Parasitology

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Could pre-infection exercise training improve the efficacy of specific antiparasitic chemotherapy for Chagas disease?

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Research Article

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Abstract

Considering a potential exercise-drug interaction, we investigated whether exercise training could improve the efficacy of specific antiparasitic chemotherapy in a rodent model of Chagas disease. Wistar rats were randomized into five groups: sedentary and uninfected (CT); sedentary and infected (SI); sedentary, infected and treated (SIT); trained and infected (TI); trained, infected and treated (TIT). After 9-weeks running training, the animals were infected with *T. cruzi* and followed up for 4 weeks, receiving 100 mg kg^{-1} day^{-1} benznidazole. No evidence of myocarditis was observed in CT animals. TI animals exhibited reduced parasitemia, myocarditis, and reactive tissue damage compared to SI animals, in addition to increased IFN-γ, IL-4, IL-10, heart non-protein antioxidant (NPA) levels and glutathione-s transferase activity ($P < 0.05$). The CT, SIT and TIT groups presented similar reductions in parasitemia, cytokines (IFN-γ, TNF-α, IL-4, IL-10, IL-17 and MCP-1), inflammatory infiltrate, oxidative heart damage and antioxidant enzymes activity compared to SI and TI animals, as well as reduced heart microstructural remodeling ($P < 0.05$). By modulating heart inflammation and redox metabolism, exercise training exerts a protective effect against *T. cruzi* infection in rats. However, the antiparasitic and cardioprotective effects of benznidazole chemotherapy are more pronounced, determining similar endpoints in sedentary and trained *T. cruzi*-infected rats.

Introduction

Chagas disease is a neglected anthropozoonosis caused by the protozoan parasite *Trypanosoma cruzi* (Pérez-Molina and Molina, 2018). It is estimated that at least 8 million people are currently infected by *T. cruzi* worldwide, and symptomatic clinical forms of Chagas disease are responsible for around 10 000 deaths every year (WHO, 2019). This disease is endemic in South and Central America, where oral and vector (triatomine insects) transmission routes are the main routes of contamination (Nogueira *et al.*, 2018; Pérez-Molina and Molina, 2018). However, there are increasing cases of infection in non-endemic areas, mainly due to the migration of infected individuals, congenital transmission (mother to fetus), and iatrogenic events related to laboratory accidents, blood transfusion and transplantation of infected organs (Bern, 2015; WHO, 2019). Recent estimates suggest at least 350 000 cases of infection in North America (Echeverria and Morillo, 2019) and 181 181 cases in European countries (Antinori *et al.*, 2017).

Chagas cardiomyopathy is the most severe and disabling manifestation of *T. cruzi* infection (Bern, 2015). It is associated with extensive inflammatory processes, oxidative damage, cardiomyocytolysis, necrosis, progressive heart fibrosis, electromechanical insufficiency, heart failure and death (Bern, 2015; Pérez-Molina and Molina, 2018). Chagas disease is the leading cause of nonischemic cardiomyopathy and the third highest indication for heart transplantation in Latin America (Mendonça *et al.*, 2018; Nogueira *et al.*, 2018). Chronic Chagas cardiomyopathy (CCC) is associated with a worse prognosis and 2.48-times greater risk of death than noninfectious cardiomyopathies (Freitas *et al.*, 2005; Nogueira *et al.*, 2018).

After more than four decades, benznidazole (Bz) is still the first-line drug for the etiological treatment of *T. cruzi* infection (Urbina, 2010; Nogueira *et al.*, 2018). However, this drug has high toxicity and low cure rates (10–20%) after the parasites have spread and established quiescent amastigote reservoirs in multiple tissues (Urbina, 2010; Mendonça *et al.*, 2018). As the prospect of new drugs for the treatment of *T. cruzi* infection is not promising, supporting drugs (e.g. antiarrhythmic, anti-inflammatory and antioxidant drugs) (Santos *et al.*, 2015; Novaes *et al.*, 2016*a*; Mendonça *et al.*, 2018) and non-pharmacological strategies (e.g. exercise training) (Novaes *et al.*, 2016*b*, 2017; Lucchetti *et al.*, 2017) have been proposed to increase host resistance against *T. cruzi*. Unlike cardiomyopathies with noninfectious etiologies, exercise training

