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ELISA LIZ BELLI CASSA DOMINGUES

IMPACT OF TRYPANOTHIONE REDUCTASE INHIBITOR ON *TRYPANOSOMA CRUZI* AND HOST CELL ANTIOXIDANT DEFENSES CHAGAS HEART DISEASE

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Tese apresentada como parte dos requisitos para obtenção do título de Doutora em Biociências aplicadas à saúde pela Universidade Federal de Alfenas. Área de concentração: Biociências Aplicadas à Saúde

Orientador: Prof. Dr. Rômulo D. Novaes

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IMPACT OF TRYPANOTHIONE REDUCTASE INHIBITOR ON TRYPANOSOMA CRUZI AND HOST CELL ANTIOXIDANT DEFENSES IN CHAGAS HEART DISEASE

O Presidente da banca examinadora abaixo assina a aprovação da Dissertação/Tese apresentada como parte dos requisitos para a obtenção do título de Doutora em Ciências pela Universidade Federal de Alfenas. Área de concentração: Biociências Aplicada à Saúde.

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RESUMO

Introdução e objetivo: As fenotiazinas inibem as enzimas antioxidantes nos tripanossomatídeos. No entanto, a homologia entre as enzimas antioxidantes do agente patogénico e da célula hospedeira é uma preocupação central na utilização destes fármacos no tratamento da miocardite infecciosa induzida pelo *Trypanosoma cruzi*. Assim, a interação da tioridazina (TDZ) com as enzimas antioxidantes do *T. cruzi* e dos cardiomiócitos, e o seu impacto na infeção celular e cardíaca foi investigada in vitro e in vivo. Métodos: Cardiomiócitos e tripomastigotas em cultura, ecamundongos tratados com TDZ e benznidazol (Bz, droga antiparasitária de referência) foram submetidos a análises microestruturais, bioquímicas e moleculares. Resultados: O TDZ foi mais citotóxico e menos seletivo contra o *T. cruzi*do que o Bz in vitro. Os cardiomiócitos tratados com TDZ desenvolveram um aumento da taxa de infeção, da produção de espécies reativas de oxigénio (ROS),da oxidação lipídica e proteica; atividade semelhante da catalase (CAT) e da superóxido dismutase (SOD), e atividade da glutationa reduzida (peroxidase - GPx, S-tranferase - GST, e redutase - GR) do que as células infectadas não tratadas. O TDZ atenuou a atividade da tripanotiona redutase no *T. cruzi* e a capacidade antioxidante das proteínas nos cardiomiócitos, tornando estas células mais susceptíveis ao estresse oxidativo induzido por H_2O_2. In vivo, o TDZ potenciou o parasitismo cardíaco, a produção total de ROS, a miocardite, a oxidação lipídica e proteica, bem como reduziu as atividades da GPx, GR e GST em comparação com os animais não tratados. O benznidazol diminuiu o parasitismo cardíaco, a produção total de ROS, a inflamação cardíaca, a oxidação lipídica e proteica em animais infectados com *T. cruzi.* Conclusões: Os nossos resultados indicam que o TDZ interage simultaneamente com alvos antioxidantes enzimáticos nos cardiomiócitos e no *T. cruzi*, potenciando a infeção ao induzir fragilidade antioxidante e aumentando a suscetibilidade dos cardiomiócitos e do coração ao parasitismo, inflamação e dano oxidativo.

Palavras-chave: Doença de Chagas; patologia cardiovascular; miocardite infecciosa; estresse oxidativo; fenotiazinas.

ABSTRACT

Background and aims: Phenothiazines inhibit antioxidant enzymes in trypanosomatids. However, the homology between pathogen and host cell antioxidant enzymes is a central concern in using these drugs to treat *Trypanosoma cruzi*-induced infectious myocarditis. Thus, the interaction of thioridazine (TDZ) with *T. cruzi* and cardiomyocytes antioxidant enzymes, and its impact on cellular and cardiac infection was investigated *in vitro* and *in vivo*. Methods: Cardiomyocytes and trypomastigotes in culture, and mice treated with TDZ and benznidazole (Bz, reference antiparasitic drug) were submitted to microstructural, biochemical and molecular analyses. Results: TDZ was more cytotoxic and less selective against *T. cruzi* than Bz *in vitro*. TDZ-pretreated cardiomyocytes developed increased infection rate, reactive oxygen species (ROS) production, lipid and protein oxidation; similar catalase (CAT) and superoxide dismutase (SOD) activity, and reduced glutathione's (peroxidase - GPx, S-tranferase – GST, and reductase – GR) activity than infected untreated cells. TDZ attenuated trypanothione reductase activity in *T. cruzi*, and protein antioxidant capacity in cardiomyocytes, making these cells more susceptible to H_2O_2-based oxidative challenge. *In vivo*, TDZ potentiated heart parasitism, total ROS production, myocarditis, lipid and protein oxidation; as well as reduced GPx,GR, and GST activities compared to untreated mice. Benznidazole decreased heart parasitism, total ROS production, heart inflammation, lipid and protein oxidation in *T. cruzi*-infected mice. Conclusions: Our findings indicate that TDZ simultaneously interact with enzymatic antioxidant targets in cardiomyocytes and *T. cruzi*, potentiating the infection by inducing antioxidant fragility and increasing cardiomyocytes and heart susceptibility to parasitism, inflammation and oxidative damage.

Keywords*:* Chagas disease; cardiovascular pathology; infectious myocarditis; oxidative stress; phenothiazines.

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1 INTRODUCTION

Chagas disease (ChD) is a primary cause of infectious cardiomyopathy worldwide; which is the most severe, disabling and deadly manifestation of *T. cruzi* infection (Novaes *et al*., 2017; Nogueira *et al*., 2018). Chagas cardiomyopathy (CC) is a complex and multifactorial condition, which is associated to cardiomyocytesdeath, cardiac autonomic denervation, autoimmune reactions, persistent inflammation, and redox imbalance determined by reactive metabolites upregulation (e.g., $O2^{\bullet-}$, $OH^{\bullet-}$, $ONOO^{\bullet-}$, H2O2, HClO, and NO) and/or antioxidant defenses (e.g., catalase, superoxide dismutase, and glutathione system) downregulation (Santos *et al*., 2015; Gupta *et al*., 2009). Oxidative stress assumes a prominent role in CC progression, manifesting itself as a response to NADPH oxidase 2 hyperactivation during the respiratory burst in recruited leukocytes, as well as nitric oxide synthase (iNOS) upregulation and disturbances in mitochondrial complexes I and III in *T. cruzi*-infected cardiomyocytes (Wen; Garg 2004; 2008; Gupta *et al*., 2009; Novaes *et al*., 2017; Sánchez-Villamil *et al*., 2020). Although these are responses primarily elicited to kill parasites, their unspecificity leads to extensive molecularoxidation and cytotoxic death of infected and uninfected host cells (Gupta *et al*., 2009; Paiva *et al*., 2018). As the pathogen is not eliminated, continuous oxidative stress depletes antioxidant enzymes and exacerbates inflammation. Thus, this process feeds on itself, amplifying myocarditis that slowly progresses to end-stage heart failure (Paiva *et al*., 2018; Sánchez-Villamil *et al*., 2020; Torrico *et al*., 2021).

The specific ChD treatment is limited to the nitroheterocyclic compounds benznidazole (Bz) and nifurtimox (NFx) (Urbina, 2015; Caldas *et al*., 2019). In addition to these drugs not ensuring infection cure, they are toxic and induce severe side-effects (e.g., bone marrow depression, polyneuropathy and dermatitis) that often determine treatment discontinuation and therapeutic failure (Urbina, 2015; Caldas *et al*., 2019). As the development of new anti-*T. cruzi* chemical entities is not a priority in the pharmaceutical industry, drugs repositioning has gained relevance in the search for more efficient ChD treatments (Ferreira; Andricopulo, 2016; Diniz *et al*., 2018). In addition, all drugs currently registered on ClinicalTrials.gov and tested for ChD treatment have been or are under evaluation within a drug repositioning context. From this perspective, drugs of the phenothiazines class such as thioridazine (TDZ) have

been identified as potential trypanocidal agents due to their ability to inhibit trypanothione reductase (TR) in trypanosomatids (Lo Presti *et al*., 2015; Mendonça *et al.*, 2018, 2019). This flavoenzyme integrates sophisticated metabolic pathways involving NADPH-reducing equivalents directed to antioxidant systems mediated by dithiol trypanothione [T(SH)2,N1,N8-bisglutathionylspermidine] and tryparedoxin (Krauth-Siegel *et al*., 2007; Flohé 2011). By interacting with FAD and NADPH binding domains in TR active site, TDZ make *T. cruzi* susceptible to oxidative stress and death (Hunter *et al*., 1992; Rivarola; Paglini-Oliva, 2002; Lo Presti *et al*., 2015). As TR is essential for *T. cruzi* survival and is not expressed in mammals,it is argued that TR inhibitors represent a valuable opportunity to identify more specific and effective ChD treatments (Lo Presti *et al*., 2015; Mendonça *et al*., 2018).

Although it is the most potent phenothiazine in irreversibly inhibiting *T. cruzi* TR, TDZ effectiveness as a ChD therapeutic is debatable (Gutierrez-Correa *et al*., 2001). Accordingly, TDZ-induced antiparasitic effects are mainly reported *in vitro*, which are not always manifested *in vivo* (Rivarola and Paglini-Oliva, 2002; Martyn *et al*., 2006; Khan *et al*., 2007; Beltran-Hortelano *et al.,* 2017; Mendonça *et al*., 2020a,b). Accordingly, negative parasitological and pathological outcomes point to the limited pharmacological safety of treating ChD with TDZ (Mendonça *et al*., 2020a,b). Although infection aggravation is not fully understood, there is evidence that *T. cruzi* TR and hots cell glutathione reductase (GR) share about 40% sequence identity, suggesting that host antioxidant enzymes may also be an inconvenient target of TR inhibitors (Krieger *et al*., 2002).

Considering that TR and GR specificity is substantially based on the substrate chemical uniqueness, rather than divergences in enzyme structure, potential interactions between TDZ and host antioxidant enzymes needs to be elucidated (Krieger *et al*., 2002; Krauth-Siegel *et al*., 2007. Thus, the present study investigated the impact of TDZ on *T. cruzi* and cardiomyocytes antioxidant enzymes *in vitro* and *in vivo*. In addition to reactive oxygen species (ROS) production, protein and lipid oxidation; parasitological and microstructural cellular and cardiac outcomes associated to TDZ treatment were evaluated and compared to Bz-based reference chemotherapy.

1.1 OBJECTIVES

1.1.1 General objective

To evaluate the impact of TDZ on cardiomyocytes and *T. cruzi* antioxidant enzymes *in vitro* and *in vivo*.

