR TS=rabbits OR TS=murine model OR TS=guinea pig OR TS=hamster OR TS=pig OR TS=animal model	*3.674.547*
Combined search: #1 AND #2 AND #3	*48*

Table S2. Analysis of methodological quality (reporting bias) of all studies included in the systematic review.

	Quality criteria / Studies included	Olmo et al. 2016	Fauro et al. 2013	Bazan et al. 2008	Rivarola et al. 2005	Lo Presti et al. 2004	Rivarola et al. 2001	Fournet et al. 2000	Baillet et al. 1996	Bazan et al. 2016	Rivarola et al. 1999	Strauss et al. 2013	Gobbi et al. 2010	Bustamente et al. 2010	Paglini-Oliva et al. 1998	Criteria completed (n)	Criteria completed (%)
	Title																
1	Accurate and concise description of the article content	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	15	100.0
	Abstract																
2	Background, research objectives, methods, principal findings and conclusions	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	15	100.0
	Introduction																
3	Sufficient scientific background	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	15	100.0
4	Explanation of the experimental approach	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	15	100.0
	Objectives																
5	Clear primary and secondary objectives	Y	Y	Y	Y	Y	Y	Y	Y	N	N	N	N	N	N	9	60.0

Y, criteria completed. N, criteria not completed.

Table S2 *(continuation).* Analysis of methodological quality (reporting bias) of all studies included in the systematic review.

	Quality criteria / Studies included	Olmo et al. 2016	Fauro et al. 2013	Bazan et al. 2008	Rivarola et al. 2005	Lo Presti et al. 2001	Rivarola et al. 2001	Fournet et al. 2000	Baillet et al. 1996	Bazan et al. 2016	Rivarola et al. 1999	Strauss et al. 2013	Gobbi et al. 2010	Bustamente et al. 2010	Paglini-Oliva et al. 1998	Criteria completed (n)	Criteria completed (%)
	Materials and Methods																
	Ethical statement																
6	Ethical permissions	Y	N	Y	Y	Y	N	N	N	Y	N	Y	Y	Y	N	9	60.0
	Study design																
7	The experiment blinded	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	0.0
8	Dosage of treatment	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	15	100.0
9	Route of administration	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	14	93.3
10	Duration of treatment	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	15	100.0
11	Period of treatment administration	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	0.0
12	Site of treatment administration	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	14	93.3
13	Rationale for dosage choice	N	N	N	N	N	Y	N	N	N	N	N	N	N	Y	2	13.3
14	Rational choice of administration rout	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	1	6.7

Y, criteria completed; N, criteria not completed.

Table S2 *(continuation).* Analysis of methodological quality (reporting bias) of all studies included in the systematic review.

	Quality criteria / Studies included	Olmo et al. 2016	Fauro et al. 2013	Bazan et al. 2008	Rivarola et al. 2005	Lo Presti et al. 2001	Rivarola et al. 2001	Fournet et al. 2000	Baillet et al. 1996	Bazan et al. 2016	Rivarola et al. 1999	Strauss et al. 2013	Gobbi et al. 2010	Bustamente et al. 2010	Paglini-Oliva et al. 1998	Citeria completed (n)	Citeria completed (%)
	Experimental animals																
15	Strain of the animals	N	Y	Y	Y	Y	Y	N	N	Y	Y	Y	Y	Y	Y	12	80.0
16	Sex of the animals	Y	Y	N	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	Y	12	80.0
17	Weight range of the animals	Y	Y	Y	Y	N	Y	N	Y	Y	Y	Y	Y	N	Y	11	73.3
18	Age of the animals	Y	N	N	N	N	N	Y	N	N	N	N	N	N	N	2	13.3
19	Previous procedures on the animals	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	0.0
	Housing and husbandry																
20	Housing of experimental animals (type of facility, cage or housing)	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	0.0
21	Experimental conditions (temperature, humidity and light cycle)	Y	N	N	N	N	N	N	N	N	N	N	N	N	N	1	6.7
22	Welfare-related assessments and interventions	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	0.0

Y, criteria completed; N, criteria not completed.

Table S2 *(continuation).* Analysis of methodological quality (reporting bias) of all studies included in the systematic review.

	Quality criteria / Studies included	Olmo et al. 2016	Fauro et al. 2013	Bazan et al. 2008	Rivarola et al. 2005	Lo Presti et al. 2001	Rivarola et al. 2001	Fournet et al. 2000	Baillet et al. 1996	Bazan et al. 2016	Rivarola et al. 1999	Strauss et al. 2013	Gobbi et al. 2010	Bustamente et al. 2010	Paglini-Oliva et al. 1998	Citeria completed (n)	Citeria completed (%)
	Sample size																
23	Report the total number of animals and animals in each group	Y	N	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	12	80.0
24	Details of sample size calculation	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	0.0
	Animals allocation																
25	Full details of animals allocation (including randomization or matching)	N	N	N	N	N	N	Y	N	N	N	N	N	N	N	1	6.7
26	Order in which the animals and groups were treated and assessed	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	0.0
	Experimental outcomes																
27	Clear experimental outcomes assessed	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	15	100.0
	Statistical methods																
28	Statistical methods used in data analysis	N	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	12	80.0
29	Assess whether the data met the assumptions of the statistical approach	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	0.00

Y, criteria completed; N, criteria not completed.

Table S2 *(continuation).* Analysis of methodological quality (reporting bias) of all studies included in the systematic review.

	Quality criteria / Studies included	Olmo et al. 2016	Fauro et al. 2013	Bazan et al. 2008	Rivarola et al. 2005	Lo Presti et al. 2001	Rivarola et al. 2001	Fournet et al. 2000	Baillet et al. 1996	Bazan et al. 2016	Rivarola et al. 1999	Strauss et al. 2013	Gobbi et al. 2010	Bustamente et al. 2010	Paglini-Oliva et al. 1998	Citeria completed (n)	Citeria completed (%)
	Results																
	Baseline data																
30	Description of health status of animals before treatment	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	0.0
	Outcomes and estimation																
32	Animals or data not included in the analysis (explanation for exclusion)	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	0.0
33	Information on parasitemia	Y	Y	Y	Y	N	Y	Y	N	Y	Y	Y	Y	N	Y	11	73.3
34	Information regarding inflammation	N	Y	Y	N	Y	Y	N	N	N	Y	Y	Y	Y	Y	10	66.7
35	Information regarding animals' mortality	N	Y	Y	Y	Y	Y	Y	N	Y	Y	Y	N	Y	N	11	73.3

Y, criteria completed; N, criteria not completed.

Table S2 *(continuation).* Analysis of methodological quality (reporting bias) of all studies included in the systematic review.

	Quality criteria / Studies included	Olmo et al. 2016	Fauro et al. 2013	Bazan et al. 2008	Rivarola et al. 2005	Lo Presti et al. 2001	Rivarola et al. 2001	Fournet et al. 2000	Baillet et al. 1996	Bazan et al. 2016	Rivarola et al. 1999	Strauss et al. 2013	Gobbi et al. 2010	Bustamente et al. 2010	Paglini-Oliva et al. 1998	Citeria completed (n)	Citeria completed (%)
	Discussion																
	Interpretation / scientific implications																
37	Interpretation of the results consider the objectives and hypotheses. current theory and relevant studies	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	15	100.0
38	Comments on the study limitations (sources of bias and limitations of the animal model)	N	N	N	N	N	N	N	N	N	N	N	N	N	N	0	0.0
	Generalizability/translation																
39	Comments on the relevance to human biology	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	N	N	Y	13	86.7
	Funding																
40	List of funding sources	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	15	100.0
	Criteria completed (n)	19	20	24	21	20	22	20	15	20	20	21	19	17	20		**19.9 ± 2.1**
	Criteria completed (%)	50.0	52.6	63.2	55.3	52.6	58.0	52.6	39.5	52.6	52.6	55.3	50.0	44.7	52.6		**52.3 ± 5.6**

Y, criteria completed; N, criteria not completed.

Table S3. General characteristics of all studies investigating the effect of trypanothione reductase inhibitors on experimental Chagas disease.

Authors	Animal species	Lineage	Sex	Age	Weight	*T. cruzi* strain
Olmo et al. 2016	Mice	Balb/C	Female	6- 8 weeks	25 - 30 g	CL Brener (?)
Lo Presti et al. 2015	Mice	(-)	(-)	(-)	(-)	Tulahuen and SGO-Z12
Fauro et al. 2013	Mice	Swiss	Male and Female	(-)	30 ± 1g	Tulahuen
Bazan et al. 2008	Mice	Swiss	(-)	(-)	30 ± 1 g	Tulahuen
Rivarola et al. 2005	Mice	Swiss	Male	(-)	30 ± 1 g	SGO-Z12
Lo Presti et al. 2004	Mice	Swiss	Male	(-)	(-)	SGO-Z12
Rivarola et al. 2001	Mice	Swiss	Male		30 ± 1 g	Tulahuen
(-)Fournet et al. 2000	Mice	Balb/C	Male and Female	6 - 8 weeks	(-)	(-)
Baillet et al. 1996	Mice	Swiss	Female	(-)	20 - 25 g	(-)
Bazan et al. 2016	Mice	Swiss	Male and Female	(-)	30 ± 1 g	Tulahuen
Rivarola et al. 1999	Mice	Swiss	Male	(-)	30 ± 1 g	Tulahuen
Strauss et al. 2013	Mice	Swiss	Male and Female	(-)	30 ± 1 g	Tulahuen
Gobbi et al. 2010	Mice	Swiss	Male	(-)	30 ± 1 g	Tulahuen
Bustamante et al. 2007	Mice	Swiss	(-)	(-)	(-)	Tulahuen and SGO-Z12
Paglini-Oliva et al. 1998	Mice	Swiss	Male	(-)	30 ± 1 g	Tulahuen

(-) Data not reported or not investigated. (?) *T. cruzi* CL Brener strain was not reported for mice infection but was quoted in PCR analysis.

Table S3 (*continuation*). General characteristics of all included studies.

Authors	Treatment tested	Administration route	Dose	Treatment (days)
Olmo et al. 2016	Tetraamina-based drugs	(-)	5 mg/kg	5
Lo Presti et al. 2015	Thioridazine	Oral	80 mg/kg	Acute: 3 / Chronic: 12
Fauro et al. 2013	Clomipramine	Intraperitoneal	5 mg/kg	60
Bazan et al. 2008	Clomipramine	Intraperitoneal	5 mg/kg	30
Rivarola et al. 2005	Clomipramine	Intraperitoneal	5 and 40 mg/kg	30 and 60
Lo Presti et al. 2004	Thioridazine	Oral	80 mg/kg	3
Rivarola et al. 2001	Clomipramine	Intraperitoneal	5 and 40mg/kg	7 and 30
Fournet et al. 2000	Bisbenzylisoquinolone alkaloids	Oral	25 mg/kg	30
Baillet et al. 1996	Diphenylsulfide (2-amino diphenylsulfides) derivatives	Oral and intraperitoneal	40 mg/kg	4
Bazan et al. 2016	Clomipramine	Intraperitoneal	5 mg/kg	60
Rivarola et al. 1999	Thioridazine	Oral	80 mg/kg	3
Strauss et al. 2013	Clomipramine	Intraperitoneal	5 mg/kg	30
Gobbi et al. 2010	Clomipramine	Intraperitoneal and Oral	5 mg/kg;	90
Bustamante et al. 2007	Thioridazine	Oral	80 mg/kg	12 and 30
Paglini-Oliva et al. 1998	Thioridazine	Oral	4, 5, 6, 16 and 80 mg/kg	3

(-) Data not reported or not investigated.

Table S4. Parasitemia and mortality in control infected mice and those treated with trypanothione reductase inhibitors.

Study	Groups of treatment / animals per group (n)	Groups	Infection stage	Parasitemia (blood tryp./mL × 10^3)		Survival, n (%)
Olmo et al. 2016	Control infected (n=6)	Untreated	Acute phase	60.24	0.05	6 (100%)
	Compound 2 (n=6)	5 mg/kg		38.27	0.05	6 (100%)
	Compound 3 (n= 6)	5 mg/kg		40.00	0.04	6 (100%)
	Compound 5 (n=6)	5 mg/kg		44.35	0.05	6 (100%)
Lo Presti et al. 2015	Control infected Tul (n=25)	Untreated	Acute phase	(-)	(-)	5 (20%)
	Infected treated Tul (n=60)	TZ (80 mg/kg)		(-)	(-)	48 (80%)
	Control infected SGO (n=42)	Untreated		(-)	(-)	26 (44%)
	Infected treated SGO (n=42)	TZ (80 mg/kg)		(-)	(-)	7 (17.5%)
Fauro et al. 2013	Control infected (n= 25)	Untreated	Chronic phase	(-)	(-)	4 (15%)
	Infected treated (n= 30)	CLO (5 mg/kg)		(-)	(-)	7 (22%)
Bazan et al. 2008	Control infected (n= 21)	Untreated	Acute phase	(-)	(-)	11 (48%)
	Infected treated (n= 37)	CLO (5 mg/kg)		101.13	13.98	33 (90%)
Lo Presti et al. 2004	Control infected (n= 42)	TZ (80 mg/kg)	Chronic phase	(-)	(-)	8 (20%)
	Infected treated (n= 42)					21 (50%)

(-) Data not reported or not investigated, TZ, Thioridazine; CLO, clomipramine; Ceph, cepharanthine; Daph, daphnoline; SGO, SGO-Z12 *T. cruzi* strain; TUL, Tulahuen *T. cruzi* strain. Parasitemia: mean and standard error (mean ± SE). Survival: absolute number and percentage, n (%).

Table S4 (*continuation*). Parasitemia and mortality in control infected mice and those treated with trypanothione reductase inhibitors.

Study	Groups of treatment / animals per group (n)	Groups	Infection stage	Parasitemia (blood tryp./mL × 10^3)		Survival, n (%)
Rivarola et al. 2005	Control infected (n= 40)	Untreated	Acute phase	455.20	52.04	12 (30%)
		CLO (5 mg/kg)		29.15	2.21	28 (70%)
	Clomipramine 5 (n= 40)	CLO (40 mg/kg)		69.66	1.62	24 (60%)
	Clomipramine 40 (n= 40)	Untreated	Chronic phase	0.00	0.00	(-)
		CLO (5 mg/kg)		0.00	0.00	(-)
		CLO (40 mg/kg)		0.00	0.00	(-)
Rivarola et al. 2001	Control infected (n= 20)	Untreated	Acute phase	93.19	19.79	14 (70%)
		CLO (5 mg/kg)		10.53	14.36	32 (80%)
	Clomipramine 5 (n= 40)	CLO (40 mg/kg)		7.34	14.36	36 (90%)
	Clomipramine 40 (n= 40)	Untreated	Chronic phase	0.00	0.00	16 (80%)
		CLO (5 mg/kg)		0.00	0.00	30 (75%)
		CLO (40 mg/kg)		0.00	0.00	28 (70%)

(-) Data not reported or not investigated, TZ, Thioridazine; CLO, clomipramine; Ceph, cepharanthine; Daph, daphnoline; SGO, SGO-Z12 *T. cruzi* strain; TUL, Tulahuen *T. cruzi* strain. Parasitemia: mean and standard error (mean ± SE). Survival: absolute number and percentage, n (%).

Table S4 (*continuation*). Parasitemia and mortality in control infected mice and those treated with trypanothione reductase inhibitors.

Study	Groups of treatment / animals per group (n)	Groups	Infection stage	Parasitemia (blood tryp./mL × 10^3)		Survival, n (%)
Fournet et al. 2000	Parasitological assays:					
	Control infected (n= 10-15)	Untreated	Acute phase	143.72	8.31	38 (85%)
	Cepharanthine (n= 10-15)	Ceph (25 mg/kg)		204.21	16.06	20 (57%)
	Daphnoline (n= 10-15)	Daph (25 mg/kg)		98.31	11.27	33 (74%)
	Mortality assays:					
	Control infected (n= 45)	Untreated	Chronic phase	0.54	0.02	30 (67%)
	Cepharanthine (n= 35)	Ceph (25 mg/kg)		3.36	0.19	20 (57%)
	Daphnoline (n= 45)	Daph (25 mg/kg)		0.27	0.013	25 (56%)
Bazan et al. 2016	Control infected (n= 10)	Untreated	Acute phase	108.81	9.85	(-)
		CLO (5 mg/kg)		(-)	(-)	(-)
	Clomipramine (n= 10)	Untreated	Chronic phase	(-)	(-)	6 (63%)
		CLO (5 mg/kg)		(-)	(-)	7 (67%)

(-) Data not reported or not investigated, TZ, Thioridazine; CLO, clomipramine; Ceph, cepharanthine; Daph, daphnoline; SGO, SGO-Z12 *T. cruzi* strain; TUL, Tulahuen *T. cruzi* strain. Parasitemia: mean and standard error (mean ± SE). Survival: absolute number and percentage, n (%).