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was contraindicated for decades for patients with Chagas disease, primarily because exercise could be limited by autonomic dysfunction, together with the belief that cardiovascular overload could potentiate cardiac deterioration (Gallo *et al.*, 1975; Bocchi, 2010). However, recent studies have shown that physical training can increase host resistance against *T. cruzi* infection (Schebeleski-Soares *et al.*, 2009; Novaes *et al.*, 2016*b*), a protective effect mainly attributed to the immunomodulatory and antioxidant mechanisms activated by exercise (Novaes *et al.*, 2016*b*; Lucchetti *et al.*, 2017). Accordingly, exercise was found to be effective in attenuating parasitemia, heart parasitism and microstructural damage in *T. cruzi*-infected rats, effects which are potentially linked to an improved Th1 immunological response and upregulation of antioxidant enzymatic defenses in trained animals (Novaes *et al.*, 2016*b*). In addition, clinical trials demonstrated that a 12-week training program was effective in reducing brain-derived neurotrophic factor levels, thereby improving autonomic modulation, oxygen consumption, exercise tolerance and quality of life in patients with CCC, with no significant adverse effects observed at 12 weeks follow-up (Lima *et al.*, 2010, 2013).

Although exercise training has emerged as a promising non-pharmacological strategy for the treatment of Chagas disease (Bocchi, 2010; Lima *et al.*, 2010; Novaes *et al.*, 2016*b*), it does not seem feasible to propose the replacement of specific chemotherapy by physical exercise programs, especially considering that there is no evidence of parasitological cure after physical training in animals or humans. On the other hand, by increasing host resistance to infection, exercise training may act as a complementary strategy to antitrypanosomal chemotherapy. Additive or synergic beneficial effects of exercise training and drug therapy have been identified in cardiovascular diseases (Lowenthal and Kendrick, 1985; Yoshizawa *et al.*, 2009; Ranjbar *et al.*, 2015); however, this combination remains unexplored in Chagas disease. Thus, we compared the isolated and combined effects of exercise training and Bz-based therapy on parasitism, inflammation and oxidative tissue damage in a murine model of *T. cruzi* infection.

Materials and methods

Experimental groups

Sixteen-week-old male Wistar rats were randomized into five groups containing 10 animals each: CT group, sedentary, uninfected and untreated controls; SI group, sedentary and infected; SIT group, sedentary, infected and treated with 100 mg kg^{-1} day^{-1} Bz; TI group, trained and infected; and TIT, trained, infected and treated with 100 mg kg^{-1} day^{-1} Bz. The sample size was calculated considering the principles described by Cruz-Orive and Weibel (1990) and Novaes *et al.* (2013). During the experiment, the animals were kept in a room with a controlled environment (12/12-h inverted dark/light cycle, humidity 60–70% and temperature 22 ± 2 °C). Water and rodent chow were provided *ad libitum*.

Exercise training and physical performance

The study design is shown in Fig. 1. A progressive running protocol until fatigue was administered to evaluate the initial (before exercise training) and final (after the 9-week treadmill training program) levels of physical performance of each animal (Novaes *et al.*, 2011). Lactate levels were determined in 5-μL peripheral blood samples collected by tail puncture every 3 min (Accutrend Lactate, Roche, Basel, Switzerland). The lactate threshold (LT) and total physical work were used as markers of physical performance. Workload (W; kg-m) was calculated using the equation W = body mass (kg) × TTF (min) × treadmill speed (m min^{-1}) × sine θ (treadmill incline), where TTF is the time until fatigue (Brooks