1.1.2 Specific objectives:

a) to compare TDZ and Bz impact on cardiomyocytes and *T. cruzi* viability;
b) to determine TDZ and Bz impact on cardiomyocytes infection rate and parasite load;
c) to evaluate TDZ and Bz impact on pro-oxidant and antioxidant markers in on cardiomyocytes and *T. cruzi*;
d) to evaluate TDZ and Bz impact on heart microstructure, parasite load, cardiomyocytes damage, pró-oxidant and antioxidant markers in *T. cruzi*- infected mice.

2 METHODS

2.1 *IN VITRO* ASSAYS

2.1.1 Drugs and Parasites

Thioridazine (TDZ - Melleril, São Paulo, SP, Brazil) and benznidazole (Bz - reference trypanocidal drug) (Sigma-Aldrich, St Louis, MO, USA) were commercially obtained. The stock solutions with both drugs were prepared in Mili-Q water and dimethyl sulfoxide (DMSO) and stored at -20 ºC. For *in vitro* assays, TDZ and Bz were diluted in fresh culture medium (Souza-Silva *et al*., 2020). The *Trypanosoma cruzi* Y strain (DTU II), was used in all assays considering its high virulent, pathogenicity and partially resistant to Bz (Diniz *et al*., 2018).

2.1.2 Parasites and Cardiomyocytes Cultures

Trypanosoma cruzi trypomastigotes (Y strain) were isolated by centrifugation at 200 ×*g* for 10 min of the peripheral blood collected from venous puncture in previously infected mice. The parasites were collected at the peak of parasitemia, which occurred 5 days post- infection (Gonçalves-Santos *et al*., 2019). Parasites were propagated in monolayers of H9c2 cardiomyocytes (American Type Culture Collection, ATCC: CRL 1446), which were cultured in Dulbecco's modified Eagle medium (DMEM) containing 2% fetal bovine serum (FBS). Propagated trypomastigotes were harvested after centrifuging the supernatant as previously reported (Tardieux *et al*., 1992). Briefly, cardiomyocytes were inoculated with fresh blood trypomastigotes. After 3 days, propagated trypomastigotes were collected together with the culture supernatant, which was centrifuged for 5 min at 4 ºC and 2500×*g*. The supernatant was removed and the parasites were resuspended in fresh DMEM. The trypomastigotes obtained were used in toxicity assays and to induce cardiomyocytes parasitism. Cardiomyocytes were kept in DMEM culture medium (Gibco, Thermo Fisher Scientific, USA) containing 0.1% penicillin (200 µg/mL), 1% 2 mM glutamine (Hepes 2%), and 10% FBS. Culture flasks with 75 cm² containing the cardiomyocytes were incubated at 5% CO2 (37 ºC) and trypsinized when 100% cell confluence was obtained (Tardieux *et al*., 1992; Gonçalves-

Santos *et al*., 2019).

2.1.3 Drug-induced Cardiomyocytes Toxicity

Uninfected cardiomyocytes were cultured as previously reported were seeded in 96-well polystyrene microplates at 2×10^3 cells/ml and incubated for 24 h at 5% CO2 (37 ºC). The culture medium was then drained and replaced with 200 µL of a fresh culture medium supplemented or not with decreasing TDZ and Bz concentrations. Cardiomyocytes cytotoxicity was investigated from three independent assays using serial dilutions, starting at 12.5 µM TDZ and 1000 µM Bz. Control cardiomyocytes were treated with culture medium alone (vehicle). Then, 20 µL of 1 mM resazurin solution was added to each well and the absorbance was read after24h incubation using a microplate reader calibrated to 600 nm and 570 nm wavelength (Mazzeti *et al*., 2019).

2.1.4 Drug-induced Trypomastigotes Toxicity

Trypomastigotes were plated at 2×10^6 parasites/well and incubated for 24h at 5% CO_2 (37 ºC). A solution based on 1 mM resazurin diluted in phosphate-buffered saline (ISO, 2009) was added at 20 µl-wells, and the reduction reaction was followed by spectrophotometry every 2h until complete reduction is reach. Absorbance was obtained in a microplate reader (Anthos Zenyth 200, Biochrom, Cambridge, UK) at 600 nm (oxidized form detection) and 570 nm (reduced form detection). The effect of TDZ on trypomastigotes was determined from the resazurin-based reaction, and Bz was applied as the reference antiparasitic (Souza-Silva *et al*., 2020). For evaluate TDZ-induced antiparasitic effect, trypomastigotes were seeded in 96-wells polystyrene microplates at 1×10^6 cells/well. Then, the parasites were treated with decreasing TDZ and Bz concentrations diluted in 100 µl culture medium. Each drug was tested in triplicate from serial dilutions, starting at 12.5 µM TDZ and 100 µM Bz. Control trypomastigotes were treated with culture medium alone (vehicle). The parasites were incubated for 24h at 5% CO_2 (37 ºC). Then, 20 µl 1 mM resazurin was added and the absorbance was read after 7h incubation using a microplate reader at 600 nm and 570 nm (Anthos Zenyth 200, Biochrom, Cambridge, UK) (Souza-Silva *et al*., 2020).

2.1.5 Cardiomyocytes Parasitism

Two pharmacological strategies were investigated to evaluate cardiomyocytes parasitism as follows: (i) Cardiomyocytes untreated and pretreated with TDZ and Bz diluted in culture medium, (ii) Trypomastigotes untreated and pretreated with TDZand Bz diluted in culture medium. For this, cardiomyocytes were seeded in 24-wells glass-lined plates at 2×10^4 cells/ml/well and were incubated for 24 h at 5% CO2 (37 ºC) (Diniz *et al*., 2018). In the first strategy (cardiomyocytes pretreatment), culture medium was drained and cells were incubated for 24h with 1 ml of fresh medium containing TDZ dose immediately below the IC50 (3.12 µM) and the minimal Bz dose associated with cardiomyocytes death (750 µM). In the second strategy (parasites pretreatment), propagated trypomastigotes were treated with TDZ (0.78 µM) and Bz (25 µM) dose immediately below the IC50. After the respective treatments, cardiomyocytes were washed with fresh culture medium and then were challenged with trypomastigotes at 20:1 ratio (parasites:cardiomyocytes). After 2h incubation, thesupernatant and non-internalized parasites were removed by washing the cells with fresh culture medium. Cells receiving culture medium alone were used as control.Cell parasitism was evaluated 48h after parasite challenge. Briefly, cultured cardiomyocytes were treated with 2% Giemsa staining solution, and parasitism was microscopically evaluated by quantifying the proportion of infected cells (Mazzeti *et al*., 2019). Parasite load was evaluated from the endocytic index, which was calculated as the average number of intracellular parasites per infected cardiomyocyte. The results were obtained from three independent experiments (Souza-Silva *et al*., 2020).

2.1.6 Cardiomyocytes ROS Assay

All measures of pro-oxidant effectors were performed in triplicate after 48h cardiomyocytes infection and treatment with TDZ and Bz. The formation of general pro-oxidants was monitored using the CM-H2DCFDA probe (Waltham, Massachusetts, USA), a broad-spectrum ROS indicator in live cells that emit fluorescence upon oxidation. Briefly, 48h after parasite challenge and treatment with TDZ and Bz, cardiomyocytes cultured in 24-wells plates were washed with PBS (pH 7.2) and treated with 10 µM CM-H2DCFDA prepared in PBS. The plates were immediately read in a spectrofluorometer at 37°C for monitoring the CM-H2DCFDA oxidization rate, with

respective excitation/emission at 485nm/520 nm, as previously reported (Dias et al., 2017). ROS levels were expressed as relative fluorescence units (RFU)/min.

2.1.7 Cardiomyocytes Molecular Oxidation Assay

The oxidation of cell lipids in unstable peroxides and malondialdehyde (MDA) was quantified using a 96-wells colorimetric kit following the manufacturer's instructions (TBARS assay kit, Cayman Chemical, Ann Arbor, MI, USA). Briefly, after 48h cardiomyocytes infection and treatment with TDZ and Bz, culture supernatantwas removed and cells were treated with lysis buffer (2% Triton X-100, 50 mM Tris [pH 7.5], 40 mM HEPES, and 1 mM EDTA supplemented with 1 mM phenylmethanesulfonyl fluoride) and sonicated for 1 min on ice. The homogenate was incubated with thiobarbituric acid, heated for 1h, cooled on ice for 10 min, and centrifuged at 1600×*g* for 10 min. The supernatant was collected (150 µl) and the absorbance was obtained in a microplate reader at 540nm (Anthos Zenyth 200, Biochrom, Cambridge, UK).

Protein carbonyl (PCN) content was measured using a modified colorimetric biochemical assay based on 2,4-dinitrophenylhydrazine (DNPH) (Mesquita et al., 2014). Briefly, cell pellets obtained after centrifuging the cardiomyocytes lysate were incubated for 15 min with 0.4 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in in 0.5 M H_3PO_4 solution. Then, 200 µL NaOH (6 M) was added and incubated for 10 min. The reaction for oxidized proteins involved derivatization of the carbonyl group with DNPH, which generates a stable 2,4-dinitrophenyl (DNP) hydrazone metabolite. The reaction was read by spectrophotometry at 450 nm (Anthos Zenyth 200, Biochrom, Cambridge, UK). The results were corrected considering the cell viability rates determined in the cytotoxicity assay for cardiomyocytes treated with the selected doses of TDZ (3.12 µM) and Bz (750 µM).

2.1.8 Trypanothione Reductase Activity Assay

Trypanothione reductase activity was investigated in trypomastigotes submitted to the same TDZ and Bz treatments previously reported by using a colorimetric biochemical method (van den Bogaart et al., 2014). Briefly, 2×10^6 parasites in culture were washed with PBS, lysed by 15 min incubation with 100 µ mL/well lysis buffer (2%

Triton X-100, 50 mM Tris [pH 7.5], 40 mM HEPES, and 1 mM EDTA supplemented with 1 mM protease inhibitor phenylmethanesulfonyl fluoride). TR activity was measured in 96-well microplates at 75 µL lysate sample/well. Thus, 25 µL/well NADPH, 75 µL/well T[S]2, and 25 µL/well DTNB were sequentially added to the sample lysate to respectively obtain 200, 75, and 100 µM (final concentrations). A blank was obtained for each sample, which was prepared from sample lysate supplemented with the reaction mixture with 0.05 M Tris buffer (pH 7.5) replacing T[S]2 substrate. The mixtures were incubated for 3h at 27°C, and the absorbance was measured by spectrophotometry at 412 nm. The blank signal subtracted from the sample absorbance corresponded to TR activity, which develops a colored reaction from 2-nitro-5-thiobenzoate production. The results were corrected considering the cell viability rates determined in the cytotoxicity assay for trypomastigotes treated with the selected doses of TDZ (0.78 µM) and Bz (25 µM).