Table S4 (*continuation*). Parasitemia and mortality in control infected mice and those treated with trypanothione reductase inhibitors.

Study	Groups of treatment / animals per group (n)	Groups	Infection stage	Parasitemia (blood tryp./mL × 10^3)		Survival, n (%)
Rivarola et al. 1999	Control infected (n= 20)	Untreated	Acute phase	52.81	11.32	8 (40%)
	Thioridazine (n= 60)	TZ (80 mg/kg)		12.49	2.06	54 (90%)
		Untreated	Chronic phase	0.00	0.00	16 (80%)
		TZ (80 mg/kg)		0.00	0.00	12 (20%)
Strauss et al. 2013	Control infected (n= 20)	Untreated	Acute phase	50.34	1.74	9 (45%)
	Clomipramine (n= 20)	CLO (5 mg/kg)		25.95	2.14	11 (55%)
Gobbi et al. 2010	Control infected (n= 20)	Untreated	Acute phase	38.07	6.40	(-)
	Clomipramine (n= 20)	CLO (5 mg/kg)		21.68	3.58	(-)
Bustamante et al. 2007	TUL infected (n= 25)	Untreated	Chronic phase	(-)	(-)	7 (30%)
	TUL inf. + Thioridazine (n= 25)	TZ (80 mg/kg)		(-)	(-)	22 (88%)
	SGO-Z12 infected (n= 20)	Untreated		(-)	(-)	8 (40%)
	SGO-Z12 inf. + Thioridazine (n= 20)	TZ (80 mg/kg)		(-)	(-)	18 (88%)
Paglini-Oliva et al. 1998	Control infected (n= 100)	Untreated	Acute phase	31.97	3.80	(-)
	Thioridazine (n= 25)	TZ (80 mg/kg)		31.20	1.80	(-)

(-) Data not reported or not investigated, TZ, Thioridazine; CLO, clomipramine; Ceph, cepharanthine; Daph, daphnoline; SGO, SGO-Z12 *T. cruzi* strain; TUL, Tulahuen *T. cruzi* strain. Parasitemia: mean and standard error (mean ± SE). Survival: absolute number and percentage, n (%).

Table S5. Parasitic load and cure rate in studies using polymerase chain reaction (PCR) or serology.

Study	Groups (mice)	Parasitic load (Parasite equivalent / ng DNA)	Cure, n (%)
Olmo *et al*. 2016*	Untreated (n=6)	(-)	0 (0%)
	Compound 2 (n=6)		2 (33.33%)
	Compound 3 (n=6)		3 (50%)
	Compound 5 (n=6)		0 (0%)
Fauro et al. 2013*	Untreated (n=25)	(-)	?
	CLO (5 mg/kg) (n=30)		
Strauss et al. 2013*	Untreated (n=20)	(-)	0 (0%)†
	CLO (5 mg/kg) (n=20)		0 (0%)†
Bazan et al. 2016*	Untreated (n=10)	90.47 ± 51.28	7 (75.87%)
	CLO (5 mg/kg) (n=10)	30.76 ± 7.69	8 (79.76%)
Fournet et al. 2000#	Untreated (n= 60)	(-)	10 (17%)
	Cepharanthine (n= 47)	(-)	24 (51%)
	Daphnoline (n= 55)	(-)	46 (84%)

*PCR, # Serology. (-) Not investigated. (?) Inconclusive analysis due to limited sample or absence of measures for each mice. †Information was reported in the results, but no graphical element was presented.

Table S6. Electrocardiographic data from control infected mice and those treated with trypanothione reductase inhibitors.

Authors	Days post-infection	Groups	Pulse (beats/min)	Axes (grade)	PQ Interval	QRS Interval
Rivarola et al. 2001	35	Untreated (n=20)	268.00 ± 5.53	57.00 ± 3.00	0.02-0.04	0.02-0.03
		CLO 5mg/kg (n=40)	260.00 ± 41.00	63.00 ± 2.84	0.02-0.04	0.02-0.04
		CLO 40mg/kg (n=40)	255.00 ± 4.43	60.00 ± 6.17	0.02-0.04	0.02-0.03
	75	Untreated (n=20)	263.00 ± 4.90	55.00 ± 3.95	0.02-0.03	0.02-0.04
		CLO 5mg/kg (n=40)	286.00 ± 3.48	65.00 ± 5.22	0.02-0.04	0.03-0.04
		CLO 40mg/kg (n=40)	282.00 ± 3.95	59.00 ± 4.27	0.02-0.05	0.03-0.04
	135	Untreated (n=20)	263.00 ± 6.48	55.00 ± 5.22	0.02-0.04	0.03-0.05
		CLO 5mg/kg (n=40)	260.00 ± 5.53	67.00 ± 4.74	0.02-0.02	0.02-0.03
		CLO 40mg/kg (n=40)	295.00 ± 4.43	57.00 ± 3.32	0.02-0.05	0.03-0.04
Bustamante et al. 2007	215	Untreated (Tul) (n=25)	570.00 ± 20.00	45.00 ± 10.00	0.027 ± 0.001	0.017 ± 0.001
		Tul + TZ 80mg/kg (n=25)	586.00 ± 10.00	31.00 ± 8.00	0.022 ± 0.001	0.015 ± 0.001
		Untreated (SGO-Z12) (n=19)	500.00 ± 15.00	50.00 ± 2.00	0.03 ± 0.002	0.035 ± 0.01
		SGO-Z12 + TZ 80mg/kg (n=20)	495.00 ± 15.00	41.00 ± 2.50	0.015 ± 0.001	0.015 ± 0.002
	350	Untreated (Tul) (n=25)	569.00 ± 29.00	38.00 ± 15.00	0.024 ± 0.001	0.015 ± 0.001
		Untreated (SGO-Z12) (n=19)	470.00 ± 12.00	48.00 ± 3.00	0.034 ± 0.01	0.035 ± 0.01
		Tul + TZ 80mg/kg (n=25)	586.00 ± 11.00	20.00 ± 6.00	0.022 ± 0.001	0.014 ± 0.001
		SGO-Z12 + TZ 80mg/kg (n=16)	515.00 ± 45.00	35.00 ± 5.50	0.02 ± 0.001	0.016 ± 0.001

Pulse and axes: mean and standard error (mean ± SE). PQ and QRS intervals: maximum and minimum.

Table S6 (*continuation*). Electrocardiographic data from infected untreated mice and those treated with trypanothione reductase inhibitors.

Authors	Days post-infection	Groups	Pulse (beats/min)	Axes (grade)	PQ Interval	QRS Interval
Rivarola et al. 1999	35	Untreated (n=10)	468.00 ± 12.10	51.00 ± 4.70	0.02-0.04	0.02-0.03
		TZ 80mg/kg (n=10)	498.00 ± 15.80	60.00 ± 5.10	0.02-0.04	0.02-0.03
	75	Untreated (n=10)	575.00 ± 4.80	44.00 ± 2.30	0.03-0.04	0.02-0.04
		TZ 80mg/kg (n=10)	519.00 ± 15.10	58.00 ± 4.90	0.02-0.04	0.02-0.04
	135	Untreated (n=10)	526.00 ± 16.90	55.00 ± 2.90	0.02-0.05	0.02-0.06
		TZ 80mg/kg (n=10)	503.00 ± 14.90	63.00 ± 3.80	0.02-0.04	0.02-0.04
Rivarola et al. 2005	55	Untreated (n=40)	501.80 ± 15.80	44.00 ± 6.00	0.015-0.03	0.02-0.04
		CLO 5mg/kg (n=40)	483.00 ± 54.70	32.00 ± 8.30	0.01-0.03	0.01-0.01
		CLO 40mg/kg (n=40)	464.00 ± 67.00	34.00 ± 27.00	0.01- 0.03	0.01-0.01
	90	Untreated (n=40)	509.70 ± 17.50	48.30 ± 8.40	0.02-0.04	0.02-0.04
		CLO 5mg/kg (n=40)	560.00 ± 54.70	36.00 ± 19.00	0.01-0.02	0.01-0.01
		CLO 40mg/kg (n=40)	560.00 ± 51.60	57.00 ± 8.20	0.01-0.02	0.01-0.01
	135	Untreated (n=40)	467.70 ± 12.00	38.20 ± 6.50	0.015-0.03	0.015-0.07
		CLO5mg/kg (n=40)	510.00 ± 22.00	57.00 ± 10.00	0.01-0.02	0.01-0.01
		CLO 40mg/kg (n=40)	517.00 ± 72.00	48.00 ± 15.20	0.01-0.02	0.01-0.01
Bazan et al. 2008	150	Untreated (n= 7)	668.00 ± 27.40	36.20 ± 4.9 0	N	N
		CLO 5mg/kg (n= 31)	622.00 ± 12.00	53.20 ± 4.12	N	N
Strauss et al. 2013	90	Untreated (n= 9)	491.25 ± 2.10	N	N	N
		CLO 5mg/kg (n=13)	500.50 ± 27.14	N	N	N

N, not investigated. Pulse and axes: mean and standard error (mean ± SE). PQ and QRS intervals: maximum and minimum.

Table S7. Electrocardiographic abnormalities and β-adrenergic receptors affinity and density in control and treated mice.

Authors	Days post-inf.	Groups	Abnormality, n (%)	β affinity (nM)	β density (mol/mg protein)
Rivarola et al. 2001	35	Untreated (n=20)	7 (35%)	5.63 ± 0.26	78.25 ± 1.67
		CLO 5mg/kg (n=40)	17 (42%)	7.46 ± 1.12	73.32 ± 4.73
		CLO 40mg/kg (n=40)	17 (43%)	4.83 ± 0.72	76.06 ± 4.18
	75	Untreated (n=20)	10 (50%)	6.86 ± 020	77.28 ± 091
		CLO 5mg/kg (n=40)	25 (62%)	0.900 ± 0.18	30.42 ± 1.59
		CLO 40mg/kg (n=40)	19 (48%)	2.93 ± 0.53	55.66 ± 4.12
	135	Untreated (n=20)	18 (90%)	11.21 ± 0.25	53.33 ± 0.71
		CLO 5mg/kg (n=40)	0 (0%)	4.56 ± 0.98	65.02 ± 4.88
		CLO 40mg/kg (n=40)	25 (63%)	4.43 ± 1.22	63.35 ± 6.01
Bustamante et al. 2007	215	Untreated (Tul) (n=20)	16 (65%)	N	N
		Tul + TZ 80mg/kg (n=40)	9 (36.7%)	N	N
		Untreated (SGO-Z12) (n=20)	12 (62%)	N	N
		SGO-Z12 + TZ 80mg/kg (n=40)	7 (33%)	N	N
	350	Untreated (Tul) (n=20)	17 (66.6%)	N	N
		Tul + TZ 80mg/kg (n=40)	11 (43.3%)	N	N
		Untreated (SGO-Z12) (n=20)	13 (63%)	N	N
		SGO-Z12 + TZ80mg/kg (n=40)	5 (33%)	N	N

N, not investigated. Affinity and density of β-adrenergic receptors: mean and standard error (mean ± SE).

Table S7 (*continuation*). Electrocardiographic abnormalities and β-adrenergic receptors affinity and density in control and treated mice.

Authors	Days post-inf.	Groups	Abnormality, n (%)	β affinity (nM)	β density (mol/mg protein)
Rivarola et al. 1999	35	Untreated (n=10)	1 (12.5%)	5.63 ± 0.27	78.25 ± 1.67
		TZ 80mg/kg (n=10)	2 (16%)	6.84 ± 0.36	76.85 ± 1.69
	75	Untreated (n=10)	7 (66%)	6.86 ± 0.21	77.28 ± 0.91
		TZ 80mg/kg (n=10)	4 (40%)	5.82 ± 0.32	74.37 ± 1.57
	135	Untreated (n=10)	6 (60%)	11.21 ± 0.26	53.33 ± 0.71
		TZ 80mg/kg (n=10)	4 (37%)	5.48 ± 0.21	72.34 ± 1.06
Rivarola et al. 2005	55	Untreated (n=40)	2 (18%)	5.71 ± 0.56	207.60 ± 8.10
		CLO 5mg/kg (n=40)	1 (11%)	9.05 ± 0.86	271.30 ± 10.10
		CLO 40mg/kg (n=40)	2 (17%)	6.06 ± 0.70	255.90 ± 10.00
	90	Untreated (n=40)	4 (36%)	6.28 ± 0.40	228.00 ± 6.02
		CLO 5mg/kg (n=40)	2 (20%)	8.10 ± 0.80	292.20 ± 11.20
		CLO 40mg/kg (n=40)	1 (10%)	5.32 ± 0.44	249.80 ± 6.60
	135	Untreated (n=40)	5 (55%)	7.32 ± 0.19	184.10 ± 2.10
		CLO 5mg/kg (n=40)	2 (18%)	7.74 ± 0.94	302.40 ± 13.6
		CLO40mg/kg (n=40)	2 (16%)	4.90 ± 0.48	230.60 ± 6.98

N. not investigated. Affinity and density of β-adrenergic receptors: mean and standard error (mean ± SE).

Table S7 (*continuation*). Electrocardiographic abnormalities and β-adrenergic receptors affinity and density in control and treated mice.

Authors	Days post-inf.	Groups	Abnormality, n (%)	β affinity (nM)	β density (mol/mg protein)
Bazan et al. 2008	150	Untreated (n= 7)	N	11.20 ± 0.26	53.30 ± 0.71
		CLO 5mg/kg	N	6.27 ± 0.23	77.20 ± 1.08
Bazan et al. 2016	90	Untreated (n=10)	4 (36%)	N	N
		CLO 5mg/kg (n=10)	3 (33%)	N	N
	180	Untreated (n=10)	5 (50%)	N	N
		CLO5mg/kg (n=10)	6 (64%)	N	N
	270	Untreated (n=10)	6 (57%)	N	N
		CLO 5mg/kg (n=10)	2 (20%)	N	N

N, not investigated. N, not investigated. Affinity and density of β-adrenergic receptors: mean and standard error (mean ± SE).

Table S8. Results obtained in infected mice treated with trypanothione reductase inhibitors compared to infected untreated mice.

Authors	Treatment	Immunological, histopathological and biochemical findings	Electrocardiographic findings
Olmo et al. 2016	Tetraamine-based compounds	Compound 3 reduced uric acid, urea and CK-MB. LDH levels (30% - 40%) in treated mice	N
Lo Presti et al. 2015	Thioridazine	Reduced necrosis, fibrosis and inflammatory infiltrate in treated mice	Prevented cardiomyopathy progression and reduced electrocardiographic abnormalities (cardiac blockades)
Fauro et al. 2013	Clomipramine	Reduced myocarditis, fibrosis, anti-*T. cruzi* antibodies serum levels and parasite load	N
Bazan et al. 2008	Clomipramine	Reduced heart inflammatory infiltrate and necrosis	Reduced cardiomyopathy progression and electrocardiographic abnormalities
Rivarola et al. 2005	Clomipramine	Similar anti-cruzipain IgG serum levels and reduced inflammatory infiltrate	Reduced electrocardiographic abnormalities (pulse, axes, PQ and QRS intervals)
Rivarola et al. 2005	Clomipramine	Similar anti-cruzipain IgG serum levels and reduced inflammatory infiltrate	Reduced electrocardiographic abnormalities (pulse, axes, PQ and QRS intervals)

*Benznidazole was used as a reference antitrypanosomal drug. N, not investigated.

Table S8 ***(continuation).*** Results obtained in infected mice treated with trypanothione reductase inhibitors compared to infected untreated mice.