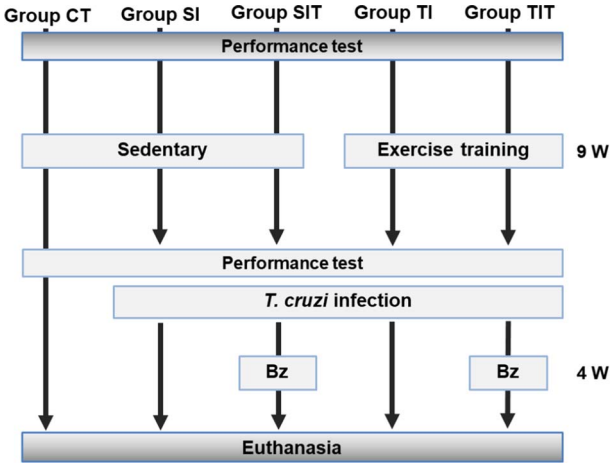

Fig. 1. Experimental groups and study design used to investigate the impact of *T. cruzi* infection in sedentary and trained rats sequentially treated or untreated with specific antiparasitic chemotherapy. Groups = CT, control sedentary, uninfected and untreated; SI, sedentary infected; TI, trained infected; SIT, sedentary infected treated with benznidazole; TIT, trained infected treated with benznidazole. 9 W: 9 weeks of treadmill running training, 4 W: 4 weeks of post-infection benznidazole(Bz)-based chemotherapy.

et al., 1984). The 9-week treadmill training program was initiated 24 h after this performance test (Novaes *et al.*, 2011, 2017).

Animals in the TI and TIT groups were trained using a motor-driven treadmill (Insight Instruments, Ribeirão Preto, Brazil). The running protocol was administered 5 days/week for 9 weeks, considering the results of LT. Thus, the exercise training was administered as follows: Week 1 and 2: 17 m min^{-1}, 0% grade for 15 min. Exercise duration was increased 5 min day^{-1} until 60 min/session by the end of week 2. Weeks 3 and 4: 17 m min^{-1}, 10% grade for 60 min. Week 5: 17 m min^{-1}, 10% grade for 8 min at (warm-up), followed by 45 min at 20 m min^{-1}, and 5 min at 17 m min^{-1} (warm-down). Weeks 6–9: 20 m min^{-1}, 10% grade for 8 min (warm-up), followed by 45 min at 23 m min^{-1}, and 5 min at 18 m min^{-1} (warm-down) (Novaes *et al.*, 2016*b*). From the lactate levels quantified at the end of each weekly exercise session, the animals were exercised at 65–75% (weeks 1 and 2), 75–85% (weeks 3–5) and 85–95% (weeks 6–9) of the initial LT.

Infection and parasitological measures

After 9 weeks of training, the exercise was completely discontinued. Forty-eight hours after the last exercise training session, animals in the TI and TIT groups were intraperitoneally infected with *T. cruzi* Y strain (150 000 blood trypomastigotes/100 g body weight) (Novaes *et al.*, 2011). Parasitological parameters (prepatent period, patent period, mean and peak parasitemia) were determined according to the Brener (1962) protocol. After confirmation of infection, animals in the SIT and TIT groups received daily treatment with Bz (100 mg kg^{-1}, administered by gavage) for 30 days (LAFEPE, Pernambuco, Brazil). The Bz dose was based on the reference dose used in preclinical models of Chagas disease supported by the Drugs for Neglected Disease initiative (DNDi, Geneva, Switzerland) (Diniz *et al.*, 2018). Fourth-eight hours after the last treatment, the animals were anesthetized (150 mg kg^{-1} ketamine and 16 mg kg^{-1} xylazine) and euthanized by cardiac puncture. The hearts were removed and weighed. The relative heart mass (hepatosomatic index) was calculated as heart mass/body mass of each animal.

Hemoculture

The efficiency of exercise training and specific chemotherapy in controlling parasite recrudescence was evaluated by hemoculture.

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In this assay, 400 μL of blood was collected by cardiac puncture during euthanasia and divided equally into two tubes containing 3 mL of sterile LIT culture medium. The tubes were incubated for 90 days at 28 °C and examined monthly for parasite detection (Novaes *et al.*, 2016*a*).