2.1.9 Cardiomyocytes Antioxidant Enzymes and Antioxidant Capacity Assay

Enzymatic and total antioxidant activity were investigated in cardiomyocytes and trypomastigotes submitted to the same TDZ and Bz treatments previously reported. Catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione reductase S-transferase (GST) activities were analyzed from 96-wells commercial kits and the manufacturer's instructions. Catalase (ThermoFisher Scientific, Waltham, Massachusetts, USA), SOD (ThermoFisher Scientific, Waltham, Massachusetts, USA) and GP (Abcam.Cambridge, UK) activities were measured from colorimetric kits. CAT, SOD and GPx activities were respectively monitored at 560 nm, 450 nm and 340 nm wavelengths, and the assays sensitivities were 0.052 U/ml (CAT), 0.044 U/ml (SOD) and 0.5 mU/ml (GPx). GR (ThermoFisher Scientific, Waltham, Massachusetts, USA) and GST (ThermoFisher Scientific, Waltham, Massachusetts, USA) activities were measured by fluorimetry using 96-wells kits following the manufacturer's instructions. GR and GST were respectively monitored at 390 nm/510 nm excitation/emission and 70 nm/460 nm excitation/emission wavelengths. The assays sensitivities were 0.009mU/ml (GR) and 2.70 mU/ml (GST). Total antioxidant capacity was analyzed in cardiomyocyte lysates using a colorimetric biochemical assay following the manufacturer's instructions (Abcam, Cambridge, UK).

In this method, Cu^{2+} ions are converted to Cu^{+} by both antioxidants. Protein masks are used to prevents $Cu2^{+}$ reduction by proteins, allowing to isolatethe effect of these molecules on the cellular antioxidant capacity. The reduced Cu^{+} ion was chelated with a colorimetric probe giving a broad absorbance peak near to 570 nm, which is proportional to the cellular antioxidant capacity. The results were corrected considering the cell viability rates determined in the cytotoxicity assay forcardiomyocytes treated with the selected doses of TDZ (3.12 µM) and Bz (750 µM).

2.1.10 Hydrogen Peroxide-Mediated Cardiomyocytes and *T. cruzi* Toxicity Assay

Cardiomyocytes (2×10^3 cells/well) and trypomastigotes (2×10^6 parasites/well) were plated in 96-well polystyrene microplates and incubated for 24 h at 5% CO_2 (37 ºC) with TDZ (3.12 µM) and Bz (750 µM) for cardiomyocytes, as well as 0.78 µM TDZ and 25 µM Bz for trypomastigotes. Then, culture medium was replaced with 200 µl fresh culture medium supplemented or not with different H_2O_2 concentrations for cardiomyocytes (0, 50, 100, 200, and 300 µM H_2O) and *T. cruzi* (0, 100, 200, 300 and 400 µM H_2O) (Gonçalves-Santos *et al*., 2023). Cardiomyocytes and trypomastigotes cytotoxicity were investigated from three independent assays. Control cardiomyocytes and parasites were treated with culture medium alone without H_2O_2 (vehicle). After 24h incubation, 20 µL of 1 mM resazurin solution was added to each well, and cell viability was measured according to the absorbance obtained from a microplate reader at 600 nm and 570 nm (Mazzeti *et al*., 2019).

2.2 *IN VIVO* ASSAY

2.2.1 Animals, Trypanosoma cruzi Infection and Treatments

Female albino *Swiss* mice with 8 weeks-old and 34.61 ± 4.79g were kept in facilities with photoperiod (12h/12h light/dark), air humidity (60–70%) and temperature (20 ± 2ºC) controlled (Sequetto et al., 2014). The animals had free access to water and rodent chow. Mice were intraperitoneally inoculated with 2000 trypomastigotes of *T. cruzi* (Y strain) isolated from the peripheral blood of infected animals (Mendonça *et al*., 2020a,b). Infection was confirmed 5 days after *T. cruzi* inoculation by microscopic

observation of trypomastigotes in 5µl blood samples (Novaes *et al*., 2017). Then, TDZ and Bz tablets were resuspended in 10 mL distilled water. The animals were treated by gavage with 100 µL TDZ at 80 mg/kg, which was effective in reducing cardiac damage in *T. cruzi*-infected mice (Bustamante *et al*., 2007). Benznidazole was administered by gavage at 100 mg/kg for 20 days, which is recommended as pharmacological control in preclinical screenings of trypanocidal drugs (Diniz *et al*., 2018; Gonçalves-Santos *et al*., 2019), and corresponds to a daily dose for human adults of 7 mg/kg based on body surface area (Mazzeti *et al*., 2018). Uninfected untreated (receiving water by gavage) and *T. cruzi*-infected untreated (receiving water by gavage) animals were concurrently followed for 20 days and used as controls (Novaes *et al*., 2017). Twenty-four hours after the last treatment (day 21), the animals were anesthetized with 30 mg/kg xylazine and 300 mg/kg ketamine and euthanized by exsanguination. Heart and blood were collected and used in molecular, biochemical and microstructural analyses.

The sample size was determined considering the probability P=1/2 to increase or decrease the variables of interest (Nogueira *et al*., 2023). Considering the significance level α=0.05, the minimal significant number of animals used in the statistical analysis was $P=(1/2)^{events}$; so, if n=5, $P=(1/2)^5$ or P=0.03; thus, P<0.05. From this result, we admitted the same probability of sample loss due to *T. cruzi* infection. Thus, the final sample size was characterized as 10 animals per group. The animals were numbered and randomized into the 5 investigated groups using the random function of the Excel software. Treatments were prepared by one researcher and administered by another blinded to the interventions. The experimental outcomes were also blindly evaluated in relation to the intervention groups. The Institutional Ethics Committee for Animals Research approved the experimental protocol (013/2016).

2.2.2 Heart Histopathology

Heart fragments were fixed for 24h with 4% paraformaldehyde prepared in 0.1 M sodium phosphate buffer (pH 7.2) (Sequetto *et al*., 2014). Then, fragments were embedded in plastic resin (glycol methacrylate) and 3-µm thick heart sections were obtained using glass knives coupled in a rotary microtome (Leica Biosystems, Wetzlar, Germany). Six heart sections were obtained in semi-series for each animal, so that one in each 30 sections were collected to avoid analyzing the same histological area. The

sections were stained at 60 ºC with hematoxylin and eosin (H&E) (Mendonça *et al*., 2020a). Ten random non-coincident microscopic images were obtained for each animal by bright field microscopy at ×400 magnification (Axioscope A1; Carl Zeiss, Germany). Heart pathological microstructural remodeling was analyzed considering: (i) parenchyma and stroma distribution, (ii) tissue necrosis, (iii) inflammatory infiltrate, (iv) myocyte hypertrophy or atrophy, (v) blood vessels distribution, and (vi) *T. cruzi* nests distribution (Rodrigues et al., 2017). These abnormalities were globally expressed using the polygonal method, which is basedon a field diagram organized in four domains: (-) normal, (- - +) mild, (+ +) moderateor (+ + +) severe damage (Felizardo *et al*., 2018). Interstitial cellularity and inflammatory infiltrate were quantified from the Image-Pro Plus software (Media Cybernetics Inc., Silver Spring, MD, USA) (Novaes *et al*., 2018). A pathologist blindedto the intervention groups performed all morphological analysis.

2.2.3 Heart Parasitism Assay

Heart parasitism was determined in heart samples by quantitative reverse transcription PCR (qPCR) as previously described (Santos *et al*., 2015). For such, heart samples were homogenized and genomic DNA was extracted using a commercial kit following the manufacturer's instructions (Promega, São Paulo SP, Brazil). DNA samples were adjusted to 25 ng/µL and PCR reactions were standardized at 10 µL volume containing 50 ng of genomic DNA, 5 µL SYBR Green (Applied Biosystems, CA, USA), 0.50 µM murine TNF-α primers or 0.35 µM 195-bp *T.cruzi* DNA primers (Table 1). From 96-wells plate, standard curve based on negative controls with mice-specific and *T. cruzi*-specific primers without DNA and with genomic DNA from uninfected mice was obtained (Santos *et al*., 2015). Parasite load (PL) based on *T. cruzi* DNA levels was normalized considering the results obtained for TNF-α as follows: Adjusted PL = (mean *T. cruzi* DNA/mean TNF-α DNA) × 1000; where the constant 1000 is the expected TNF-α concentration in 30 mg heart samples. Amplification efficiency (*E*) was calculated as $(E) = 10^{-1/\text{slope}}$ (StepOne Software, ThermoFisher, MA, USA) (Stordeur *et al*., 2002).

Table 1 - Primers used to quantify heart *Trypanosoma cruzi* load by quantitative polymerase chain reaction (qPCR)

TNF-α*	Forward	5′-GCTCTTGCCCACAMGGGTGC-3′
	Reverse	5′-CCAAGCAGCGGATAGTTCAGG-3′
TCZ DNA*	Forward	5′-TCCCTCTCATCAGTTCTATGGCCCA-3′
	Reverse	5′-CAGCAAGCATCTATGCACTTAGACCCC-3′

Source: Author
Note: *All primers were validated: Infect Immun. 2012; 123:170-7. TNF-α primers amplify a 170-bp productand *T. cruzi* (TCZ) DNA primers amplify a 182-bp fragment: Infect Immun. 04;72:40819.

2.2.4 Heart Oxidation, Antioxidant Enzymes and Antioxidant Capacity Assay

Total ROS production, lipid and protein oxidation, total antioxidant capacity and activity of antioxidant enzymes in cardiac tissue from uninfected and infected animals were analyzed using the same protocols described to quantify oxidative and antioxidant markers in cardiomyocytes. For such, heart samples were frozen in liquid nitrogen (-196°C) and pulverized in a crucible. Then, the macerated tissue was homogenized in the respective buffers provided by the biochemical kits and centrifuged according to the manufacturer's instructions. Supernatants and residual pellets were used to quantify of malondialdehyde and carbonyl protein heart levels, cardiac antioxidant capacity, as well as the activity of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione s-transferase (GST). CAT, SOD and GR activities were analyzed by spectrophotometry, while GR and GST activities were measured by fluorescence spectroscopy, as previously reported.

2.2.5 Cardiac Troponin I Assay

Cardiac troponin I (cTnI) serum levels were quantified as a marker of structural cardiac damage (Bonney *et al*., 2013). For such, 300 µL blood samples obtained during euthanasia were centrifuged at 3000 ×*g* at 4ªC for 15 min in the presence of protease

inhibitor (Sigma-Aldrich, San Luis, MO, USA), and the serum was collected. cTnI was quantified from an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions (MyBioSource, San Diego, CA, USA). cTnI was quantified by spectrophotometry at 450 nm by using a 99-wells microplate reader (Anthos Zenyth 200, Biochrom, Cambridge, UK). This method has detection limits ranging from 7.813 to 500 pg/mL.