Authors	Treatment	Immunological, histopathological and biochemical findings	Electrocardiographic findings
Lo Presti et al. 2004	Thioridazine	Similar anti-cruzipain IgG serum levels and reduced inflammatory infiltrate	Reduced β-receptors affinity and higher β-receptors density
Rivarola et al. 2001	Clomipramine	Reduced anti-cruzipain IgG antibody serum levels in treated groups	Reduced receptor density, affinity and electrocardiographic abnormalities (pulse, axes, PQ and QRS intervals)
Fournet et al. 2000	Bisbenzylisoquin-olone alkaloids	N	N
Baillet et al. 1996	Diphenylsulfide derivatives	N	N
Bazan et al. 2016	Clomipramine	Similar anti-*T. cuzi* antigens serum levels. Reduced parasitic DNA in blood samples	Attenuation of intra-ventricular block and arrhythmias
Rivarola et al. 1999	Thioridazine	Similar anti-cruzipain IgG serum levels, reduced inflammatory infiltrate and absence of amastigote nests	Attenuation of intra-ventricular block and arrhythmias. Reduced receptor affinity and higher density

N, not investigated.

Table S8 ***(continuation).*** Results obtained in infected mice treated with trypanothione reductase inhibitors compared to infected untreated mice.

Authors	Treatment	Immunological, histopathological and biochemical findings	Electrocardiographic findings
Strauss et al. 2013	Clomipramine	Absence of necrosis, similar fibrosis and inflammatory infiltrate. No evidences of liver and kidney toxicity.	No prevented prolonged PR interval (atrioventricular block)
Gobbi et al. 2010	Clomipramine	Similar anti-cruzipain IgG serum levels and reduced myocardial damage	Reduced β-receptor affinity and higher density. No electrocardiographic abnormalities
Bustamante et al. 2007	Thioridazine	Reduced heart necrosis, fibrosis and inflammatory infiltrate	Reduced β-receptor density, affinity, and electrocardiographic abnormalities (pulse, axes, PQ and QRS intervals)
Paglini-oliva et al. 1998	Thioridazine	Reduced inflammatory infiltrates and no amastigote nests	Prevented cardiomyopathy progression

N, not investigated.

CHAPTER 2

COMPARISON BETWEEN MONOTHERAPY AND COMBINATION THERAPY BASED ON BENZNIDAZOLE AND THIORIDAZINE FOR THE TREATMENT OF EXPERIMENTAL *TRYPANOSOMA CRUZI* INFECTION

Chapter II

ABSTRACT

Although phenothiazine's exhibit antitrypanosomal effect *in vitro*, the relevance of these drugs in the treatment of *Trypanosoma cruzi* infection *in vivo* is poorly explored, especially in combination with reference antitrypanosomal drugs. Thus, we used a murine model of Chagas disease to compare the antiparasitic potential of thioridazine (TDZ) and benznidazole (Bz) administered in monotherapy and combined. Female Swiss mice were randomized into six groups: (i) uninfected untreated, (ii) infected untreated, infected and treated with (iii) Bz (100 mg/kg), (iv) TDZ (80 mg/kg), (v) Bz (100 mg/kg) + TDZ (80 mg/kg), (vi) Bz (50 mg/kg) + TDZ (80 mg/kg). Infected animals were intraperitoneally inoculated with 2000 *T. cruzi* trypomastigotes (Y strain) and treated by gavage during 20 days. The animals treated only with thioridazine presented the highest levels of parasitemia, parasitic load, anti-*T. cruzi* immunoglobulin G plasma titers, plasma and/or cardiac cytokine levels (IFN-γ, TNF-α, IL-10 and IL-17), as well as cardiac, skeletal muscle and hepatic damage when compared to the other groups ($P<0.05$). These parameters were significantly reduced in the group treated with Bz-based monotherapy compared to the other groups ($P <0.05$). However, the combination of TDZ with Bz at the therapeutic dose (100 mg/kg) and mainly at half of this dose attenuated the response to treatment, worsening parasitological control, systemic and tissue inflammation, as well as microstructural lesions of all organs investigated compared to the group treated with Bz alone ($P<0.05$). Taken together, our results indicated that when administered alone, TDZ potentiated tissues injuries in animals infected with a virulent and pathogenic *T. cruzi* strain and partially resistant to Bz. So TDZ attenuated the antiparasitic effect of Bz, impairing parasitological control and potentiating inflammation and pathological remodeling of the heart, skeletal muscle and liver. Our findings reinforce the evidence that Bz-based monotherapy remains a better option for the treatment of experimental Chagas disease.

Keywords: American trypanosomiasis. Experimental chemotherapy. Trypanotione reductse. Antitrypanosomal drugs. Chagas disease.

1 INTRODUCTION

Chagas disease is a potentially fatal parasitic disease caused by the protozoan *Trypanosoma cruzi* (WHO, 2019). Although this disease is endemic in Latin America countries, an increasing number of positive cases of infection have been reported in North America, Europe, Oceania and Asia (SANTOS et al., 2018; WHO, 2019). Recent estimates indicate that 6-7 million people live with Chagas disease worldwide and about 120 million people are at risk of infection (DAMASIO, 2019), especially in poor communities with low socio-economic development and limited access to health care (GASPE, 2015). In general, patients are diagnosed in the late chronic phase of disease, in which about 30-40% cases develops a symptomatic form of infection that courses severe and irreversible cardiac, gastrointestinal and/or neurological damage (MC CALL, 2018). At this stage, etiological chemotherapy is not successful in achieving parasitological cure, which occurs in about 10-20% of cases (MENDONÇA et al., 2018).

Although *T. cruzi* is capable of infecting all nucleated mammalian cells and organs, it has intense tropism by muscle tissues, making the heart a primary target of parasitism and immunomediated damage (PEREZ-MOLINA and MOLINA, 2018). During the evolution to the symptomatic chronic phase, direct cell parasitism, autoimmune and pro-oxidant events trigger pathological changes such as vascular insufficiency, hyalinization, cardiomyocytolysis, myonecrosis and fibrosis. So, the accumulation of tissue damage determines progressive microstructural and electromechanical heart deterioration (SIMÕES, 2018; CALDAS, 2019) causing at least 50,000 deaths each year due to cardiovascular failures (SILVA et al., 2019).

Benznidazole (Bz) and nifurtimox (Nfx) are the only drugs available for the etiological treatment of Chagas disease (LIDANE et al., 2019). The mechanism of action of Nifurtimox relates to the reduction of the nitro group with subsequent production of free radicals (mainly hydroxyl radicals, $OH^{\bullet -}$), which is toxic to the parasite (URBINA and DOCAMPO, 2003). Benznidazole is a prodrug that exerts its trypanocidal effects after enzymatic activation by trypanosomal type I nitroreductases (NTRs), resulting in dialdehyde glyoxal biosynthesis (CALDAS et al., 2019). As glyoxal-thiols adducts inhibits DNA biosynthesis and *T. cruzi* antioxidant system, the parasite highly susceptible to oxidative damage (CALDAS, 2019). These drugs are highly toxic and do not guarantee cure after parasite dissemination and the establishment of *T. cruzi* reservoirs in vertebrate host tissues and organs (SALES JUNIOR et al., 2017). As access to nifurtimox is limited and this drug is associated with more pronounced systemic toxicity and side effects (i.e., hypersensitivity reactions, anorexia,

vomiting, polyneuritis and bone marrow suppression), benznidazole is the first-line treatment of *T. cruzi* infection (MUÑOZ et al., 2011; CALDAS et al., 2019).

Despite therapeutic limitations, reference chemotherapy (Bz and Nfx) has been used since its development for over forty years (CALDAS et al., 2019). During this period, advances in the understanding of parasite biology and host pathogen interaction, new molecular targets have been identified as potentially relevant for the development of more selective and efficient antitrypanosomal drugs (RAJASEKARAN and CHEN, 2015). Due to the rudimentary antioxidant metabolism in trypanosomatides, disruptors of redox metabolism are potential candidates to be used as new antiparasitic drugs against *T. cruzi* infection (PAGLINI-OLIVA and RIVAROLA, 2003; MACHADO-SILVA, 2016). In a context of drug repositioning, tricyclic drugs of the phenothiazines class has proved to be effective in inhibiting trypanothione reductase (TR), the central antioxidant enzyme of *T. cruzi* (PAGLINI-OLIVA and RIVAROLA, 2003).

Thioridazine (TDZ) is the most potent phenothiazine in inhibiting *T. cruzi* TR, increasing parasite susceptibility to leucocytes-derived or drug-induced pro-oxidant effectors, especially nitric oxide, superoxide anion, hydroxyl radical and hydrogen peroxide (LO PRESTI et al., 2015; MENDONÇA et al., 2018). *In vitro* evidence indicates that TDZ also exerts direct antitrypanosomal effects, especially from its anti-calmodulin action, condensation of cytoplasmic organelles, mitochondrial and kinetoplast disruption in epimastigotes and trypomastigotes (RIVAROLA et al., 1999; RIVAROLA and PAGLININI-OLIVEIRA, 2002; PAGLINI-OLIVA and RIVAROLA, 2003). From an comprehensive systematic review, we identify that the preclinical *in vivo* evidence support the applicability of thioridazine as an antitrypanosomal drug, whose *in silico* modeling corroborates its interaction with TR catalytic site (MENDONÇA et al., 2018). By using a meta-analysis framework, it was also clear that TDZ-based chemotherapy is beneficial for attenuating morphofunctional heart damage and mortality in murine models of *T. cruzi* infection (MENDONÇA et al., 2018).

Considering the existence of *T. cruzi* strains partially resistant to benznidazole (PALACIO, et al., 2018) and that TDZ benefits are not associated with parasitological cure (MENDONÇA et al., 2018), we believed that the blockage of a single metabolic pathway may be related to the limited efficacy of monotherapy with these drugs. Currently, the antitrypanosomal potential of combination chemotherapy that includes Bz and TDZ is unclear. However, it is know that Bz exerts pro-oxidant effects (ZEFERINO et al., 2019; PAIVA, et al., 2018) and TDZ acts as a redox disruptor in *T. cruzi* (MENDONÇA et al.,

2018; BELTRAN-HORTELANO et al., 2017), so a combined therapy using this drugs becomes rational and desirable. Thus, Bz and TDZ co-administration may be relevant to simultaneously block multiple metabolic pathways associated with parasitism and tissue damage, representing a promising strategy in the treatment of Chagas disease.

2. MATERIAL AND METHODS

2.1. Animals and infection

Eighty eight-weeks-old female Swiss mice weighting 35.24g ± 5.53g were used in this study. The animals were kept in collective polypropylene cages in an environment with 12h/12h light/dark cycles, temperature (21 ± 2ºC) and humidity (60-70%) controlled. Water and ration were provided *ad libitum*. *T. cruzi* infection was induced by means of an intraperitoneal inoculum containing 2000 trypomastigotes (Y strain). The parasites were obtained from blood collected in previously infected animals (CALDAS et al., 2008). This project was approved by the Institutional Ethics Committee for the Use of Laboratory Animals (registration, 013/2016).

2.2. Treatment strategy and experimental groups

To evaluate the effect of Bz and TDZ administered in monotherapy and combined, the animals were treated orally by gavage with 0.1 mL of each treatment during 20 consecutive days (CALDAS et al., 2008). Benznidazole (LAFEPE, Recife, Pernambuco, Brazil) and thioridazine (Melleril, São Paulo, SP, Brazil) tablets were macerated and resuspended in 10 mL of water using dimethyl sulfoxide as suspending agent. The experiments were performed considering the therapeutic dose of Bz for mice, which is 100 mg/kg body weight (CALDAS et al., 2008). From this dose, the following therapeutic schemes were tested: (i) therapeutic dose and (ii) half the therapeutic dose alone or combined with TDZ. Thioridazine was administered at 80 mg/kg, which demonstrated a trypanocidal effect for *T. cruzi* strain SGO-Z12 (LO PRESTI et al., 2004). The treatments started at day 5 post-inoculation, 24h after confirming the infection through the microscopic identification of trypomastigotes in the peripheral blood of all animals (CALDAS et al., 2008). The groups were distributed as follows: Group 1: uninfected untreated; Group 2: infected untreated; Group 3: infected + Bz (100 mg/kg); Group 4: infected + TDZ (80 mg/kg); Group 5: infected + Bz (100 mg/kg) + TDZ (80 mg/kg); Group 6: infected + Bz (50 mg/kg) + TDZ (80 mg/kg).

2.3. Analysis of parasitemia and mortality

The parasitemia was evaluated daily according to technique described by Brener (1962). Briefly, 5µl of peripheral blood collected by venipuncture in the tail of the animals was transferred to a glass slide and a coverslip (22×22 mm). Then, trypomastigotes were quantified in 50 random fields in a bright field microscope with a ×40 objective lens (×400 magnification). The parasitemia curve of all infected groups was plotted (CALDAS et al., 2008), and parameters such as initial, mean, final and peak of parasitemia were additionally calculated (FELIZARDO et al., 2018). All mice were monitored during the experimental time and weighed weekly. The mortality rate was calculated by counting all animals that died during the experimental period.

2.4. Parasitic load molecular assay

The parasite load in the heart, skeletal muscle and liver were determined from the quantification of *T. cruzi* DNA by quantitative reverse transcription PCR (RT-qPCR). Briefly, total genomic DNA was extracted in organs samples using a commercial genomic DNA purification kit (Promega, São Paulo SP, Brazil) according to the protocol described by Caldas et al. (2012). DNA samples were analyzed in spectrophotometer and adjusted to 25 ng/µl (GeneQuant; Pharmacia Biotech, Piscataway, NJ, USA). All analysis were standardized at 10-µl volume containing 5µl of SYBR Green PCR Mastermix (Applied Biosystems, Carlsbad, CA, USA), 50 ng of genomic DNA, 0.35 µM *T. cruzi* 195-bp-repeat DNA-specific primers or 0.50 µM murine-specific TNF-α primers. The primers for murine TNF-α (TNF-5241, 5′-TCCCTCTCATCAGTTCTATGGCCCA-3′, and TNF-5411, 5′-CAGCAAGCATCTATGCACTTAGACCCC-3′) amplify a 170-bp product. The primers for *T. cruzi* repetitive DNA (TCZ-F, 5′-GCTCTTGCCCACAMGGGTGC-3′, where M is A or C, and TCZ-R, 5′-CCAAGCAGCGGATAGTTCAGG-3′) amplify a 182-bp fragment (CUMMINGS and TARLETON, 2003). The molecular assay was developed according previously described analytical conditions (time, temperature, and cycles) (SANTOS et al., 2015). In each 96-well reaction plate was used a standard curve and two negative controls containing *T. cruzi*-specific or mice-specific primers without DNA and tissue DNA from uninfected mice. The mean quantification values for *T. cruzi*-specific DNA were normalized by the results of mice-specific (TNF-α) primers as follows: normalized value = (mean *T. cruzi* DNA/mean TNF-α DNA) × 1,000. The value 1,000 corresponds to the expected result for TNF-α in 30 mg of organs sample. The efficiencies of amplification were determined

following calculation: efficiency (E) = $10^{-1/slope}$ (StepOne Software v2.0, ThermoFisher, São Paulo SP, Brazil) (STORDEUR et al., 2002).

2.5. Cytokines immunoassay

Twenty-four hours after the last treatment (day 25), the animals were euthanized by cardiac puncture under anesthesia (150 mg/kg ketamine and 16 mg/kg xylazine). Blood was collected with heparin and centrifuged (3500 ×g for 15 min at 4°C) to obtain the plasma. Plasma samples were used to quantity the following cytokines: interferon gamma (IFN-γ), tumor necrosis factor (TNF), interleukin-10 (IL-10), and interleukin-17 (IL-17). These cytokines were quantified by using a commercial cytometric bead array (CBA) mouse Th1/Th2/Th17 cytokine kit, according to the manufacturer's instructions (BD Biosciences, San Diego, CA, USA). The data were obtained in the flow cytometer FACSVerse and analyzed with the FCAP 3.0 software (Biosciences, San Diego, CA, USA). Standard curves were obtained for each cytokine, from a range of 20-5000 pg/mL. In this CBA method, the lower limit of cytokines detection was 2.5-52.7 pg/ml, according to the cytokine analyzed (RODRIGUES et al., 2017).