Enzyme-linked immunosorbent assay for cytokines

The cytokine levels in cardiac muscle were measured by enzyme-linked immunosorbent assay (ELISA). Briefly, heart fragments (80 mg) were homogenized in sodium phosphate buffer (pH 7.2) and centrifuged at 3500 ***g*** for 10 min at 4 °C. The homogenate was collected and the concentration of TNF-α, IFN-γ, IL-4, IL-10, IL-17 and MCP-1 cytokines were determined according to the manufacturer's instructions (Promega, Madison, WI, USA). The reaction was revealed by a peroxidase-conjugated streptavidin method (Vector Lab., CA, USA) and a substrate containing 3,3′,5,5″-tetramethylbenzidine (Promega, WI, USA). The reactions were stopped with 50 μL hydrochloric acid (1 N) and read in a spectrophotometer at 450 nm. Cytokine levels were determined by comparing the optical densities (OD) obtained with a standard curve developed from recombinant cytokines (Novaes *et al.*, 2016*b*).

Heart histopathology

Heart fragments were fixed for 24 h in 10% formaldehyde. The fragments were dehydrated in ethanol, embedded in glycol methacrylate histological resin and cut into 3-μm thick sections using a rotary microtome (Leica Biosystems, Wetzlar, Germany). Five histological sections were collected in semi-series (one out of every 50) and stained with toluidine blue and basic fuchsine. Histological sections were analyzed using a bright field microscope (Axioscope A1, Carl Zeiss, Germany). Fifty microscopic fields (400× magnification) were randomly sampled, and a total myocardial area of 1.38×10^6 μm^2 was analyzed for each group (Novaes *et al.*, 2011).

The severity of heart inflammation was assessed by comparing the distribution of interstitial cells in the myocardium for all groups (Novaes *et al.*, 2013). Tissue cellularity was evaluated by bright field microscopy (40 × objective lens, 400 × magnification; Axioscope A1, Carl Zeiss, Germany) using a standardized test area (At = 25×10^3 μm^2) applied to 20 randomly sampled myocardial fields for each animal. A total tissue area of 25.0×10^5 μm^2 was analyzed for each group. Interstitial cellularity was analyzed using the image analysis software Image-Pro Plus® (version 4.5; Media Cybernetics Inc., Silver Spring, MA, USA). The results were expressed through a polygonal field diagram sectorized in domains according the intensity of myocardial cellularity: (−) minimum, (− − +) mild, (+ +) moderate, or (+ + +) intense cellularity/inflammatory infiltrate (Felizardo *et al.*, 2018).

Heart microstructural remodeling

Using the same histological images, a stereological method was applied to estimate the distribution of cardiomyocytes (parenchyma), connective tissue (stroma) and inflammatory cells. A test area (A_T) of 3.25×10^3 μm^2 with 100 test points (P_T) was applied for all histological images. The volume density of cardiomyocytes (Vv_{cmy}), connective tissue (Vv_{cnt}), and blood vessels (Vv_{bvs}) were estimated as $Vv = P/P_T$, where P is the number of test points on the structure of interest. The relationship between blood vessels and cardiomyocytes ($Vv[_{bvs}]/Vv[_{cmy}]$) was used as a morphological index of myocardium vascularization. The relationship between structural and functional heart compartments was estimated as $Vv[_{cnt}]/Vv[_{cmy}]$ (Novaes *et al.*, 2013). The number density (QA) of mononuclear (MN) and polymorphonuclear (PMN) interstitial cells per histological area was estimated as $QA = \Sigma Q/A_T$; where ΣQ is the number of MN or PMN in the microscopic focal plane, and A_T is the dimensions of the test area ($A_T = 8.56 \times 10^3$ μm^2). Volume density was estimated from 20 randomly sampled histological fields for each animal using a 100× objective lens (1000× magnification; Axioscope A1, Carl Zeiss, Germany) (Novaes *et al.*, 2013). All counts were performed using the corresponding image analysis software (AxionVision; Carl Zeiss, Germany).

Biochemical assays for nitric oxide, hydrogen peroxide, lipid and protein oxidation

The same heart homogenate used to quantify cytokines was used for the biochemical analysis of reactive stress. Hydrogen peroxide (H_2O_2) levels in the heart were analyzed using a 96-well colorimetric commercial kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MI, USA). This method is based on a chromogenic Fe^{3+}-xylenol orange reaction, in which a purple complex is formed when Fe^{2+} is oxidized to Fe^{3+} by peroxides present in the sample, generating a colorimetric (585 nm) result proportional to tissue H_2O_2 levels.