2.3 STATISTICAL ANALYSIS

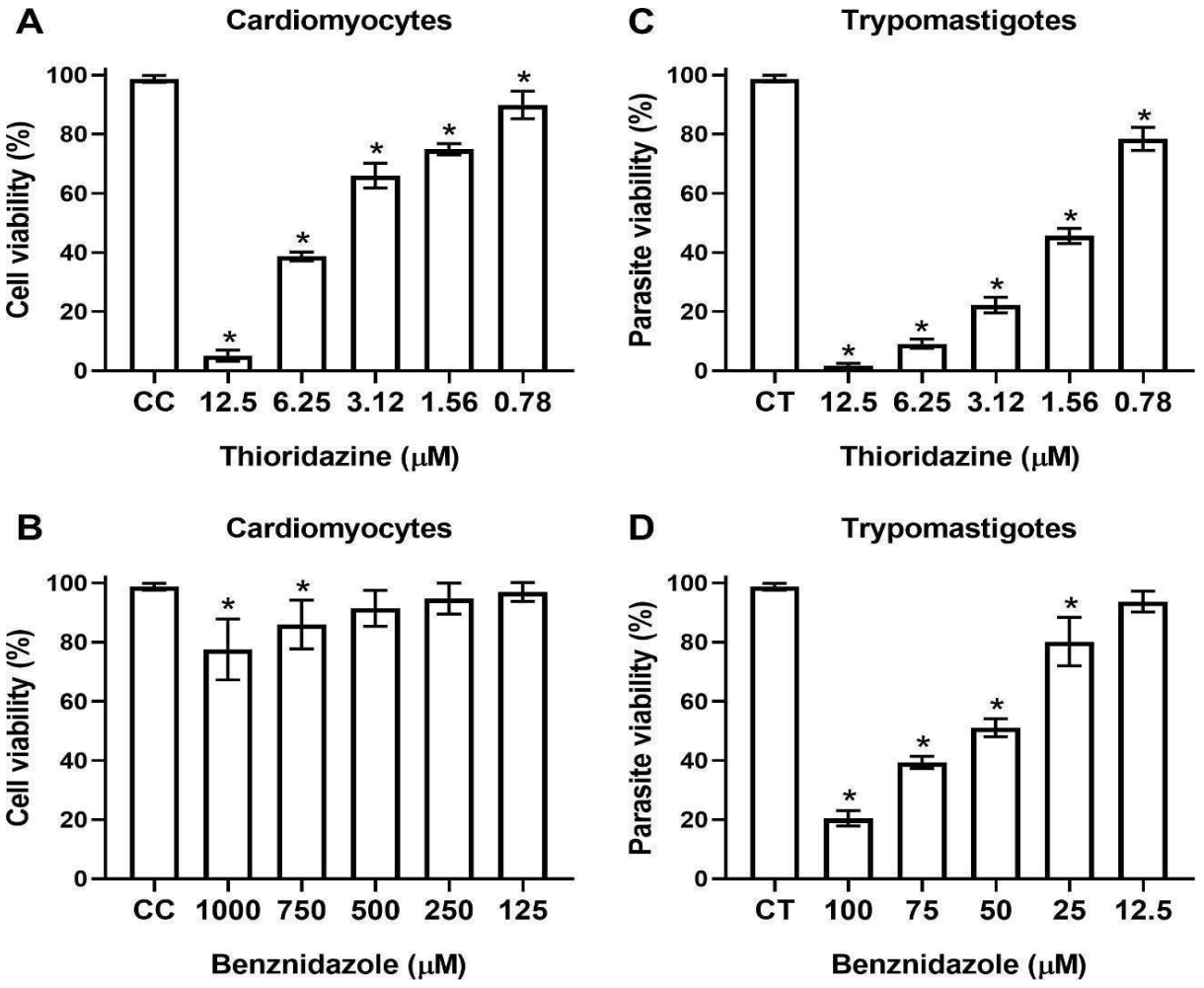
The results were presented as mean and standard deviation (mean ± S.D.). Data distribution was verified according to the D'Agostino-Pearson's K2 normality method. Data with normal distribution were compared using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls *post-hoc* test. Non- parametric data were compared using the Kruskal-Wallis one-way ANOVA on Ranks test followed by the Student-Newman-Keuls method. Results with $P \leq 0.05$ were statistically significant.

3 RESULTS

3.1 IN VITRO FINDINGS

As indicated in Fig. 1, TDZ treatment exerted a marked dose-dependent toxicity on cardiomyocytes and trypomastigotes, which was more prominent than Bz. Benznidazole was well tolerated by cardiomyocytes, which exhibited some degree of cytotoxicity from 750 µM. Bz was highly toxic to trypomastigotes, reducing parasite viability in a dose-dependent manner at concentrations from 25 µM.

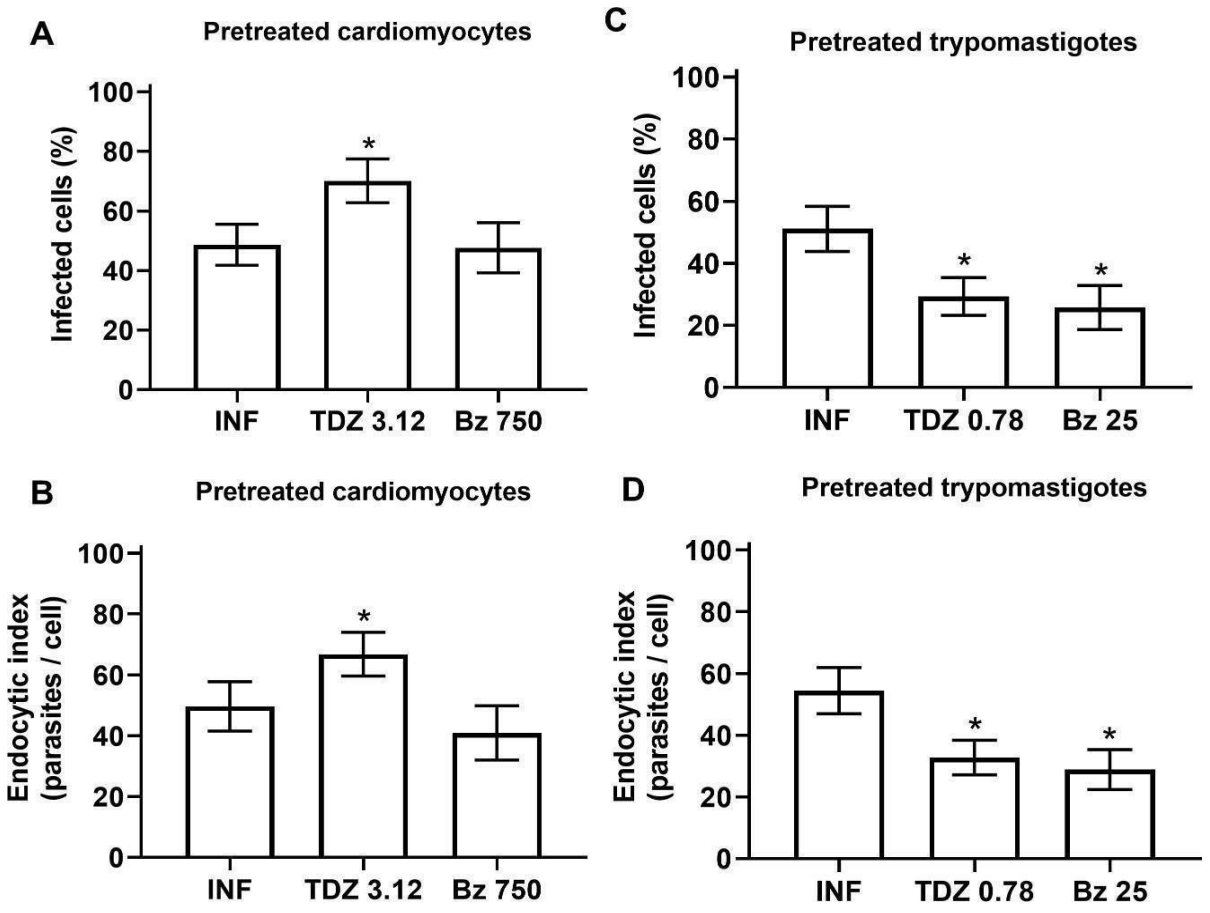
Figure 1 - Effect of thioridazine (TDZ) and benznidazole (Bz) on H9c2 cardiomyocytes and *Trypanosoma cruzi* viability



Source: Author.

As indicated in Fig. 2, increased cell parasitism rate and higher parasite load were observed in cardiomyocytes pretreated with TDZ but not with Bz compared to untreated cardiomyocytes. Conversely, the rate of infected cells and parasite load were similarly reduced in cardiomyocytes challenged with TDZ- and Bz-pretreatedtrypomastigotes compared to cardiomyocytes challenged with untreated parasites.

Figure 2 - Effect of thioridazine (TDZ) and benznidazole (Bz) on H9c2 cardiomyocytes infection rate and parasite load (endocytic index) 48h after *Trypanosoma cruzi* challenge



Source: Author.

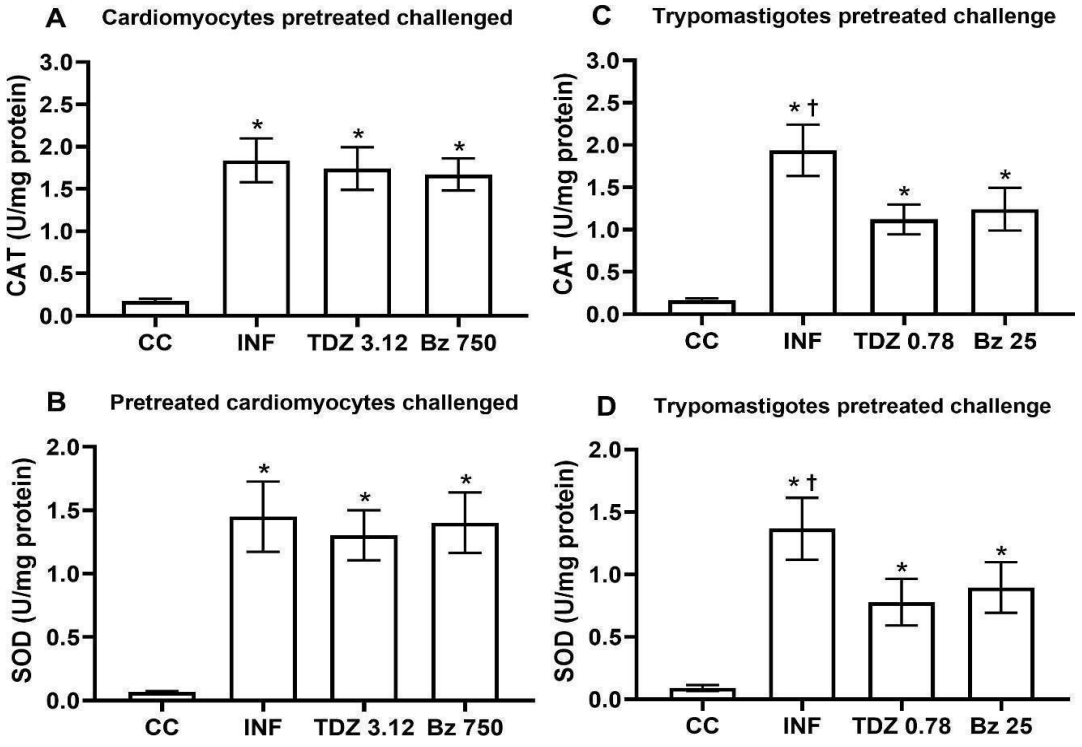
Total ROS levels were similarly increased in infected untreated and Bz- pretreated cardiomyocytes compared to uninfected untreated cardiomyocytes ($P<0.05$).