2.6. Anti-*T. cruzi* immunoglobulin G immunoassay

Anti-*T. cruzi* immunoglobulin G (IgG) plasma levels were quantified by enzyme-linked immunosorbent assay (ELISA, Bethyl Laboratories, Montgomery, USA) according Novaes et al. (2016). In this method, 96 wells polystyrene microplates with high-affinity to proteins were coated with 3µg *T. cruzi* antigens. Then, each well was incubated during 12h with 5µl of plasma from each animal. The reaction was followed by the treatment with anti-mouse immunoglobulin G peroxidase conjugate antibodies, which were used as detection probes (BethylLaboratories, Montgomery, TX, USA). The optical density (OD) was registered at 490 nm in a plate spectrophotometer (Anthos Zenyth 200; Biochrom, Cambridge, UK). Plasma obtained from uninfected animals were used as negative controls. Negative and positive results were discriminated considering the absorbance detected from 10 negative-control samples plus 2 standard deviations (RODRIGUES et al., 2017).

2.7. Histological processing

Fragments of heart, liver, and anterior tibialis skeletal muscle were fixed for 48 hours in buffered 4% paraformaldehyde solution (0.2M sodium phosphate buffer, pH= 7.2). The fragments were dehydrated in ethanol and included in glycol methacrylate histological resin

(Historesin, Leica Biosystems, Sao Paulo, SP, Brazil). Semi-serial histological sections with 3-μm thickness were obtained in a rotary microtome with 100 μm intervals to avoid analyze the same tissue area (SANTOS et al., 2018). For general histopathology and stereology, all tissue sections were stained with hematoxylin (1 hour at 60 °C), toulidine blue and basic fuchsine (15 seconds at room temperature), and mounted with entellan (Merk, Sao Paulo SP, Brazil). Liver sections were additionally stained by the Periodic acid-Schiff (PAS) staining for glycogen (CERRI and SASSO-CERRI et al., 2003). For each staining method, tissue fragment and animal, histological images were obtained using a bright field photomicroscope and a ×40 objective lens (Axio Scope.A1, Carl Zeiss, Germany) (RODRIGUES et al., 2017).

2.8. Histopathology and distribution of liver glycogen storages

In the histopathological analysis, the following morphological characteristics were observed from the microscopic examination of all organs investigated: Presence and distribution of inflammatory cells, cell degeneration (volumetric changes and cytoplasm vacuolization), necrosis, vascular abnormalities (collapse, dilatation and congestion), stromal expansion, fibrosis, cell parasitism (presence and distribution of amastigote nests). The histopathological findings comparatively described among the groups, considering organs obtained from uninfected and untreated animals as a negative control (normal heart, liver, and skeletal muscle microstructure) (NOVAES et al., 2015, 2016).

In the liver images stained with the PAS method, glycogen distribution in hepatocytes was estimated from a computational method based on a color segmentation function. This method was applied by using the ImageJ image analysis software (SCHNEIDER et al., 2012). Briefly, digital liver images were converted to a black and white 8-bit pixelated picture. Then, a threshold toll was used to apply a black and white segmentation, removing the non-stained background. From the segmented image, glycogen distribution was quantified by the proportion of marked areas in the total histological area (GONÇALVES et al., 2019).

2.9. Quantitative microstructural analysis by second-order stereology and morphometry

All stereological parameters were quantified in 20 randomly non-coincident histological imagens obtained from each tissue and animal. Tissue cellularity/inflammation was analyzed by comparing the distribution of interstitial cells in the myocardium, liver and skeletal muscle for all groups. The number density of interstitial cells (QA_{IC}) and amastigote nests (QA_{AN})was estimated as $QA_{IC\ or\ AN} = \Sigma_{ICN\ or\ AN} / T_A$, where $\Sigma_{ICN\ or\ AN}$ is the number of interstitial cell nuclei

or amastigote nests counted in the microscopic focal plane, and T_A is the dimension of the test area used as a test system at tissue level ($T_A = 25 \times 10^3$ μm^2) (NOVAES et al., 2013).

The volume density (Vv, %) occupied by tissue parenchyma (cardiomyocytes, skeletal myocytes, and hepatocytes) and stroma (connective tissue) was estimated by point counting according to the following formula $Vv = \Sigma P[\text{structure}]/\Sigma P_T$, where ΣP[structure] is the number of points that hit the interest structure (parenchyma or stroma) and ΣP_T is the total number of test points. A test system with 1.38×10^4 μm^2 and 42 points at tissue level was used. All stereological analysis was performed using the image analysis software Image Pro-Plus 4.5 (Media Cybernetics, Silver Spring, MD, USA) (NOVAES et al., 2013; RODRIGUES et al., 2017).

The thickness and sarcomere length of cardiac and skeletal myocytes was analyzed by computational planimetry. These parameters were quantified in digital images stained with hematoxylin/toulidine blue/basic fuchsine by using the linear tool of the image analysis software Image Pro-Plus 4.5 (Media Cybernetics, Silver Spring, MD, USA) (CHRISTENSEN et al., 2006; FELIZARDO et al., 2018).

2.10. Biochemical assay for transaminases

The circulating levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were quantified in plasma samples by using a commercial colorimetric kit and the manufacturer instructions (Human in Vitro Diagnostics, Belo Horizonte, MG, Brazil). Transaminases levels were used as marker of morphofunctional hepatocytes damage (NOVAES et al., 2016).

2.11. Statistical analysis

Data were presented as the mean and standard deviation (mean ± SD) or median and interquartile range. Data distribution was verified by the Kolmogorov-Smirnov test. Parametric data were compared using one-way analysis of variance (one-way ANOVA) followed by Student-Newman-Keuls *post-hoc* test. Non-parametric data were compared using the Kruskal-Wallis test. All results with $P \leq 0.05$ (95% confidence index) were considered statistically significant.

3. RESULTS

Circulating trypomastigotes were detected in all groups on the fifth day of infection. The peak parasitemia occurred on the eighth day of infection, and was highest in the groups T80 and CI, followed by group B50+T80. The group T80 presented slower parasites clearance, while animals in the groups B100 and B100+T80 showed fast parasite control with undetectable parasitemia (Figure 1).

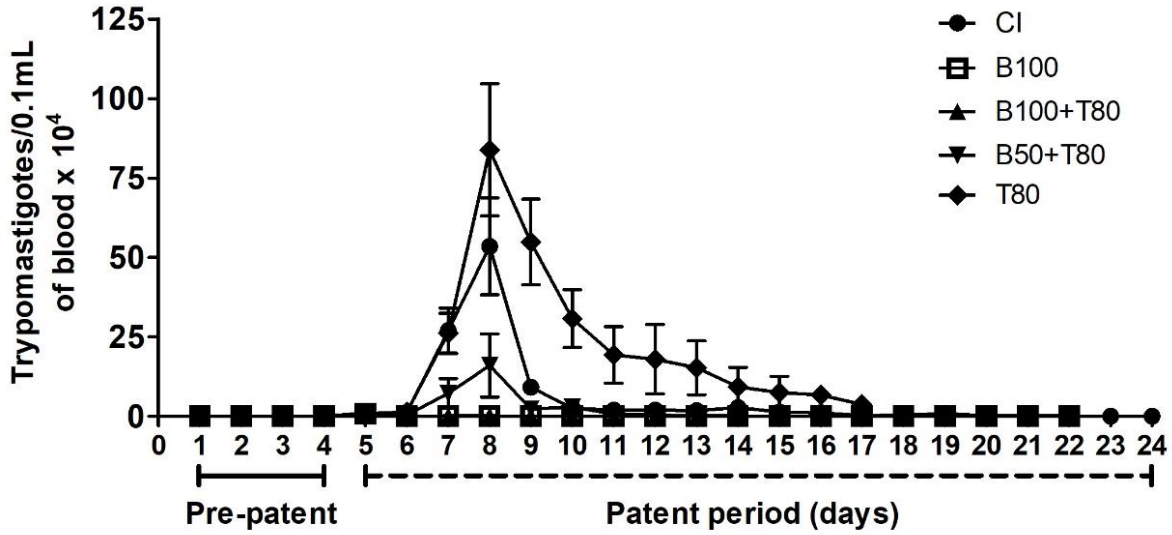

Fig. 1. Parasitemia curve in *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined. Groups: CI= untreated, B100= 100 mg/kg Bz, T80= 80 mg/kg TDZ, B100+T80= 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= 50 mg/kg Bz + 80 mg/kg TDZ. Data are represented as mean and standard deviation.

Initial, mean, final parasitemia were higher in the group T80, as well as the peak of parasitemia compared to the other groups ($P<0.05$). The combined treatment was ineffective in effective in abolishing parasitemia in the group B50+T80, which was reduced compared to CI ($P<0.05$) and increased compared to B100+T80 ($P<0.05$). In the group B100+T80, undetectable parasitemia was obtained only at the end of treatment. Persistent negative parasitemia was obtained only in the group B100 (Table 1).

Table 1. Initial, mean, peak and final parasitemia in *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined.

	IP (Tryp. / 0.1 mL blood 10^4)	PP (Tryp. / 0.1 mL blood 10^4)	MP (Tryp. / 0.1 mL blood 10^4)	FP (Tryp. / 0.1 mL blood 10^4)
CI	14.72 ± 20.54a	57.25 ± 59.40a	2.16 ± 1.18a	0.38 ± 0.37a
B100	0.11 ± 0.18b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.0b
T80	29.07 ± 26.96c	83.81 ± 77.82c	18.70 ± 9.86c	0.0 ± 0.0b
B50+T80	3.86 ± 13.00d	2.30 ± 3.06d	0.13 ± 0.18d	0.01 ± 0.08b
B100+T80	0.22 ± 0.39b	1.16 ± 0.64d	0.0 ± 0.0e	0.0 ± 0.0b

IP= initial parasitemia, PP: parasitemia peak, MP: mean parasitemia, FP: final parasitemia. Tryp: Trypomastigotes. Groups: CI= untreated, B100= 100 mg/kg Bz, T80= 80 mg/kg TDZ, B50+T80= 50 mg/kg Bz + 80 mg/kg TDZ; B100+T80= 100 mg/kg Bz + 80 mg/kg TDZ. Data are represented as mean and standard deviation. In each column, groups with different letters (a, b, c, d, e) exhibit statistical difference ($P \leq 0.05$), and columns with the same letter indicate that there is no statistical difference ($P \geq 0.05$).

Initial and final body mass, as well as heart mass was similar in all groups ($P>0.05$). Liver and spleen mass was higher in the group T80 compared to the other groups ($P<0.05$). All infected animals presented increased spleen mass compared to the group CNI ($P<0.05$). All groups treated with Bz alone or combined with TDZ presented reduced spleen mass compared to CI animals ($P<0.05$) (Figure 2).

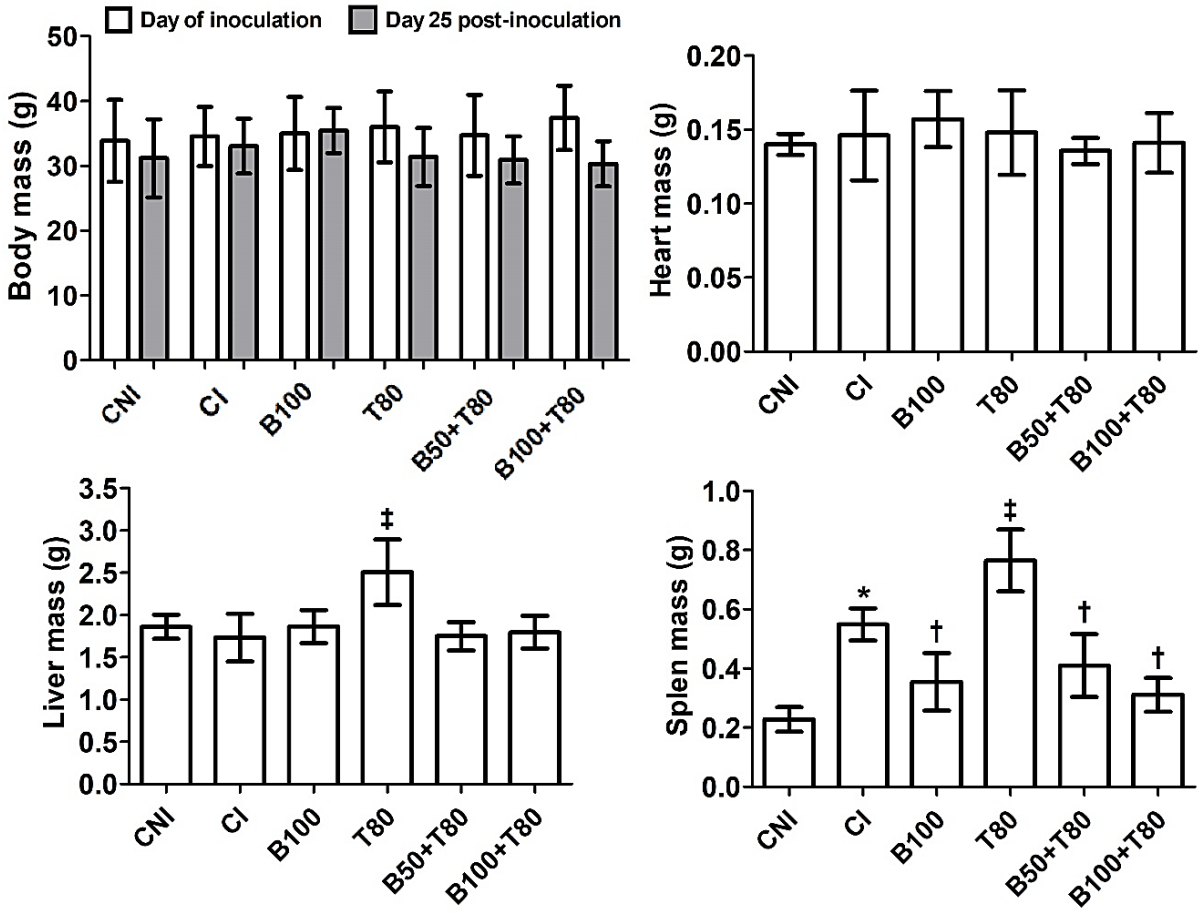

Fig. 2. Body and organs mass in control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined. Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ. Data are represented as mean and standard deviation. The symbols indicate statistical difference (P≤0.05), compared to *(CNI); †(CNI, CI and T80), and ‡(CNI, CI, B100, B50+T80 and B100+T80).

Quantitative PCR assay indicated that the groups CI and T80 exhibited the higher parasite load in the heart, skeletal muscle and liver compared to the other groups (P<0.05). Treatments with B50+T80 and B100+T80 reduced significantly the parasite load compared to CI, B100 e T80 (P<0.05). However, Bz-based monotherapy (B100) was even more effective in attenuates tissue parasitism compared to the groups CI and T80 (P<0.05) (Fig. 3).