This increase was even greater in TDZ-pretreated cardiomyocytes compared to untreated or Bz-treated cells ($P<0.05$). Total ROS levels were reduced in cardiomyocytes challenged with TDZ- and Bz-pretreated trypomastigotes compared to

cardiomyocytes infected with untreated parasites.

CAT and SOD activities were similarly increased in infected untreated, TDZ- and Bz-pretreated and infected cardiomyocytes compared to uninfected untreated cardiomyocytes ($P<0.05$). These parameters were reduced in cardiomyocytes challenged with TDZ- and Bz- pretreated trypomastigotes compared to cardiomyocytes infected with untreated parasites (Fig. 3).

Figure 3 - Effect of thioridazine (TDZ) and benznidazole (Bz) on catalase (CAT) and superoxide dismutase SOD) activities in H9c2 cardiomyocytes 48h after *Trypanosoma cruzi* challenge

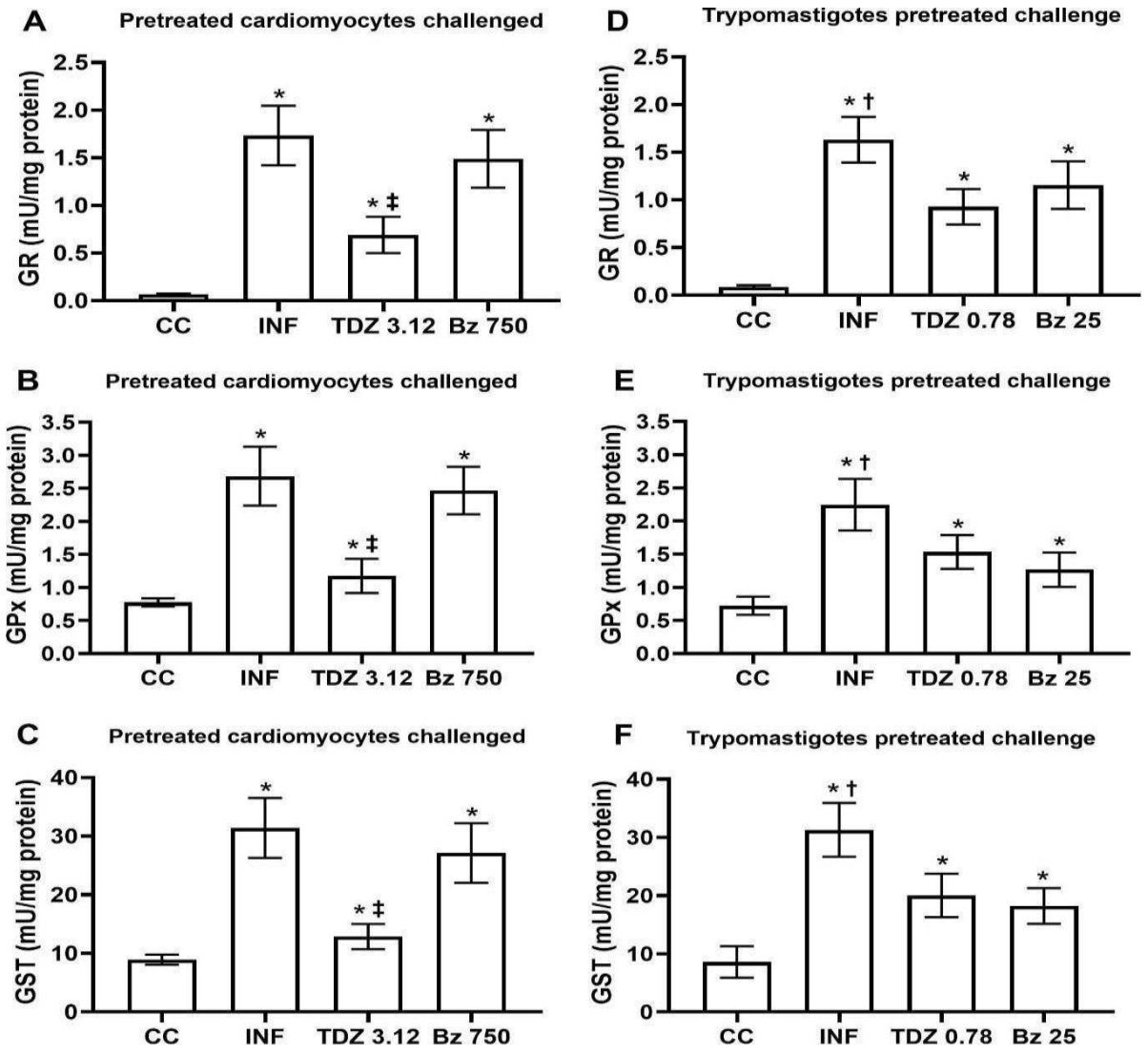


Source: Author

Glutathione reductase, GPx, and GST activities were increased in all infected cardiomyocytes compared to uninfected untreated cells ($P<0.05$). These parameters were lower in TDZ-pretreated cardiomyocytes compared to uninfected and Bz-pretreated cardiomyocytes challenged with *T. cruzi* ($P<0.05$).

These parameters were similarly reduced in cardiomyocytes challenged with TDZ- and Bz-pretreated trypomastigotes compared to cardiomyocytes infected with untreated parasites ($P<0.05$), but they were also higher compared to untreated uninfected cardiomyocytes ($P<0.05$) (Fig. 4).

Figure 4 - Effect of thioridazine (TDZ) and benznidazole (Bz) on glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione s- transferase (GST) in H9c2cardiomyocytes 48h after *Trypanosoma cruzi* challenge



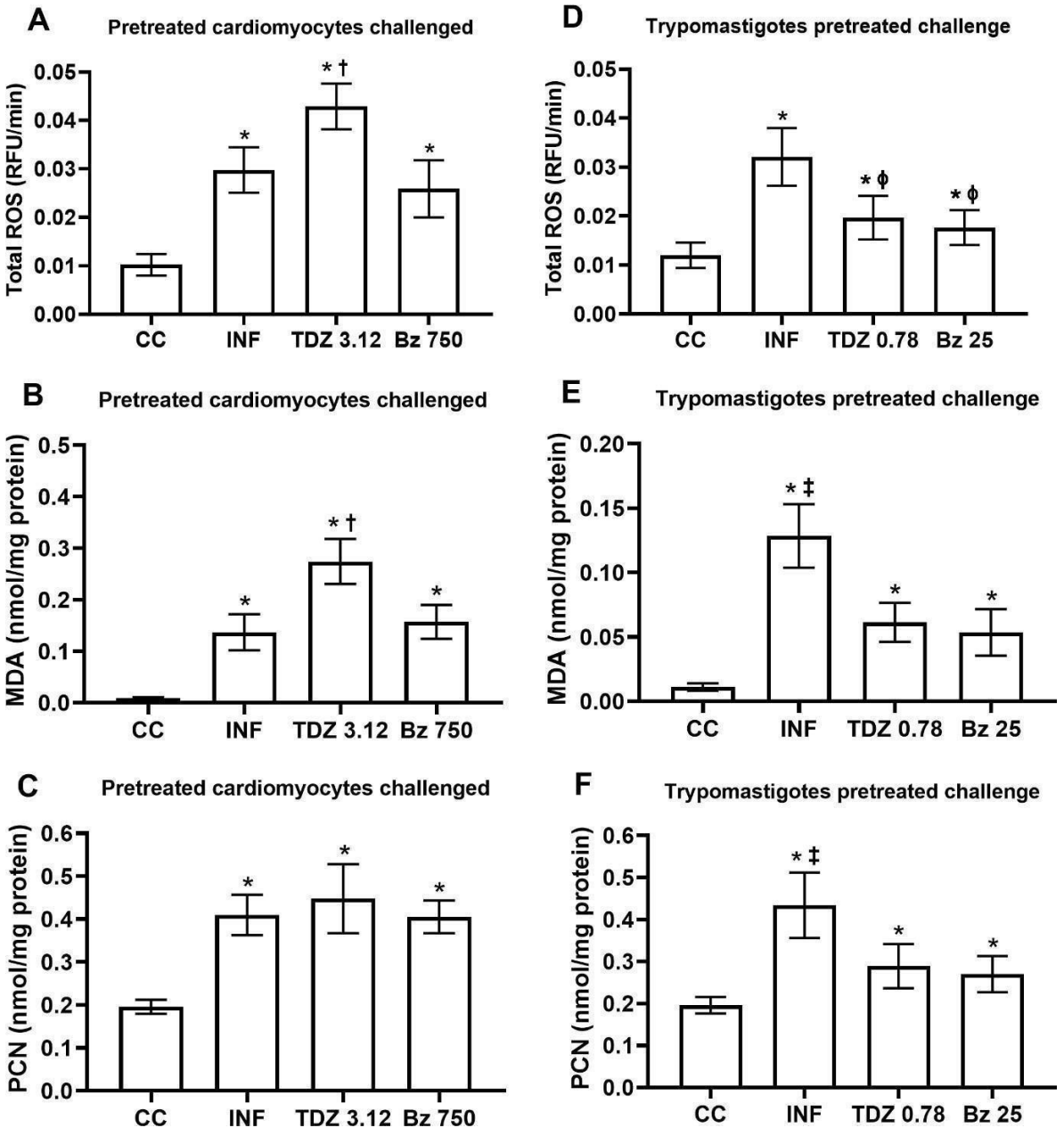
Souce: Author.

Lipid (MDA) and protein (PCN) oxidation were higher in all infected cardiomyocytes compared to uninfected untreated cells ($P<0.05$).

Malondialdehyde levels were even greater in TDZ-pretreated infected cardiomyocytes compared to untreated or Bz-treated infected cells ($P<0.05$). Protein carbonyl levels were similar in infected untreated, TDZ- and Bz-pretreated cardiomyocytes ($P<0.05$). These parameters were similarly reduced in cardiomyocytes challenged with TDZ- and Bz- pretreated trypomastigotes compared to cardiomyocytes infected with untreated parasites ($P<0.05$) (Fig. 5).

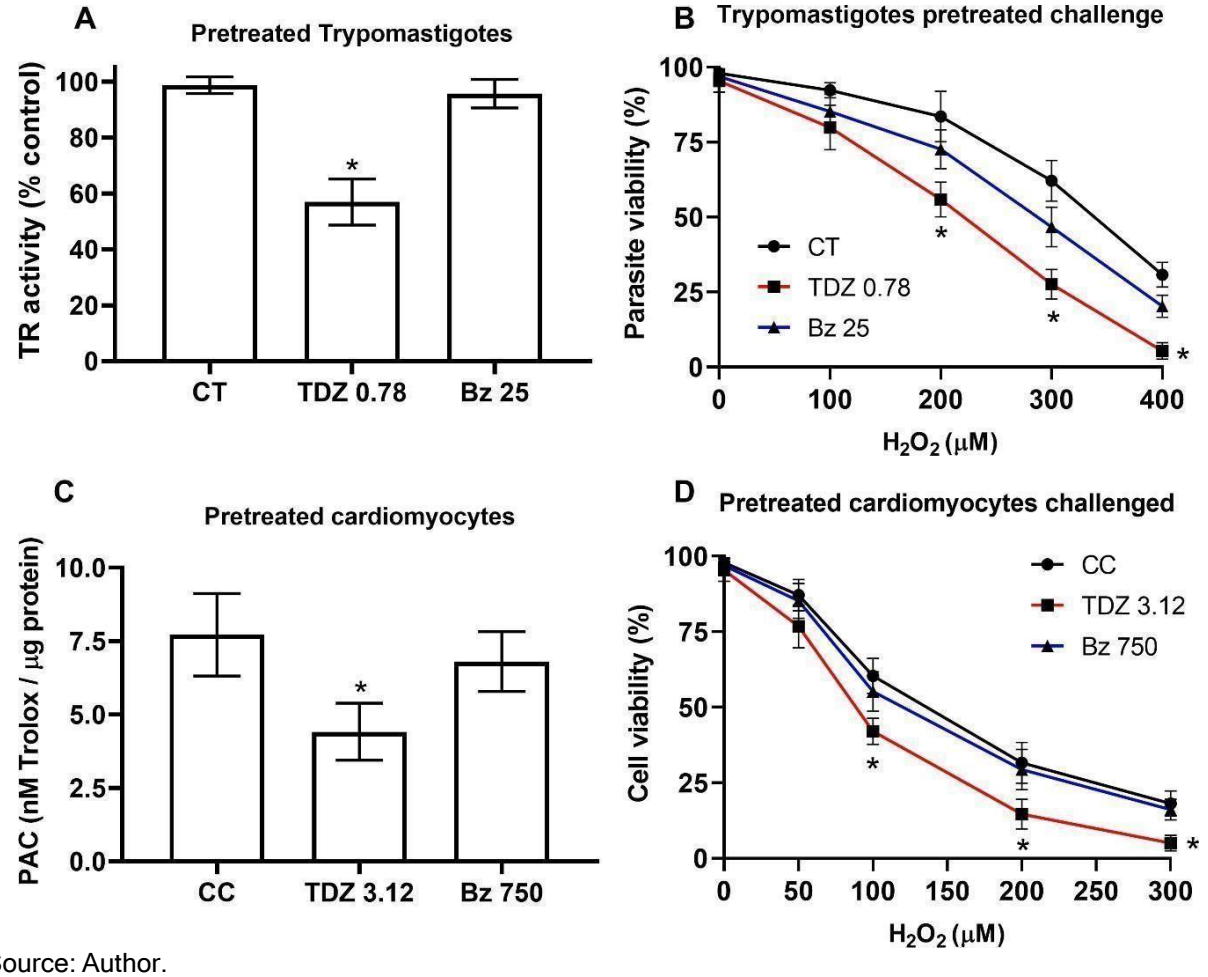
Reduced TR activity and total protein antioxidant capacity were respectively observed in TDZ-treated trypomastigotes and cardiomyocytes compared to untreated and Bz-treated trypomastigotes and cardiomyocytes ($P<0.05$). Trypomastigotes pretreated with both drugs exhibited reduced resistance to H_2O_2 challenge compared to untreated trypomastigotes. Conversely, only TDZ-pretreated cardiomyocytes exhibited reduced resistance to H_2O_2 challenge compared to untreated cells ($P<0.05$) (Fig.6).

Figure 5 - Effect of thioridazine (TDZ) and benznidazole (Bz) on total reactive oxygen species (ROS) production, lipid (MDA – malondialdehyde) and protein (PCN –protein carbonyl) oxidation in H9c2 cardiomyocytes 48h after Trypanosoma cruzi challenge



Source: Author .

Figure 6 - Effect of thioridazine (TDZ) and benznidazole (Bz) on trypanothione reductase (TR) activity, protein antioxidant capacity (PAC), and hydrogen peroxide (H_2O_2) resistance in H9c2 cardiomyocytes and trypomastigotes



Source: Author.

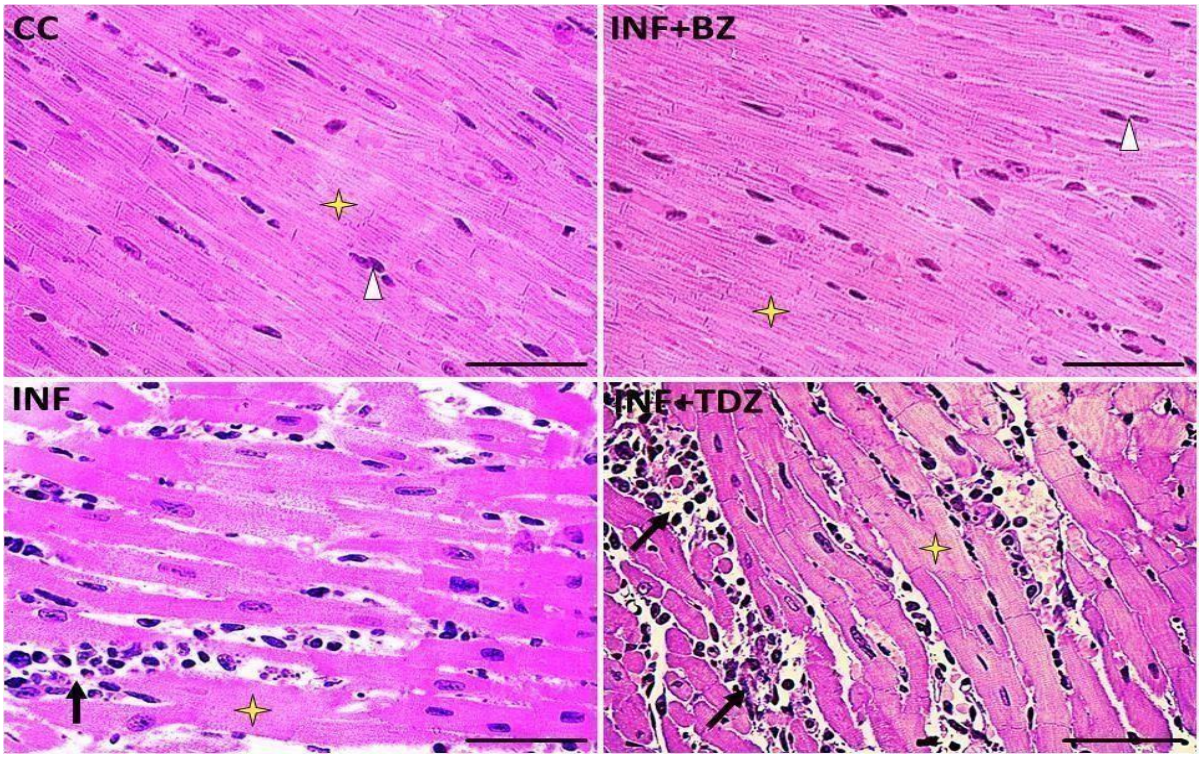
3.1 IN VIVO FINDINGS

As indicated in Fig. 7, the histopathological analysis indicated that control uninfected animals and *T. cruzi*-infected mice receiving Bz exhibited a typical myocardial structure with parallel and well-defined cardiomyocytes, scarce connective tissue and interstitial cellularity with no evidence of myocarditis and cell degeneration.

Conversely, infected untreated mice exhibited marked myocarditis, with evident inflammatory infiltrate rich in mononuclear cells and connective tissue expansion.

These pathological changes were more pronounced in *T. cruzi*-infected and TDZ-treated mice, which presented diffuse inflammatory infiltrate with predominance on mononuclear cells. Polymerase chain reaction confirmed infection in all *T. cruzi*-inoculated animals (Fig. 8). Parasite load was higher in infected mice receiving TDZ ($P<0.05$), andreduced in Bz-treated mice ($P<0.05$) compared to infected untreated animals. Myocardial cellularity was similar in uninfected and Bz-treated mice ($P>0.05$), but reduced compared to the other groups ($P<0.05$). This parameter was increased in infected and TDZ-treated compared to infected untreated mice ($P<0.05$). Histopathological analysis indicated extensive global microstructural damage in TDZ-treated mice, followed by infected untreated animals compared to the other groups. This response was consistent with increased cTnI serum levels in all infected compared to uninfected mice ($P<0.05$). This parameter was higher in TDZ-treated mice compared to the other groups ($P<0.05$). cTnI levels was reduced in Bz-treated compared to infected untreated animals ($P<0.05$) (Fig. 8).

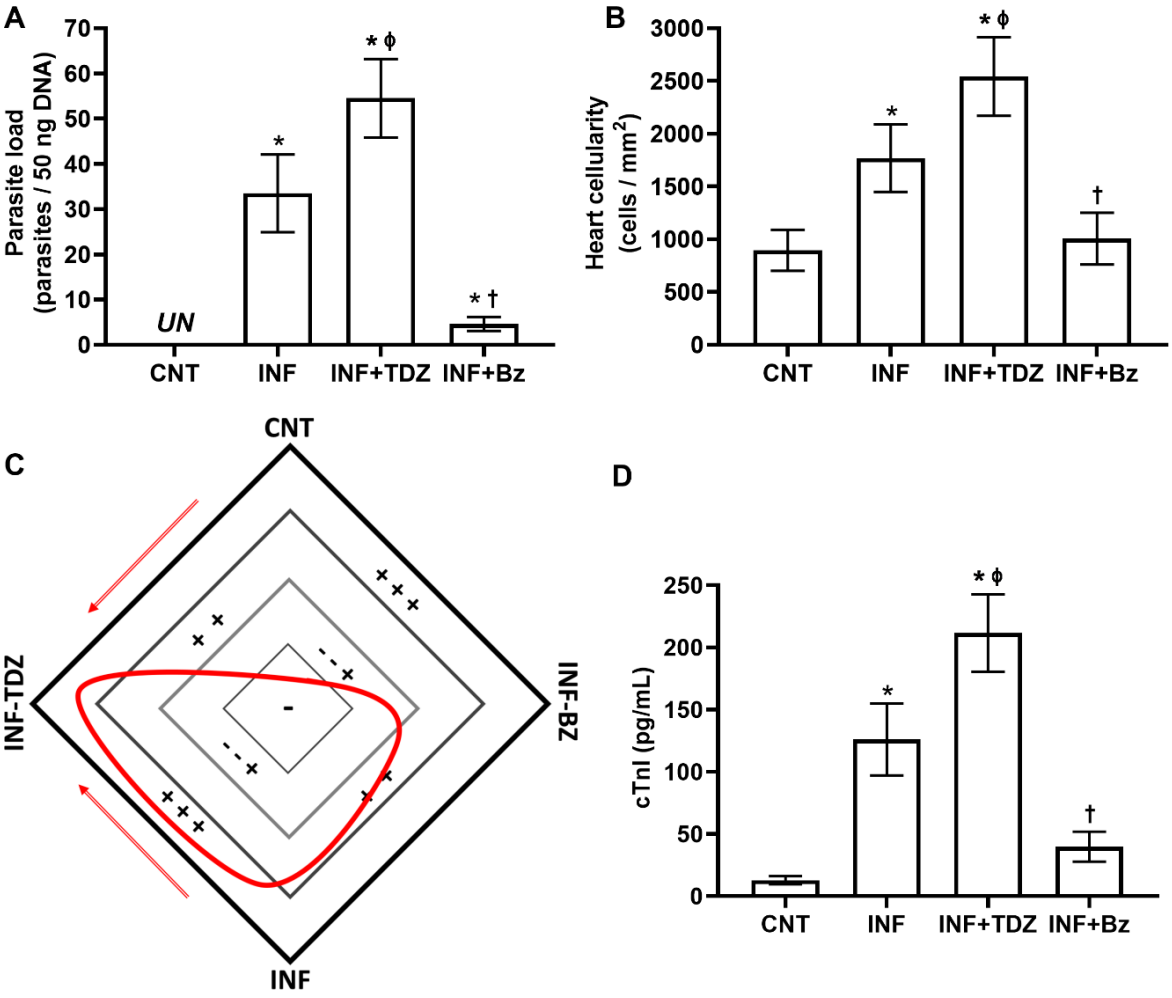
Figure 7 - Microscopic images of the heart from uninfected and *Trypanosoma cruzi*-infected mice treated with thioridazine (TDZ) and benznidazole (BZ)



Source: Author.