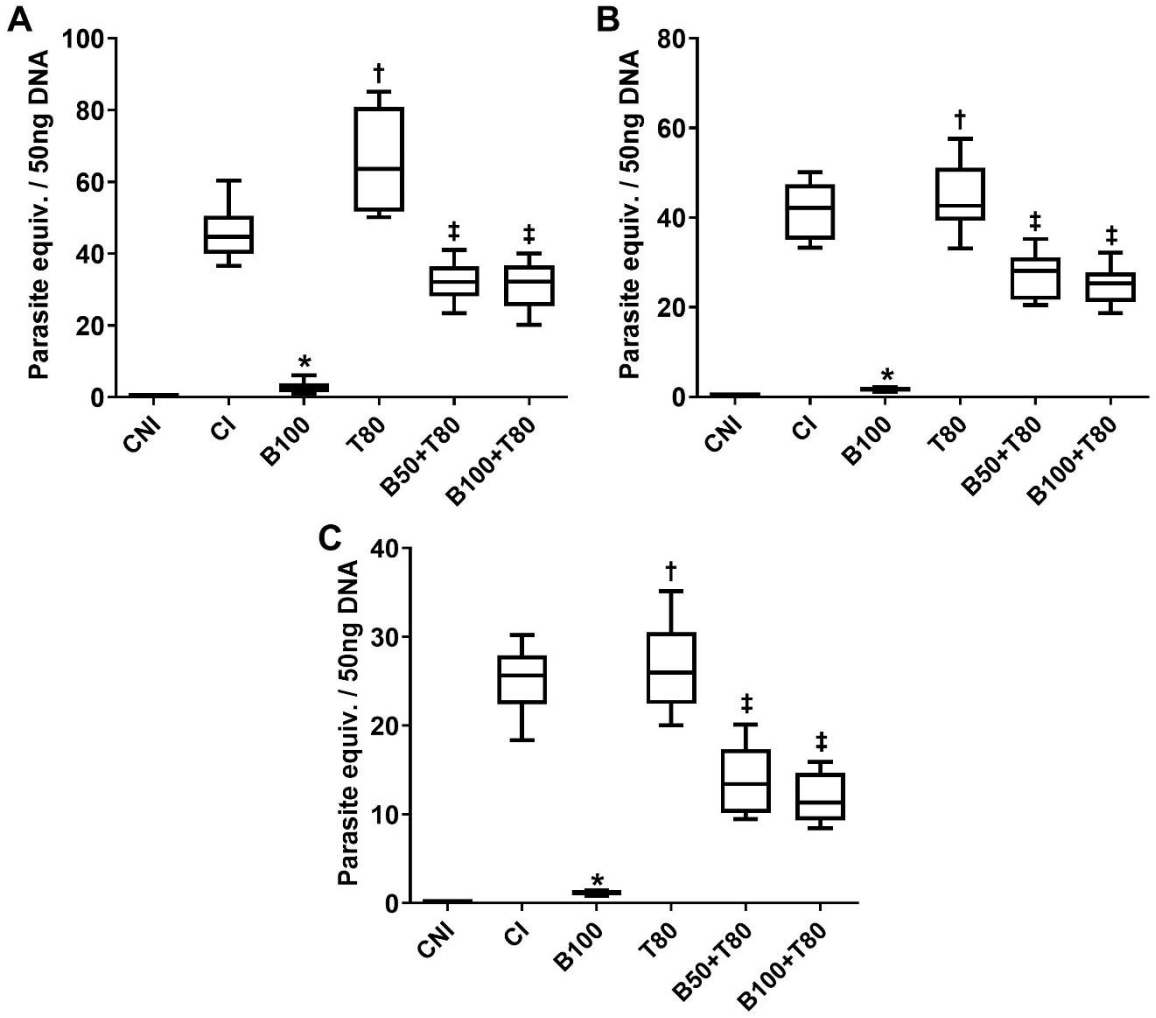

Fig. 3. Parasitic load in heart (A), skeletal muscle (B) and liver (C) of control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined. Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ. Data are represented as mean and standard deviation. The symbols indicate statistical difference ($P \leq 0.05$), compared to *(CI); †(CI, B100, B50+T80, B100+T80), and ‡(CI, B100, T80, B50+T80).

Increased cytokines plasma levels were detected in all infected groups compared to the group CNI ($P<0.05$). In general, animals in the groups B50+T80, B100+T80, and especially B100 presented reduced IFN-γ, TNF-α, IL-10, and IL-17 plasma levels compared to the groups CI and T80 ($P<0.05$), which exhibited similarly increased these cytokine levels

(P>0.05). Only IL-10 was reduced in the group B100+T80 compared to the group B50+T80 (P<0.05) (Fig. 4).

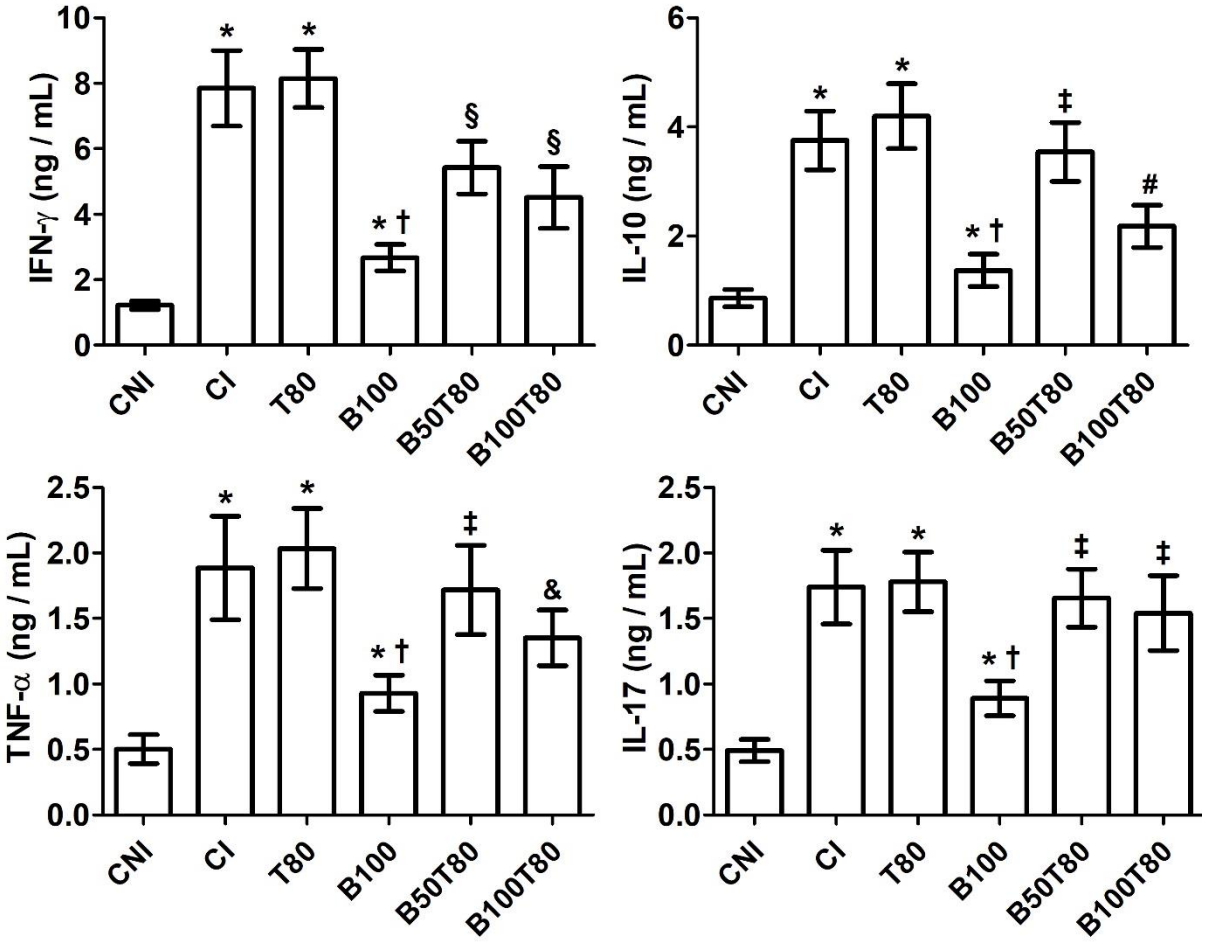

Fig. 4. Cytokine plasma levels in control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined. Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ. Data are represented as mean and standard deviation. The symbols indicate statistical difference (P≤0.05), compared to *(CNI), †(CNI, CI, T80, B50+T80 and B100+T80), §(CNI, CI, T80, B100), ‡(CNI, B100), &(CNI, CI, T80, B100), and #(CNI, CI, T80, B100, B50+T80).

In general, a similar cytokines profile was observed in the heart when compared to plasma. However, IFN-γ, TNF-α levels were higher in the group T80 compared to CI group

(P<0.05). Only TNF-α was reduced in the group B100+T80 compared to the group B50+T80 (P<0.05). In addition, CNI and B100 animals presented similar IL-17 heart levels (P>0.05) (Fig. 5).

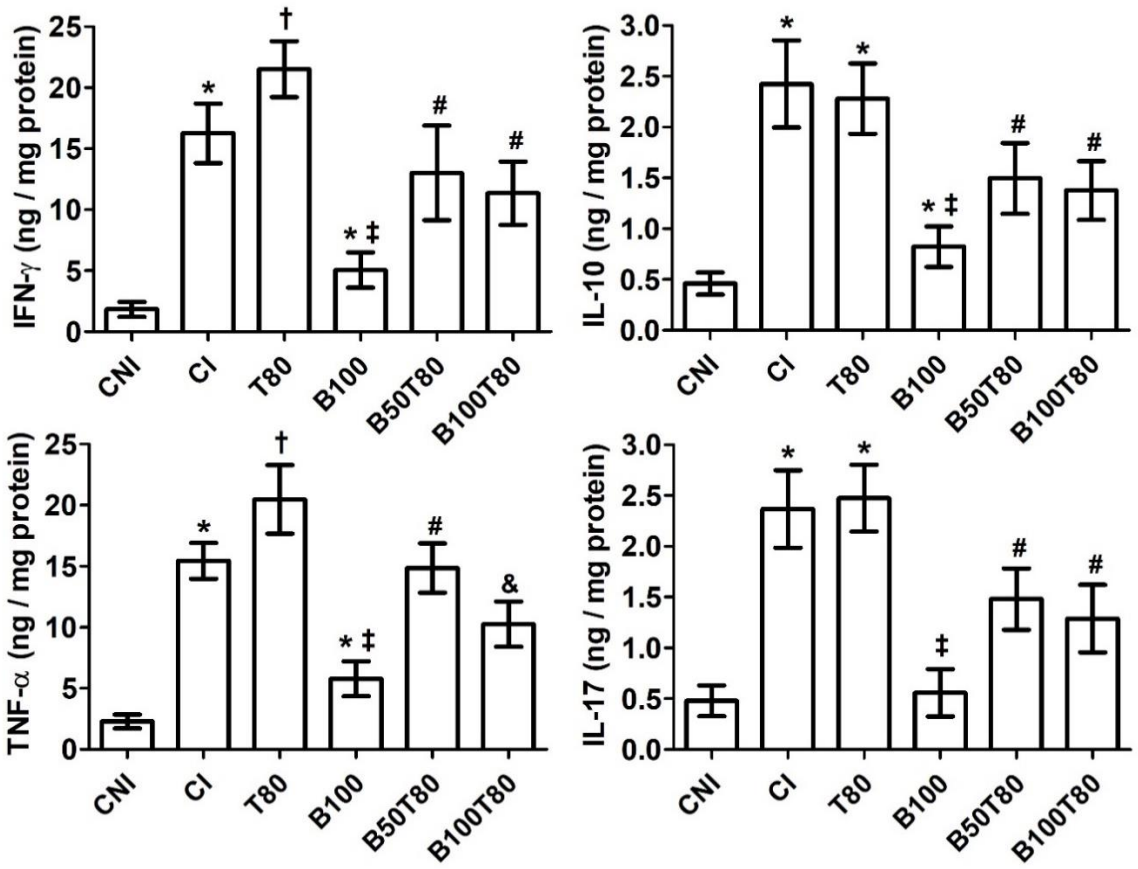

Fig. 5. Cytokine heart levels in control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined. Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ. Data are represented as mean and standard deviation. The symbols indicate statistical difference (P≤0.05), compared to *(CNI), †(CNI, CI, B100, B50+T80 and B100+T80), ‡(CNI, CI, T80, B50+T80 and B100+T80), #(CNI, CI, T80, B100), and &(CNI, CI, T80, B50+T80 and B50+T80).

As expected, anti-*T. cruzi* IgG was not detected in CNI animals. All infected animals exhibited high IgG titles. The groups B100, B50+T80 and B100+T80 presented reduced total anti-*T. cruzi* immunoglobulins (total IgG, IgG 1, IgG 2a and IgG 2b) compared to CI animals

(P<0.05). Higher IgG1 titles were detected in the group T80 compared to the other groups (P<0.05). Reduced total IgG and IgG1 titles were identified in the groups B100, B50+T80 and B100+T80 compared to the group T80 and CI (P<0.05) (Fig. 6).

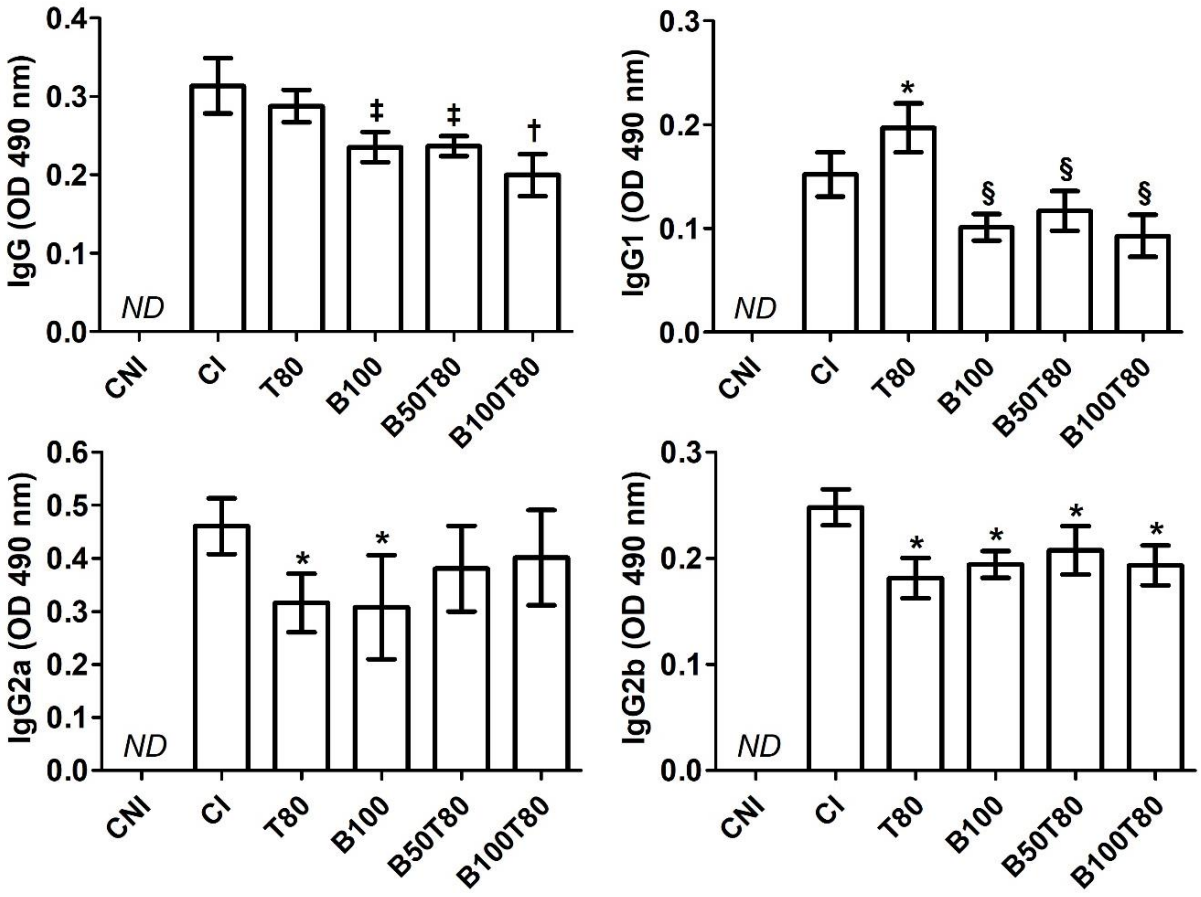

Fig. 6. Anti-*T-cruzi* immunoglobulin G (IgG) plasma levels in control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined. Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ. Data are represented as mean and standard deviation. The symbols indicate statistical difference (P≤0.05), compared to *(CI), ‡(CI, T80 and B100+T80), †(CI, T80, B100, and B50+T80), and §(CI and T80).

The microscopic analysis indicated a well-organized heart microstructure in control uninfected mice, with paralleled cardiomyocytes, scarce connective tissue, and reduced interstitial cellularity. CNI and T80 animals exhibited diffuse leucocytes infiltrate and stromal

expansion. Animals in the group B100 presented myocardial morphology similar to uninfected animals. Conversely, the groups B50+T80 and B100+T80 evidence of connective tissue expansion and myocarditis was also detected (Fig. 7).