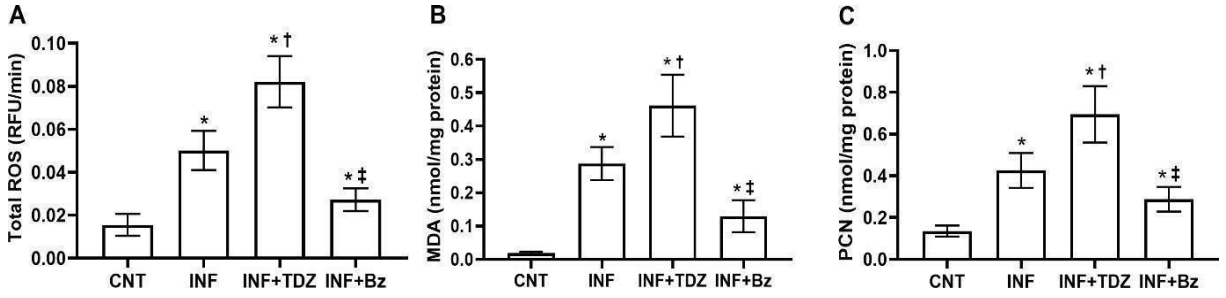
As indicated in Fig. 10; CAT, SOD, GR, GPx, and GST cardiac activities were increased in all infected compared to uninfected animals ($P<0.05$). CAT and SOD activities were attenuated in Bz-treated mice ($P<0.05$) compared to both infected untreated and TDZ-treated mice, which exhibited similar results ($P>0.05$). GR, GPx, and GST activities were reduced in infected and TDZ-treated compared to infected untreated animals ($P>0.05$), but increased compared to Bz-treated mice ($P<0.05$).

Figure 8 - Cardiac parasite load (A), heart cellularity (B), global microstructural damage (C) and cardiac troponin I - cTnI serum levels (D) in Trypanosoma cruzi-infected micetreated with thioridazine (TDZ) and benznidazole (BZ)



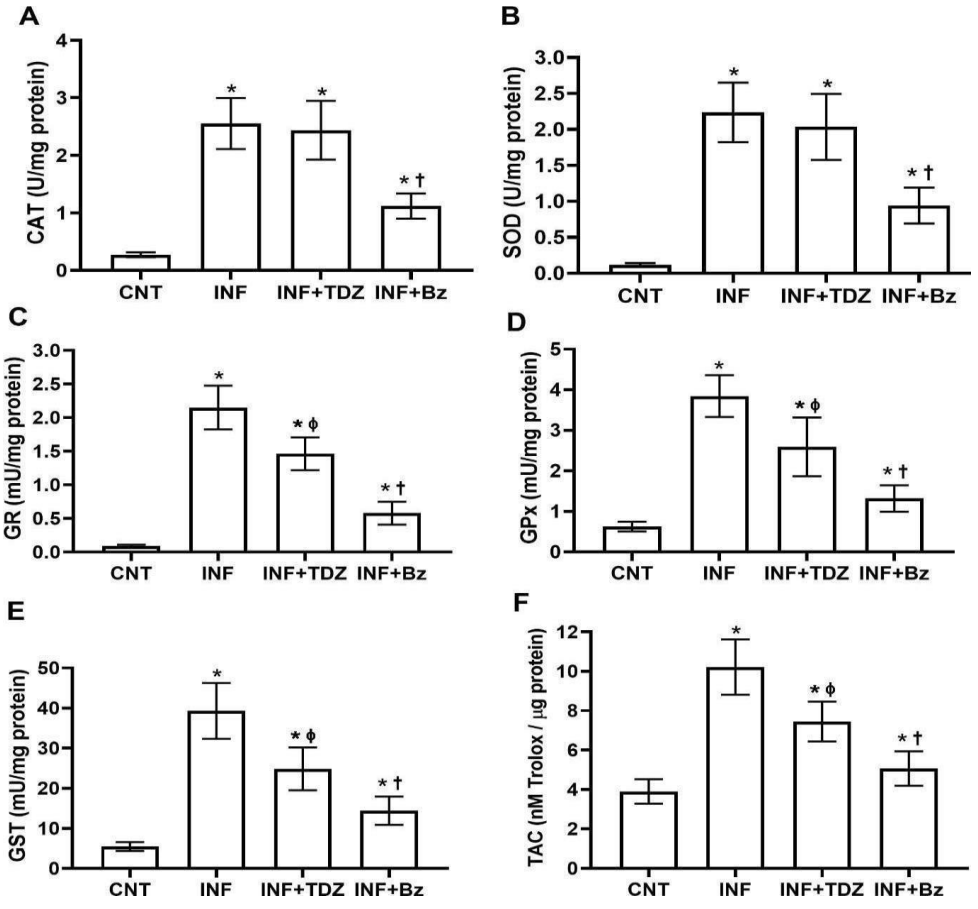
Source: Author.

Figure 9 - Reactive oxygen species (ROS), malondialdehyde (MDA) and protein carbonyl(PCN heart levels in *Trypanosoma cruzi*-infected mice treated with thioridazine (TDZ) and benznidazole (BZ).



Source: Author.

Figure 10 - Activity of antioxidant enzymes and total antioxidant capacity (tac) in the heart from uninfected and *trypanosoma cruzi*-infected mice treated with thioridazine (tdz) and benznidazole (bz)



Source: Author.

4 DISCUSSION

From integrated *in vitro* and *in vivo* analysis, we identified for the first time a marked divergence in therapeutic effects when *T. cruzi* and cardiomyocytes were differentially exposed to TDZ. Although this drug had a superior trypanocidal effect than Bz in reducing parasites viability at lower concentrations, TDZ also induced notorious toxicity in cardiomyocytes. Furthermore, cardiomyocytes were resistant while trypomastigotes were clearly susceptible to Bz, indicating that this reference drug has greater pharmacological selectivity. The mechanism associated to TDZ- induced mammalian cells cytotoxicity is still poorly understood. However, it is admitted that it is potentially related to the imbalance of calcium and magnesium dynamics, anti-calmodulin effect, as well DNA damage induced by this drug (Pantazaki; Lialiaris, 1999; de Faria *et al*., 2015). Accordingly, TDZ can inhibit CA^{2+}- and Mg^{2+}-dependent molecular transporters and opening of the mitochondrial membrane permeability transition pores (MPTP), compromising cell viability by destabilizing the respiratory chain and mitochondrial energy production (de Faria *et al*., 2015). From a pharmacological safety perspective, low toxicity and high selectivity are desirable characteristics in the screening of new and repositioned drugs (Diniz *et al*., 2018; Mazzeti *et al*., 2018, 2019). Accordingly, an ideal antiparasitic drug should be able to neutralize the parasite, causing no or minimal damage to host cells (Diniz *et al*., 2018; Mazzeti *et al*., 2018, 2019). In this case, dose adjustment can be preponderant to optimize therapeutic results, which manifest primarily as adequate parasitological control of the infection (Bahia *et al*., 2014; Bustamante *et al*., 2023).

Interestingly, after adjusting TDZ and Bz concentrations, we found that cellular parasitism was markedly divergent when cardiomyocytes and trypomastigotes were exposed to these drugs before interacting in a co-culture infection assay. Accordingly, cellular infection rate and intracellular parasites load (e.g., endocytic index) were increased only in TDZ-pretreated cardiomyocytes. Conversely, these parameters were attenuated when cardiomyocytes were challenged with TDZ- and Bz- pretreated trypomastigotes. These findings reinforce the selectivity profile of both drugs, and reveals that TDZ can increase the cardiomyocytes susceptibility to *T. cruzi* infection, a process previously suggested *in vivo* that is still poorly understood (Mendonça *et al*., 2020a).

Considering the differential parasitism in cardiomyocytes, both treatments were investigated in view of the antiparasitic mechanism centrally attributed to TDZ, which induces redox imbalance in *T. cruzi* (Khan *et al*., 2007; Lo Presti *et al*., 2015; Beltran-Hortelano *et al*., 2017). As expected, acute infection increased the activity of all investigated antioxidant enzymes (e.g., CAT, SOD, GR, GPx and GST). Notably, this increase is part of a counter-regulatory mechanism in an attempt to protect infected host cells from oxidative stress triggered by *T. cruzi* (Novaes *et al*., 2017; Mendonça *et al*., 2019). Interestingly, the activity of these enzymes was not influenced in infected cardiomyocytes pretreated with Bz, a characteristic consistent with the high cardiomyocytes' tolerability to Bz. Thus, this response reinforces that Bz-induced antiparasitic effect does not depend on direct interaction with the antioxidant enzymes of the host cell, although it may indirectly stimulate antioxidant mechanisms *in vivo* and *in vitro* via NRF2 activation (Pedrosa *et al.,* 2001; Lambertucci *et al*., 2017). In this case, the mechanism of action itself seems to partially explain Bz selectivity. Accordingly, Bz antiparasitic effect is mainly mediated glyoxal dialdehyde formation by *T. cruzi* NADH-dependent trypanosomal type I nitroreductases, which kills the parasite by damaging its DNA and neutralizing its thiol-based antioxidant effectors (Trochine *et a*l., 2014; Caldas *et al*., 2019).