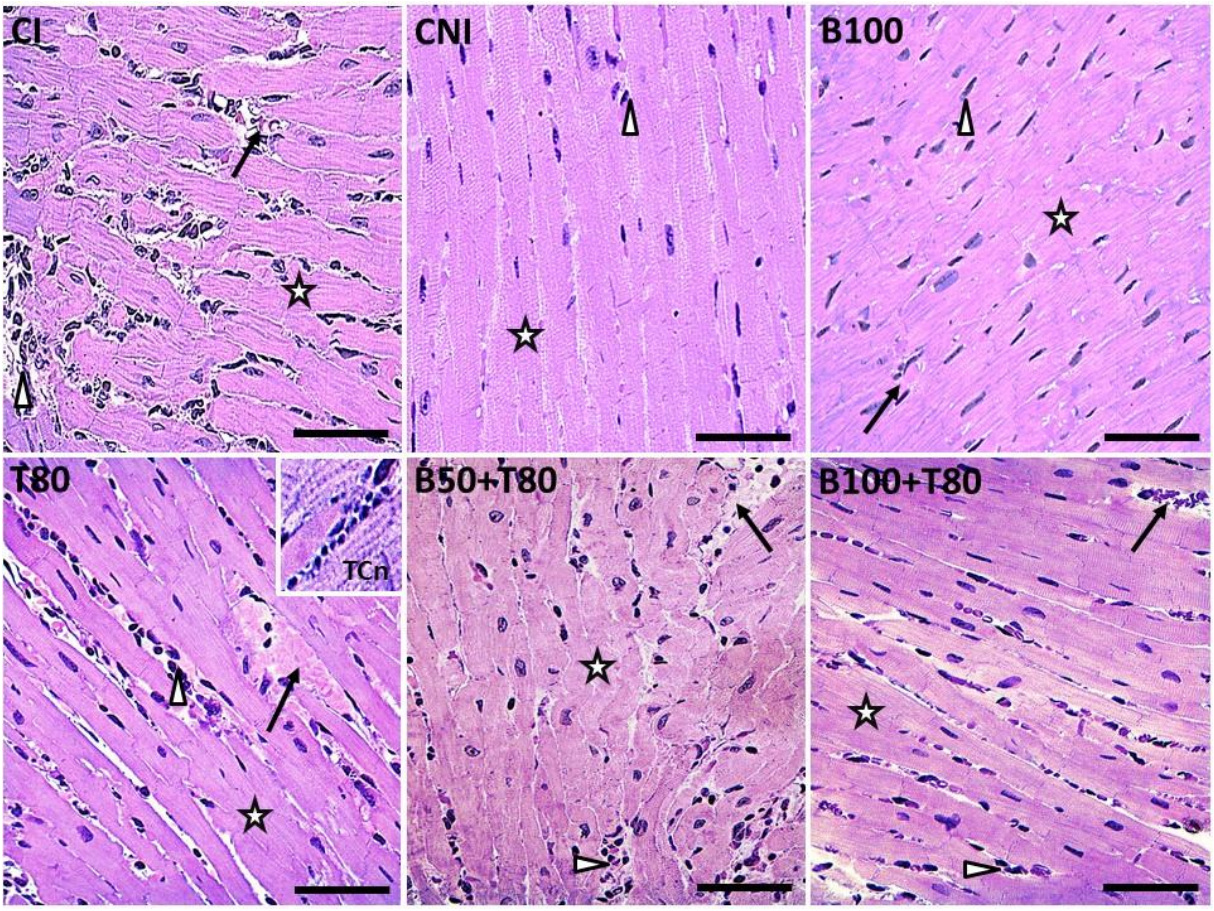

Fig. 7. Microscopic images of the heart from control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined (Staining: hematoxylin, toluidine blue and basic fuchsine; bright field microscopy, scale bars= 50µm). Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ (Scale bars= 50µm). Arrow= blood vessel, arrowhead= interstitial/inflammatory cells, star= cardiomyocyte, Tcn= *T. cruzi* amastigote nest.

Quantitative microstructural analysis indicted that CNI and B100 animals similarly reduced myocardial cellularity, cardiomyocytes thickness, parenchymal and stromal

distribution compared to the other groups (P<0.05), in which all these parameters were similar. Sarcomere length was similar in all groups (P>0.05), Fig. 8.

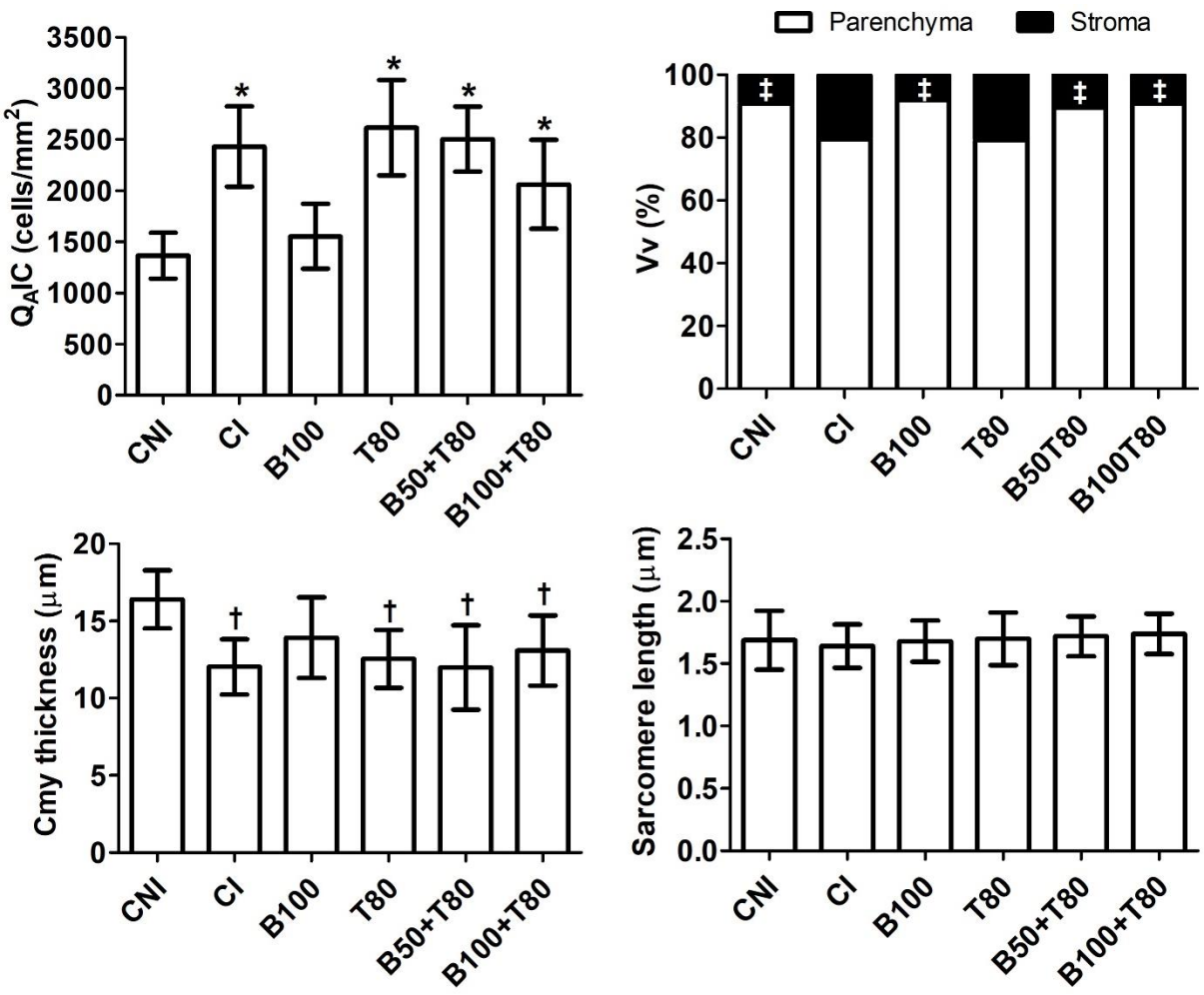

Fig. 8. Cardiac microstructural parameters in control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined. Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ. Q_A= number density, IC= interstitial cellularity, Vv= volume density, Cmy= cardiomyocytes. Data are represented as mean and standard deviation. The symbols indicate statistical difference (P≤0.05), compared to *(CNI and B100), †(CNI), and ‡(CI and T80).

A well-organized skeletal muscle microstructure with reduced distribution of interstitial cells and connective tissue, as well as evident cytoplasmic cross-striation in skeletal myocytes

were identified in the groups CNI and B100. Accentuated skeletal myositis was evident in the groups CI and T80. Slight leukocyte infiltrate was additionally observed in the groups B50+T80 and B100+T80 (Fig. 9).

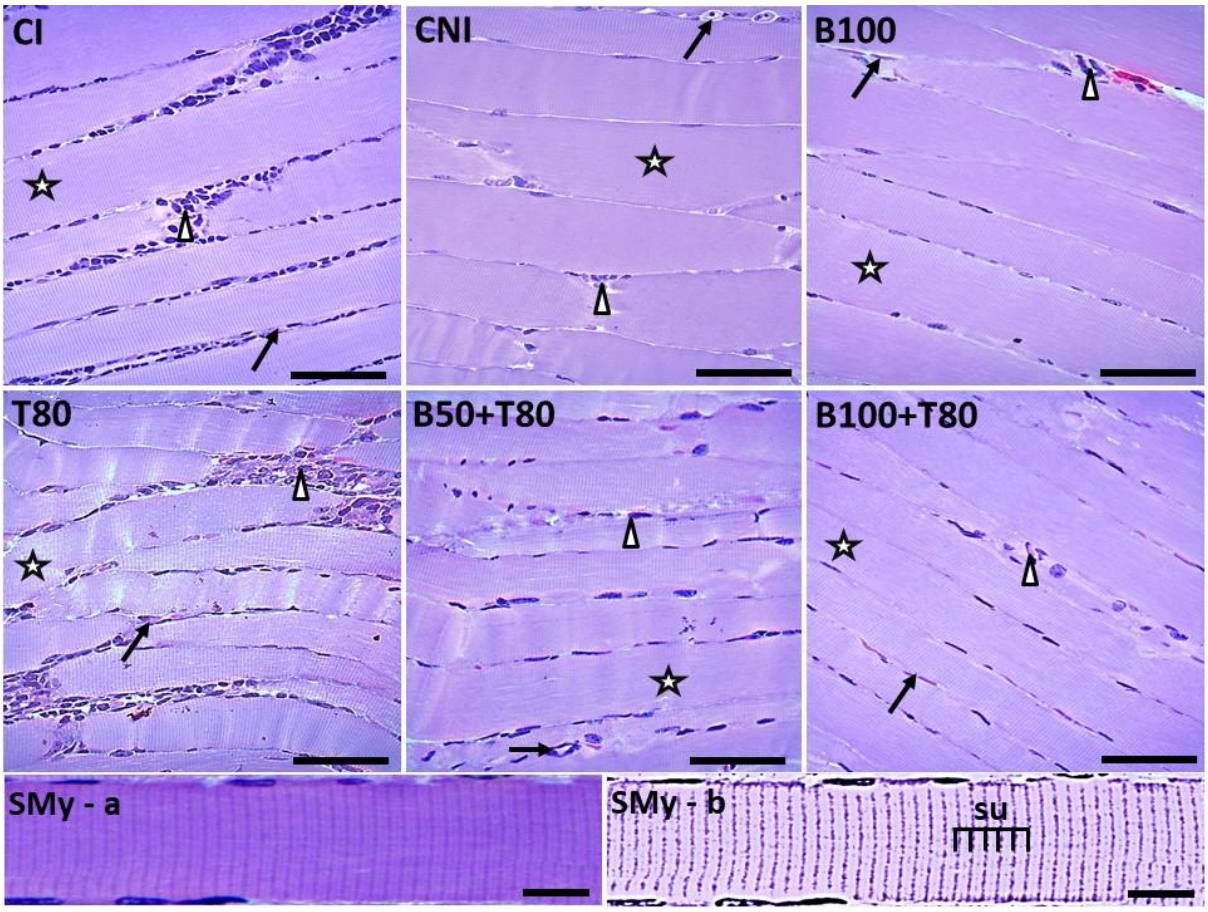

Fig. 9. Microscopic images of the skeletal muscle from control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined (Staining: hematoxylin, toluidine blue and basic fuchsine; bright field microscopy). Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ (Scale bars= 50µm). Arrow= blood vessel, arrowhead= interstitial/inflammatory cells, star= skeletal myocyte, Tcn= *T. cruzi* amastigote nest. Smy-a= Detail of a skeletal myocyte indicating cytoplasmic cross striations, Smy-b= Detail of the same skeletal myocyte after color segmentation applied to increase the definition of sarcomeric units (su) (Scale bars= 25 µm).

Animals in the groups CI and T80 presented increased interstitial cellularity compared to the other groups (P<0.05). This parameter was similarly higher in the groups B50+T80 and B100+T80 compared to CNI and B100 animals (P<0.05), which showed reduced and similar tissue cellularity (P>0.05). Significant stromal expansion was identified in the groups CI and T80 compared to the other groups (P<0.05). Only T80 animals presented reduced skeletal myocytes thickness when compared to other groups (P<0.05), and sarcomere length was similar in all groups (P>0.05), Fig. 10.

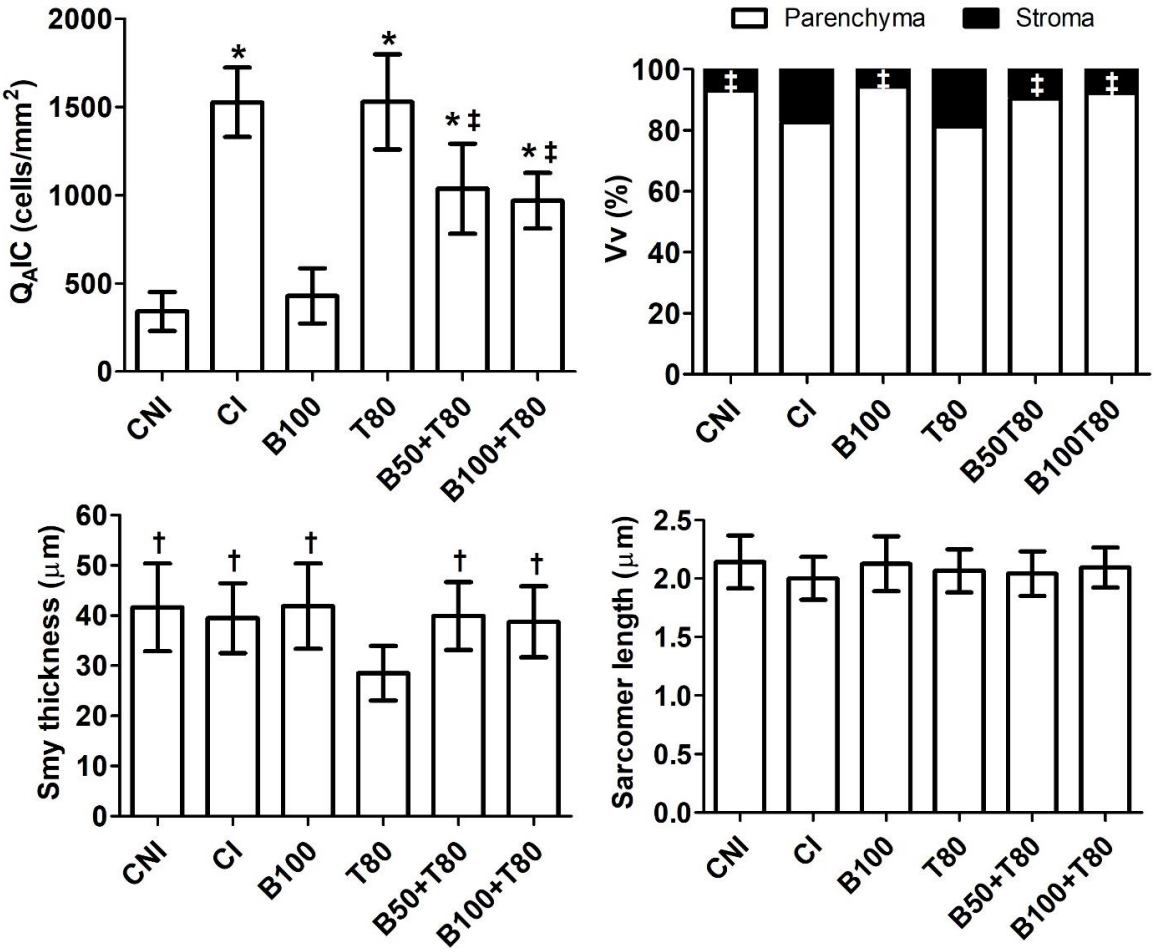

Fig. 10. Skeletal muscle microstructural parameters in control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined. Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ. Q_A= number density, IC= interstitial cellularity, Vv= volume density, Smy= skeletal myocytes. Data are represented as

mean and standard deviation. The symbols indicate statistical difference (P≤0.05), compared to *(CNI and B100), ‡(CI and T80), and †(T80).