Unlike Bz pretreatment, while CAT and SOD activity remained high, glutathione-based enzymes (e.g., GR, GST and GPx) was down-regulated in TDZ-pretreated cells; suggesting a differential impact of TDZ on specific enzymatic antioxidant effectors in cardiomyocytes. Notably, this enzymatic downregulation seems consistent with increased susceptibility to parasitism in TDZ-exposed cardiomyocytes, indicating a potential pharmacological risk factor for *T. cruzi* infection treatment with this phenothiazine. On the other hand, the activity of all investigated antioxidant enzymes was attenuated in cardiomyocytes infected with Bz- and TDZ- pre-treated trypomastigotes. This is a well-characterized finding due to the direct Bz toxicity on *T. cruzi*. However, it reveals that TDZ is also effective in reducing the infective potential of the parasite. Accordingly, as cardiomyocytes were not exposedto TDZ and Bz in this experimental scenario, the down-regulation of antioxidant enzymes shows a response potentially linked to the direct attenuation of the infection rate and parasite load induced by these drugs. Despite the interest traditionally focused on trypanosomatids redox metabolism, attenuation in *T. cruzi* infective viability by TDZ cannot be conceived as a

restricted response to TR inhibition (Rivarola; Paglini-Oliva, 2002; Lo Presti *et al*., 2015; Mendonça *et al*., 2018). In this sense, there is evidence that TDZ also interacts with *T. cruzi* membrane and exerts anti- calmodulin action, favoring cytoplasmic organelles condensation, mitochondrial and kinetoplast disruption, compromising parasite viability and its infectious potential (Rivarola; Paglini-Oliva, 2002; Lo Presti *et al*., 2015).

As an integrated response, alterations in the activities ofMantioxidant enzymes had repercussions on ROS, lipid and protein oxidation levels in *T. cruzi*-infected cardiomyocytes (Gupta *et al*., 2009). Accordingly, ROS production and lipid oxidation were enhanced in *T. cruzi*-challenged cardiomyocytes pretreated with TDZ, but not with Bz compared to infected untreated cardiomyocytes. Apparently, this response seems to be potentially linked to antioxidant defenses attenuation in host cells, especially the glutathione enzymes. In the glutathione system, reduced glutathione (GSH, the thiol form) contributes to maintaining a protective reduced intracellular environment, whose preservation depends on glutathione enzymes (Hunter *et al*., 1992; Krauth-Siegel *et al*., 2007; Lo Presti *et al*., 2015). Thus, GPx, GR, GST have a critical relevance in scavenging free radicals and protecting cardiomyocytes against oxidative injury (Gupta *et al*., 2009; Sánchez-Villamil *et al*., 2020). In addition, we parallelly identified that cardiomyocytes infected with TDZ- and Bz-pretreated trypomastigotes exhibited reduced levels of ROS, oxidized proteins and lipids compared to infected untreated cardiomyocytes. There is evidence that *T. cruzi* infection destabilizes mitochondrial complexes I and III, generating a primary source of ROS that induce oxidative molecular damage and eventual death of parasitized cells (Wen; Garg 2004; 2008; Gupta 2009). Thus, it is consistent that drugs with antiparasitic potential are efficient in down-regulating cellular pro-oxidant markers by restricting the infection rate and parasite load (Novaes *et al*., 2016; Nogueira *et al.,* 2023).

From enzymatic assay, we confirmed previous evidence that TDZ was effective in inhibiting TR activity in *T. cruzi*, which did not occur in Bz treatment (Gutierrez-Correa *et al*., 2001; Rivarola; Paglini- Oliva, 2002; Lo Presti *et al*., 2015). However, TDZ also exhibited an undesirable promiscuous nature by interacting with and inhibiting total protein antioxidant activity in cardiomyocytes. These findings reinforce the proposition of a cross-interaction of TDZ with host cell antioxidant enzymes, increasing their susceptibility to *T. cruzi*-induced oxidative stress.

Interestingly, both Bz and TDZ treatments reduced *T. cruzi* resistance to pro-oxidant H_2O_2 challenge in a dose-dependent manner. Notably, this response is

potentially related to the ability of these drugs in attenuating *T. cruzi* antioxidant defenses by complexing dialdehyde glyoxal with thiol-rich effectors following Bz treatment and irreversible TR inhibition by TDZ (Gutierrez- Correa et al., 2001; Trochine et al., 2014; Caldas et al., 2019). In parallel, TDZ treatment also decreased cardiomyocytes resistance to H_2O_2, which did not occur in Bz-exposed cells. This finding indicates that TDZ weakens cardiomyocytes antioxidant machinery. Accordingly, this condition can trigger and/or aggravate redox instability, which is known to favor *T. cruzi* replication during acute infection (Wen; Garg 2004; 2008; Gupta *et al*., 2009; Paiva *et al*., 2018).

In line with the *in vitro* results, our *in vivo* findings indicated a protective effect of Bz against heart parasitism (e.g., parasite load) and morphological damage in *T. cruzi*-infected animals. However, TDZ treatment potentiated heart parasitism andoverall cardiac pathological remodeling, increasing myocarditis and cTnI circulating levels, a classical marker of microstructural cardiomyocytes damage (Hammarsten *et al*., 2022; Nogueira *et al*., 2023). There is no doubt that CC is the most severe condition of *T. cruzi* infection, which is primarily determined by parasite tropism and pathogenicity in muscle tissues (Rodriguez *et al*., 2014; Queiroga *et al*., 2021). Accordingly, cardiomyocytes are primary targets of infection, suffering direct and indirect injuries caused by cell lysis associated with continuous *T. cruzi* invasion and intracellular replication, as well as oxidative aggressions coupled with the parasite- induced cardiac inflammatory response (Gupta *et al*., 2019; Souza-Silva *et al*., 2020).

Interestingly, microstructural cardiac damage was accompanied by elevated ROS, lipids and oxidized proteins levels in all infected groups, especially in TDZ-treated animals. These findings recapitulate Chagas myocarditis pathophysiology, reinforcing that parasitism and oxidative stress intensity are closely related to the severity of inflammation and acute cardiac injury in Chagas disease (Novaes *et al*., 2016; Wesley *et al*., 2019). In this context, TDZ pro-inflammatory potential reveals an antiparasitic disadvantage of this drug compared to Bz. Accordingly, in addition to direct trypanocidal activity, Bz also has a recognized anti-inflammatory effect, which is mediated by NF-κB pathway inhibition and IL- 10/STAT3/SOCS3 axis activation in cardiomyocytes (Ronco *et al*., 2011; Cevey *et al.,* 2019).

Considering that exacerbated pro-inflammatory and pro-oxidant reactions are directly involved in the deterioration of cardiac structure and function in ChD, combined trypanocidal and anti-inflammatory properties are highly relevant and desirable in anti-

T. cruzi drugs (Santos *et al*., 2019; Mendonça *et al*., 2019). Conversely, the antiparasitic benefits may be attenuated when trypanocidal drugs have concurrent pro-inflammatory and pro- oxidant properties, as they may aggravate cardiac lesions by acting as a pharmacological risk factor (Mendonça *et al*., 2020a,b).

As expected in acute *T. cruzi* infection, our findings indicated that redox imbalance was accompanied by antioxidant enzymes upregulation (Novaes *et al*., 2015, 2018; Santos *et al*., 2019). However, this counter-regulatory response was not sufficient to counteract ROS and cardiac oxidative damage, reinforcing the evidence that excessive ROS production can overcome host antioxidant defenses in ChD (Gupta *et al*., 2009; Novaes *et al*., 2015, Santos *et al*., 2019). Although mitochondrial dysfunction in infected cardiomyocytes is the primary source of *in vitro* pro-oxidant effectors, cardiac recruitment of immune cells provides an additional source of ROS *in vivo*, which is associated with the respiratory burst in activated leukocytes (Wen; Garg 2004; 2008; Gupta *et al*., 2009). Accordingly, theantioxidant system is even more demanding *in vivo*, which may be related to the enzymatic exhaustion/depletion observed throughout Chagas heart disease progression (Gupta *et al*., 2009; Sánchez-Villamil *et al*., 2020). Although TDZ did not interfere with the CAT and SOD cardiac activity, all glutathione-based enzymes were down-regulated in animals treated with this drug. Thus, it is assumed that cardiac oxidative damage can be partially attributed to the combination of antioxidant defenses inhibition and increased ROS production in a pro-inflammatory setting (Gupta *et al*., 2009; Wen; Garg, 2008; Sánchez-Villamil *et al*., 2020). Especially considering that parasitism and myocarditis were aggravated by TDZ treatment. On the other hand, there is no doubt that Bz-induced cardioprotective effects were associated with a combination of better parasitic, inflammatory and oxidative control (Novaes *et al*., 2016, 2018; Gonçalves-Santos *et al*., 2023). In this sense, antioxidant enzymes down-regulation represents a consequence naturally associated with the trypanocidal and anti-inflammatory properties of Bz. Therefore, better parasitological control attenuates antigenic load- dependent immune activation, tissue inflammatory reaction (e.g., leukocyte recruitment and activation) and ROS production; resulting in less activation of the cardiac antioxidant system (Santos *et al.,* 2015; Novaes *et al*., 2015, 2016; 2018; Santos *et al*., 2019; Sánchez- Villamil *et al*., 2020). Taken together, our findings indicate that due to the inability in controlling events centrally associated with ChD pathogenesis, TDZ may act as a risk factor for acute Chagas heart disease. Guided by Bz-based reference chemotherapy, this undesirable

response to TDZ treatment is partially related to poor pharmacological selectivity, marked cytotoxicity, and redox imbalance upregulation in cardiomyocytes and in the heart. Accordingly, these structures develop prominent enzymatic antioxidant fragility and greatersusceptibility to cell parasitism, heart inflammation and oxidative damage triggered by*T. cruzi* infection when exposed to TDZ. Furthermore, the present study suggests that TDZ can simultaneously interact with different enzymatic antioxidant targets in cardiomyocytes and *T. cruzi*, determining a heterogeneous infection profile when cells and parasites are differentially exposed to this drug. Considering that cardioprotective effects were previously attributed to TDZ treatment, it is not possible to disregard the influence of different *T. cruzi* strains of on therapeutic response (Lo Presti *et al*., 2015) . In this sense, genetic and metabolic variability of previously investigated strains (e.g., Tulahuen and SGO-Z12) determine different infectivity, virulence, tropism, and pathogenicity profiles; which trigger pathological manifestations predominantly associated with hepatosplenic parasitism (Rivarola; Paglini-Oliva, 2002; Zingales et al., 2012; Lo Presti *et al*., 2015; Erdmann et al.,2016). Despite these divergences, there is consensus on the potent TDZ effectagainst *T. cruzi in vitro*. Thus, this drug may be relevant as a structural platform for the synthesis of more selective antiparasitic and cardioprotective molecules, whose therapeutic efficacy and safety must be determined from further *in vitro* and *in vivo* mechanistic studies.

5 FINAL CONSIDERATIONS

Findings indicate that TDZ simultaneously interact with enzymatic antioxidant targets in cardiomyocytes and *T. cruzi*, potentiating the infection by inducing antioxidant fragility and increasing cardiomyocytes and heart susceptibility to parasitism, inflammation and oxidative damage.

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