Animals in the group CNI presented normal liver microstructure, with well-defined hepatocytes cords and sinusoidal capillaries. Pericellular and perivascular leucocytes infiltrate were clearly identified in CI and T80 animals. In addition, the T80 group showed microvesicular steatosis distributed in all tissue. In the groups B100 and B100+T80 was observed evident obliteration of sinusoidal capillaries. (Fig. 11).

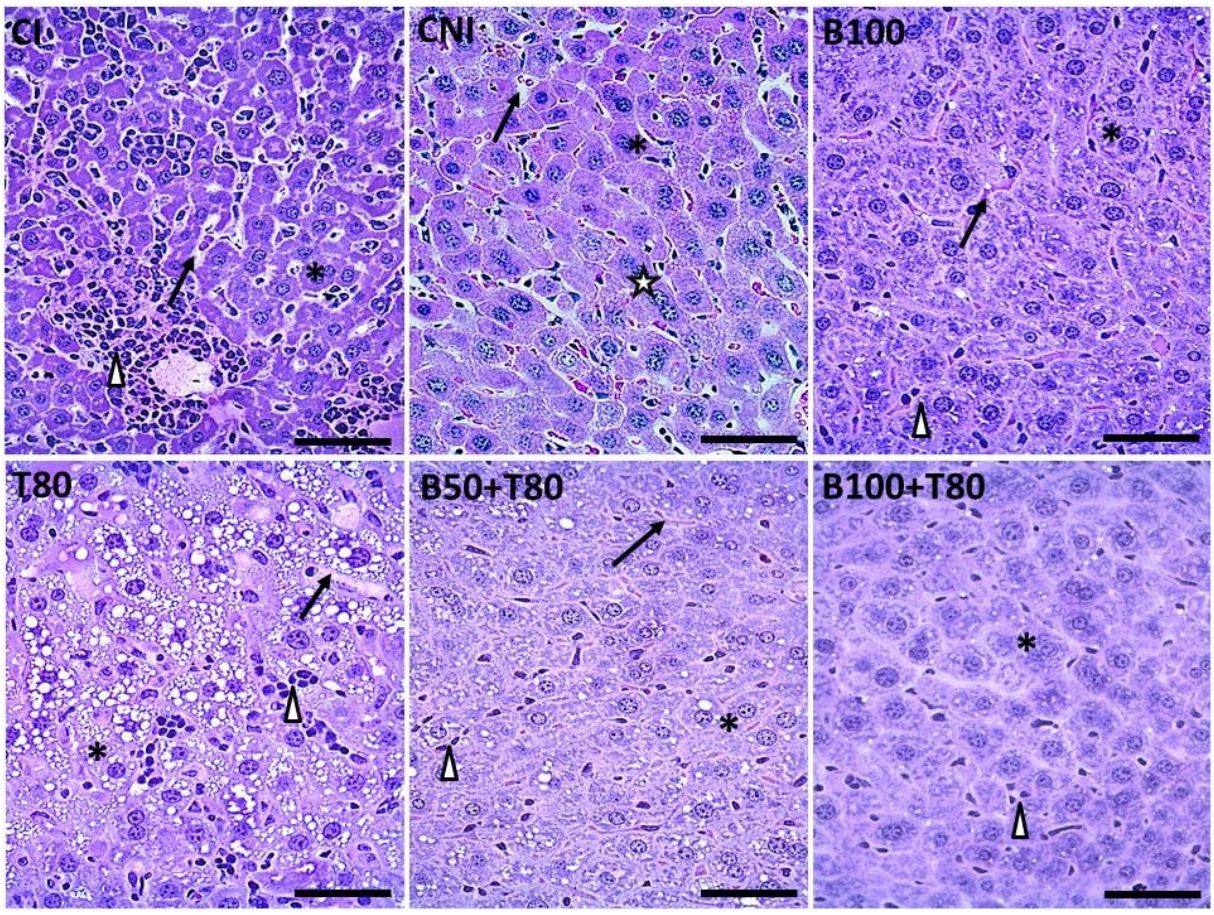

Fig. 11. Microscopic images of the liver from control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined (Staining: hematoxylin, toluidine blue and basic fuchsine; bright field microscopy, scale bars= 50µm). Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ. Arrow= sinusoidal capillaries, arrowhead= interstitial/inflammatory cells, asterisk= hepatocytes.

By using an integrated method based on glycogen histochemistry and computational analysis (Fig. 12), we identified that hepatocytes of animals from CNI, B100 and B100+T80 groups showed a wide distribution of glycogen cytoplasmic inclusions. Marked depletion of glycogen storages was identified in the groups CI and T80 (Fig. 13).

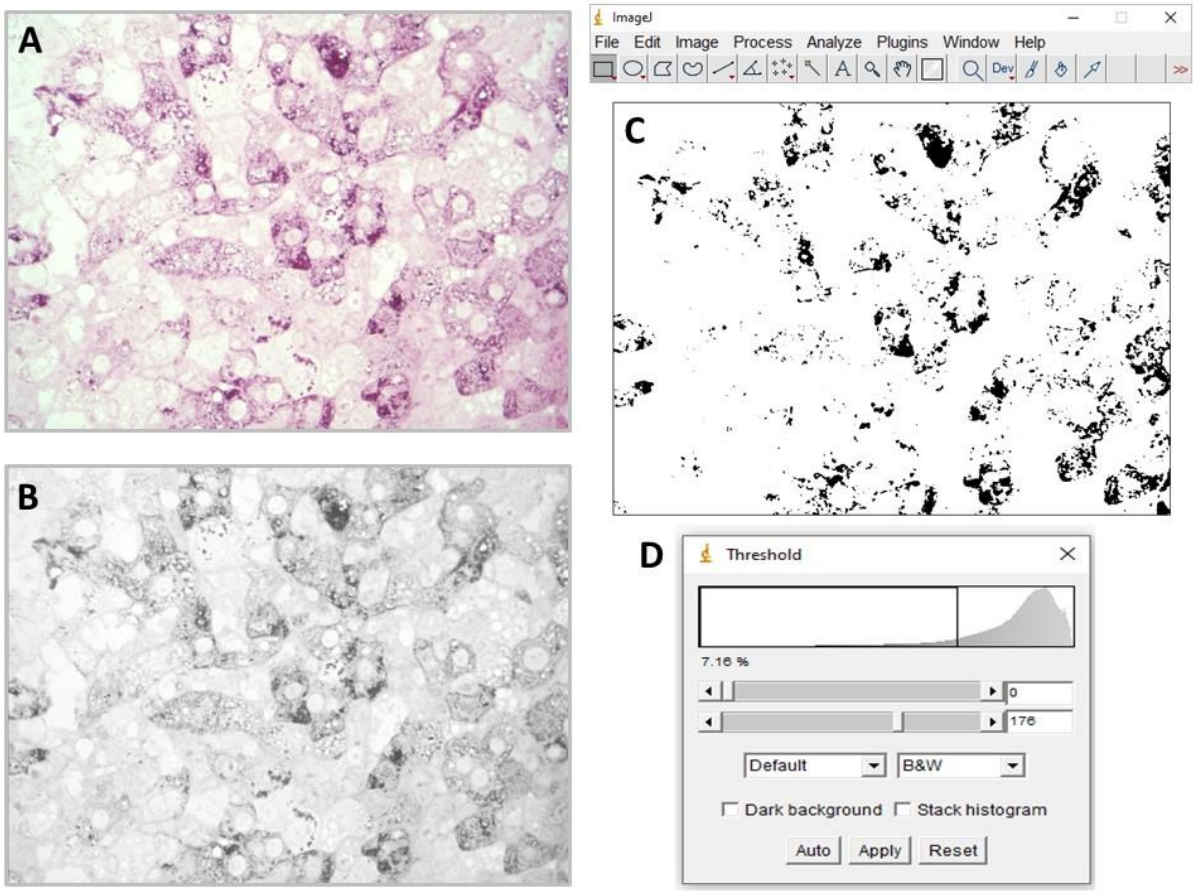

Fig. 12. Three-steps computational method for glycogen quantification. (A) Microscopic image of liver sections stained with Periodic acid-Schiff histochemistry for glycogen (deposits marked a purple-magenta color). (B) The same image converted in an 8-bit grayscale. (C) Background removal by black and white segmentation on ImageJ image processing software. (D) Automatic quantification of glycogen distribution in hepatocytes/tissue area by threshold method.

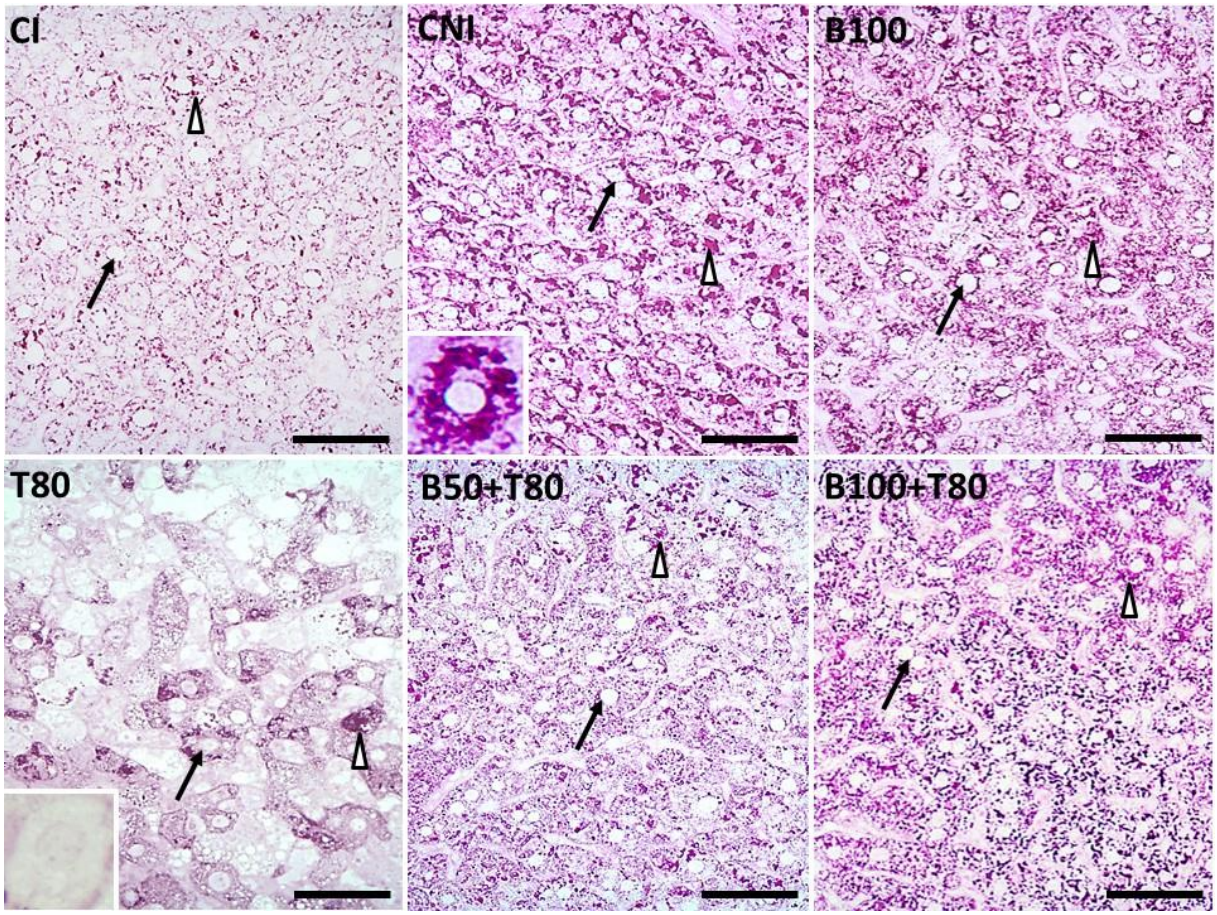

Fig. 13. Microscopic images of the liver indicating glycogen cytoplasmic deposits in hepatocytes from control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined (Staining: PAS histochemistry, bright field microscopy, scale bars= 50µm). Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ. Arrow= hepatocytes nuclei, arrowhead= glycogen cytoplasmic inclusions, asterisk= hepatocytes. In CNI, a hepatocyte with well-stained glycogen inclusions is highlighted (bottom left image). In T80, a depleted glycogen hepatocyte is highlighted (bottom left image).

In the liver, CI and T80 animals presented increased interstitial cellularity and stromal expansion compared to the other groups ($P<0.05$). These parameter was similar and higher in the groups B50+T80 and B100+T80 compared to CNI and B100 animals ($P<0.05$), which showed reduced and similar tissue cellularity ($P>0.05$). T80 animals exhibited reduced

number density of hepatocytes compared to the other groups (P<0.05). Glycogen depletion was confirmed in the groups CI and T80 compared to the other groups (P<0.05), Fig. 14.

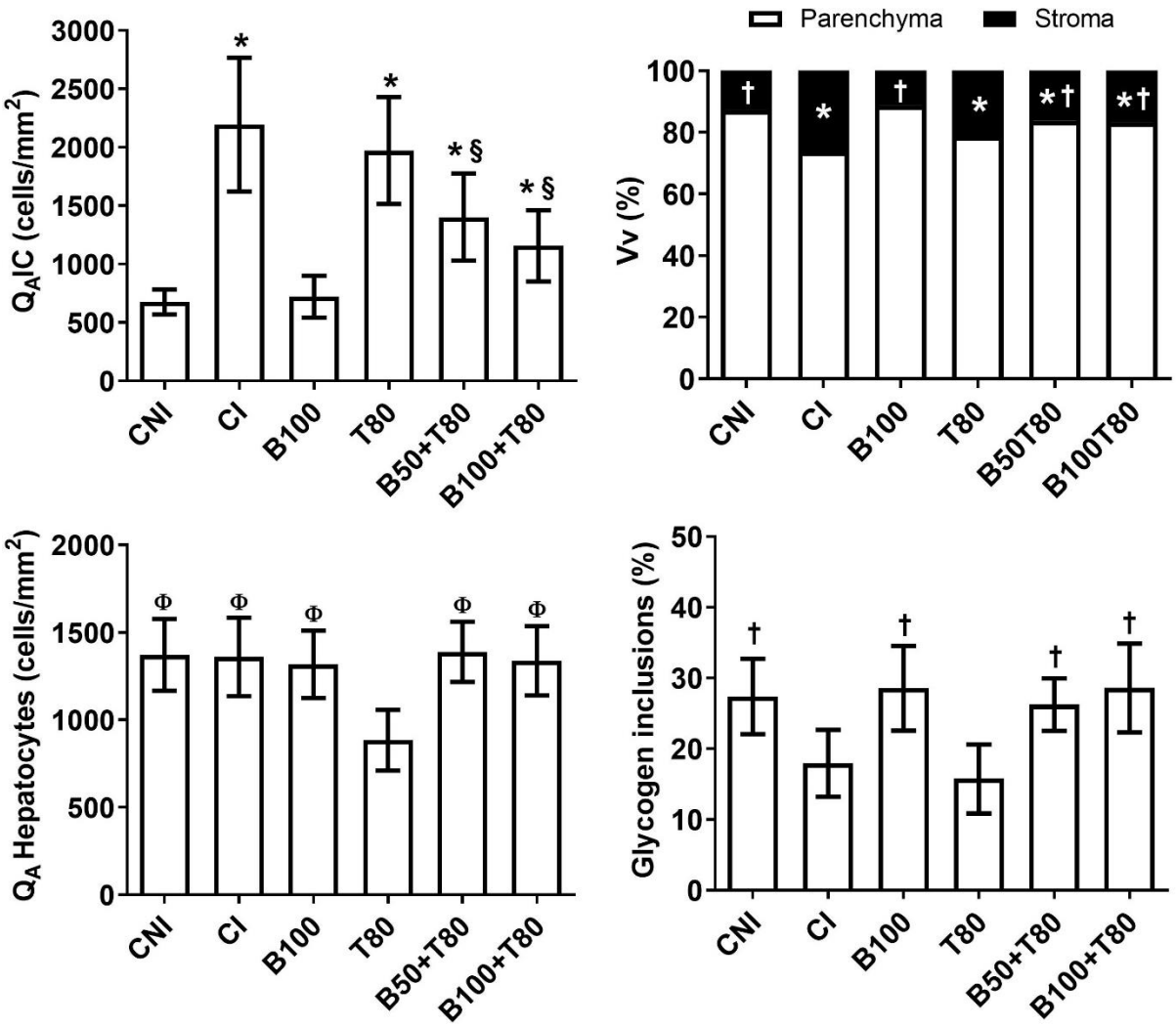

Fig. 14. Hepatic microstructural parameters in control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined. Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ. Q_A= number density, IC= interstitial cellularity, Vv= volume density. Data are represented as mean and standard deviation. The symbols indicate statistical difference (P≤0.05), compared to *(CNI and B100), †(CI and T80).

All groups infected and treated presented increases AST and ALT circulating levels compared to the groups CNI and CI ($P<0.05$), which exhibited similar values ($P>0.05$). The groups T80 and B50+T80 had the highest levels of these transaminases compared to the other groups ($P<0.05$) (Fig. 15).

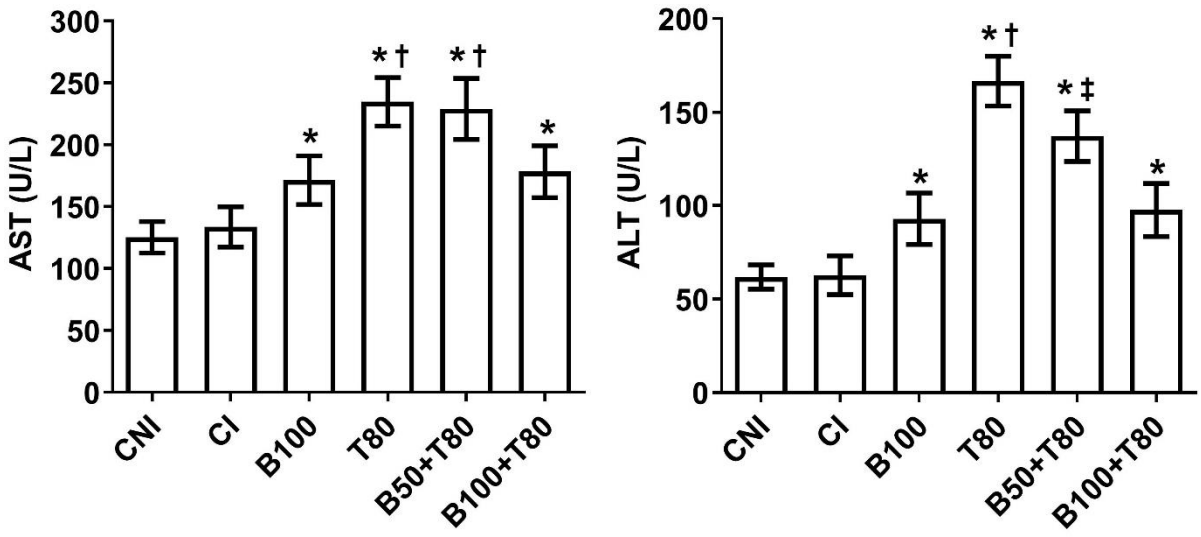

Fig. 15. Circulating levels of hepatic transaminases in control and *Trypanosoma cruzi*-infected mice treated with benznidazole (Bz) or thioridazine (TDZ) in monotherapy or combined. Groups: CNI= uninfected untreated, CI= infected untreated, B100= infected + 100 mg/kg Bz, T80= infected + 80 mg/kg TDZ, B100+T80= infected + 100 mg/kg Bz + 80 mg/kg TDZ, B50+T80= infected + 50 mg/kg Bz + 80 mg/kg TDZ. Q_A= number density, IC= interstitial cellularity, Vv= volume density. Data are represented as mean and standard deviation. The symbols indicate statistical difference ($P\leq0.05$), compared to *(CNI and CI), †(B100 and B100+80), ‡ (B100, B50+T80 and B100+T80).

4. DISCUSSION

In this study, we compared the impact of TDZ and Bz alone and combined in the treatment of *T. cruzi* infection. When administered alone, both dugs exerted opposite impact on blood parasitism. While Bz was effective in control parasitemia early and accelerates parasite clearance, TDZ prolonged the patent period and determined a high peak of parasitemia and mean parasitemia. Although the combination of TDZ and the therapeutic dosed of Bz (100 mg/kg) was also effective in controlling parasitemia, additional effectiveness was not detected for analyzed of other parameters when compared to Bz

monotherapy. As the parasite control is closely correlated with the efficacy of antiparasitic drugs (CALDAS et al., 2014), our findings indicated that TDZ can increase host susceptibility to *Trypanosoma cruzi* infection. Since the results obtained from Bz monotherapy were superior or similar to drug combination, the antiparasitic potential of this treatment strategy was exclusively dependent on Bz, especially considering that parasitemia was markedly attenuated when TDZ was combined with on half of Bz dose.

Based on the results of parasitemia, our findings indicated that the *in vitro* trypanocidal potential attributed to TDZ (LO PRESTI et al., 2015; OLIVA et al., 2015) may not manifest in *in vivo* systems. In a recent meta-analysis conducted by our research group (MENDONÇA et al., 2018), we identified limited evidence that TDZ can exert antiparasitic and cardioprotective potential *in vivo*. However, even using animal model and TDZ dose (80 mg/kg) similar to those described in the studies included in meta-analysis, we observed divergent results. Unfortunately, we clearly identified that the current evidence *in vivo* is exclusively based on Tulahuen and SGO-Z12 strains (MENDONÇA et al., 2018). These strains belong to discrete typing units VI (DTU VI), which exhibits a reticulotropic profile, infecting mainly spleen and liver cells (ZINGALES et al., 2012; ERDMANN et al., 2016). In addition, these strains determine delayed peak of parasitemia (30-40 days post-infection) (BUSTAMENTE et al., 2007) and increased susceptibility to Bz (DNDi, 2013). Conversely, we investigated for the first time the *in vivo* effect of TDZ on a DTU II strain-induced infection (Y strain), which is highly virulent and pathogenic, shows rapid parasite multiplication (ANDRADE et al., 2006) and peak of parasitemia (8-10 days post-infection) (ROMANHA et al., 2002; Aleixo et al., 2012), is partially resistant to Bz (FILARDI and BRENER, 1987; BAHIA et al., 2012), and induce high mortality rates in the acute phase of infection (ROMANHA et al., 2002; ANDRADE et al., 2006). It is recognized that the high degree of structural and functional intraspecific heterogeneity is directly related to the adaptability, survival and pathogenicity of different *T. cruzi* strains (MARTÍNEZ-DÍAZ et al., 2001, MIELNICZKI-PEREIRA et al., 2007). As the effect of phenothiazine's on *T. cruzi* infection remains overlooked and the effect of this drug on parasitic strains with different phenotypic and pharmacological resistance profiles is unknown, it is not possible to disregard that therapeutic effect of TDZ may be influenced by the characteristics of parasite strain. Thus, in addition to our initial findings with a DTU II strain, studies with *T. cruzi* isolates expressing different genotypic and phenotypic profiles are required to determine the potential of TDZ as an anti-*T. cruzi* drug.

Our results of parasitemia were also coherent with hepatomegaly and splenomegaly, which was more pronounced in infected animals treated with TDZ. Although hepatomegaly was not evidenced in the other groups, Bz alone or combined with TDZ was effective in attenuates splenomegaly. Hepatomegaly and specially splenomegaly is a common manifestation of *T. cruzi* infection, which is directly related to the intensity of the immune response that is established proportionally to the parasitic and antigenic load (FREITAS et al., 2016). In this sense, is not surprising that drugs that induce a better parasitic control is also effective in attenuates splenomegaly. This proposition was also aligned with the serum and heart levels of cytokines, indicating that infected untreated animals and those receiving TDZ presented a more intense and similar immunological response, which was evidenced by upregulation of Th1, Th2 and Th17 cytokines. Although the levels of all cytokines investigated were markedly reduced by Bz monotherapy, this reduction was attenuated by TDZ co-administration. These findings are consistent with the lower parasitic control induced by the combined treatment, indicating a direct relationship between immune response, parasitemia and parasitic load; which has been proven in preclinical studies (SANTOS et al., 2015; NOVAES et al., 2016).

Attenuation of cellular and humoral immune response has been used as a relevant marker of therapeutic efficacy of antitrypanosomal drugs (CALDAS et al., 2019). Reduction in cytokines levels represents an early event associated with an efficient parasitological control (GONÇALVES-SANTOS et al., 2019). Although this response is partially determined by a direct anti-inflammatory effect of Bz (MENDONÇA et al., 2019; SANTOS et al., 2015), it is recognized that the trypanocidal of Bz exerts a preponderant impact on the reduction of antigenic load, downregulating the activation immune cells (PENITENTE et al., 2015). As observed in our assays, there is consistent evidence indicating reduction in IFN-γ and TNF-α levels in response to Bz-based chemotherapy (GONÇALVES-SANTOS et al., 2019, MENDONÇA et al., 2019). As typical Th1 effector molecules, IFN-γ and TNF-α are directly involved in host resistance, against *T. cruzi*, especially by stimulating the recruitment of lymphocytes and monocytes recruitment in parasitized organs and nitric oxide-mediated parasite death (FELIZARDO et al., 2018; SILVA et al., 2015). As a regulatory cytokine, IL-10 exerts a potent anti-inflammatory role in *T. cruzi* infections (EBERHARDT et al., 2019; RODRÍGUEZ et al., 2019). Thus, deficiencies in this molecule have been closely correlated with increased host susceptibility to *T. cruzi* infection, developing intense organ damage and high mortality rates (GONÇALVES-SANTOS et al. 2019). Although not yet extensively investigated, previous evidence indicates that deficiency in IL-17 production is associated

with prolonged and more intense parasitemia, impaired differentiation and activation of Th1 immune cells, as well as higher mortality rates in *T. cruzi*-infected mice (MIYAZAKI et al., 2010; TOSELLO BOARI et al., 2018). Thus, IL-17 is also involved in protective mechanisms against *T. cruzi*, especially by trapping parasites in the endolysosomal compartment of macrophages (ERDMANN et al., 2013) and by stimulates CD8+ T cells survival in a more prolonged defensive antiparasitic response (TOSELLO BOARI et al., 2018). Taken together, our findings of cytokines rather than indicating impaired host resistance to infection, attenuation of this cytokine is linked to a better parasite control (TOSELLO BOARI et al., 2018; RODRÍGUEZ et al., 2019). Thus, although IL-10 and IL-17 was reduced in animals treated with Bz combined to TDZ, this effect was potentially linked to Bz, reinforcing the applicability of this drug as a reference treatment to control the inflammatory response associated with *T. cruzi* infection (CARNEIRO et al., 2019; URBINA, 2015).

Consistent with the specific antiparasitic immunological response profile, in our study all infected groups had high levels of anti-*T. cruzi* immunoglobulins. Interestingly, infected untreated animals and those treated with TDZ exhibited similar total IgG plasma levels. However, increased IgG1 and reduced IgG2a and IgG2b titers were identified in TDZ animals. In addition, Bz alone or combined with TDZ was effective in reduce total IgG levels, a finding mainly determined by a marked impact of these treatments on IgG1 downregulation. Although cell-mediated immune response is the main defense line against *T. cruzi* infection, humoral mechanisms reinforces host defense by acting on extracellular parasite forms (PYRRHO et al., 1998; BRYAN et al., 2010). In this sense, IgG2a is the main effector molecule in the humoral response against *T. cruzi*. However, similar lytic activities previously attributed to IgG2a and IgG1 reinforces the evidence that both immunoglobulins are effective in control parasitemia in *T. cruzi*-infected hosts (PYRRHO et al., 1998; BRYAN et al., 2010). However, intense humoral activation and high *T. cruzi*-induced antibodies titers are not invariably protective to the host. Thus, by interacting with irrelevant antigens, non-neutralizing anti-*T. cruzi* antibodies can impair the activity of lytic antibodies, determining an ineffective humoral response (POWELL and WASSOM, 1993; BRYAN et al., 2010). Corroborating previous evidence (SANTOS et al., 2015; NOVAES et al., 2016), the downregulation of anti-*T. cruzi* IgG titers observed in this study was coherent with a more effective parasite control and reduced parasitic load obtained when Bz was administered alone or combined with TDZ.

The increase cytokines and antibody levels were also consistent with heart and skeletal muscle microstructure. From a quantitative analysis, all infected groups presented marked

evidence of myocarditis and reduced cardiomyocytes thickness, except Bz-treated animals that exhibited similar tissue cellularity and cellular dimension compared to uninfected control animals. Infected untreated animals and those receiving TDZ also presented reduced cardiac parenchymal loss distribution and stromal expansion. Parenchymal degeneration is a typical manifestation of Chagas disease. In acute infections, this manifestation is associated with cardiomyocytolysis and myonecrosis, two events induced by direct cardiomyocytes parasitism and secondary immunomediated tissue damage (NOVAES et al., 2017; VIZCAÍNO-CASTILLO et al., 2014). Secondary tissue damage is also closed correlated with the progression of cardiac deterioration in Chagas cardiomyopathy, which is associated with parasite persistence in parasitized organs, low-grade immunological activation, accumulation of reactive tissue damage and cross-reactive autoimmune processes directed to heart antigens (BERMUDEZ et al., 2016; BONNEY et al., 2019). Similar pathological manifestations were observed and are expected in skeletal muscle, especially considering that most *T. cruzi* strains exhibits some degree of tropism by muscle cells (VIZCAÍNO-CASTILLO et al., 2014; BAÉZ et al., 2015; NOVAES et al., 2017). Despite the limited effect of the treatments on heart inflammation, Bz alone or combined with TDZ were effective in attenuating skeletal myositis. However, TDZ reduced the efficacy of the reference treatment, since the lowest tissue cellularity was obtained from Bz-based monotherapy. Although the heterogeneous effect of antiparasitic chemotherapy on different target organs is poorly understood, previous evidence suggest a potential influence of divergent profiles of drug biodistribution and pharmacological efficiency (MENDONÇA et al., 2018; BAHIA et al., 2014). However, still remain debatable if Bz biodistribution could be associated with the distinct injury patterns observed in cardiac and skeletal muscle, an issue that requires further investigation.

Liver microstructure also revealed that *T. cruzi* infection was accompanied by liver inflammation. In addition, animals treated with TDZ alone or co-administered with half the therapeutic dose of Bz also exhibited evidence microsteatosis. As this process was absent in the other groups treated with the highest dose of Bz, it is possible that liver damage is determined by a cumulative effect of TDZ toxicity and immunomediated damage associated with a more intense inflammatory process in animals with a attenuated parasitological control. Previous assays indicated that infection and antiparasitic chemotherapy are independently associated with liver damage (NOVAES et al., 2015). Thus, it is not surprising that drug toxicity is exacerbated in systemic inflammatory conditions, including *T. cruzi* infection, where the liver is simultaneously challenged by two potentially damaging stimuli (NOVAES et al., 2015, 2016; PAVAN et al., 2018). This proposition was reinforced by our findings of

hepatic glycogen and serum transaminases, indicating that the infection determined a negative functional impact on hepatocytes, especially in animals treated with TDZ alone. Thus, in addition to the potential increase in hepatocytes energy metabolism determined by the infection, the high ALT and AST plasma levels in animals treated with TDZ alone or combined with half Bz dose indicated that a worse parasitic control and a more intense inflammatory response can potentiates chemotherapy-induced hepatotoxicity. Although the infection alone is not always associated with significant variations in transaminases levels, antiparasitic chemotherapy often changes this parameters, representing a relevant marker of drug toxicity, including Bz (NOVAES et al., 2015, 2016; PAVAN et al., 2018).

5. CONCLUSIONS

Taken together, our findings indicated that when administered alone, TDZ potentiated parasitemia, parasitic load, inflammatory response, as well as heart, skeletal muscle, and liver injury in animals infected with a *T. cruzi* Y strain partially resistant to Bz. In combination therapy, TDZ attenuated the antiparasitic effect of Bz, aggravating the parasitemia, systemic inflammation and pathological remodeling of investigated organs. Thus, our findings reinforce the evidence that benznidazole monotherapy remains as a better treatment option in an experimental Chagas disease model.

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