Nitric oxide was estimated from the nitrite/nitrate levels, which were determined using a 96-well colorimetric commercial kit according to the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA, USA). This reaction converts nitrate to nitrite using the enzyme nitrate reductase. Nitrite is then detected at 540 nm as a colored product of the Griess reaction. The optical density obtained from the reaction was read using a microplate spectrophotometer (Anthos Zenyth 200, Biochrom, Cambridge, UK).

For analysis of lipid peroxidation, heart homogenate was reacted with thiobarbituric acid solution (15% trichloroacetic acid, 0.375% thiobarbituric acid and 0.25 N HCl) for 15 min. Heart levels of malondialdehyde were monitored in a spectrophotometer at 535 nm, as described previously (Gutteridge and Halliwel, 1990). Protein carbonyl (PCN) was measured in heart pellets by adding 0.5 mL of dinitrophenylhydrazine (DNPH, 10 mM). The reaction involved the formation of a 2,4-dinitrophenyl (DNP) hydrazone product from a derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine. The optical density was measured in spectrophotometer at 370 nm (Levine *et al.*, 1990).

Assays for non-protein and enzymatic antioxidant defenses

Non-protein antioxidant defenses in the heart homogenate were analyzed using a total antioxidant capacity colorimetric kit, according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MI, USA). This method is based on the inhibition of endogenous antioxidant enzymes and blocking Cu^{2+} oxidation by small non-protein antioxidant molecules, which were detected in a spectrophotometer at 570 nm. The antioxidant capacity was estimated from a standard curve, using trolox as the antioxidant reference (Novaes *et al.*, 2018).

The activity of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) was analyzed by spectrophotometry in heart samples. Samples were prepared by homogenizing heart tissue in ice-cold sodium phosphate buffer (pH 7.0), which were then centrifuged at 3500 ***g*** for 15 min at 5 °C. SOD activity was analyzed using the xanthine oxidase method, which is based on the reduction of nitroblue tetrazolium (NBT) and the production of H_2O_2 (Sarban *et al.*, 2005). CAT activity was measured by a kinetic method based on H_2O_2 decomposition, where the velocity is proportional to enzyme activity in tissue samples (Aebi, 1984). GST activity was

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determined using a method based on the NADPH oxidation rate (Habig *et al.*, 1974). The results were normalized by protein levels measured using the Bradford method (Bradford, 1976).

Statistical analysis

Data are presented as the mean and standard deviation (mean ± S.D.) or median and interquartile range. Data distribution was verified by the Kolmogorov-Smirnov test. Parametric data were compared using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls *post hoc* test. Non-parametric data were compared using the Kruskal–Wallis test. A probability of $P < 0.05$ was considered statistically significant.

Results

As indicated in Fig. 2, trained animals (TI and TIT) exhibited improved physical performance, which was evidenced by a prolonged lactate threshold and total physical work compared to all sedentary animals ($P < 0.05$).

As expected, no evidence of infection was observed in CT animals. All infected groups exhibited a similar prepatent period, but TI rats presented reductions in the patent period and peak and mean parasitemia compared to SI animals ($P < 0.05$). These parameters were similar in the SIT and TIT groups, but were reduced when compared to the SI and TI groups ($P < 0.05$). Trypomastigotes were detected by hemoculture in all SI and TI animals (100%), while SIT and TIT rats presented 50% and 60% positive hemocultures, respectively (Table 1).

Hepatosomatic index was similar in all groups (CT = 3.67 ± 0.44, SI = 3.71 ± 0.58, TI = 3.80 ± 0.65, SIT = 3.64 ± 0.49, TIT = 3.70 ± 0.55 mg g^{-1}) ($P > 0.05$). All cytokines investigated (INF-γ, TNF-α, IL-10, IL-4, IL-17, and MCP-1) were present at increased levels in the hearts of animals in the SI and TI groups compared to the CT, SIT and TIT groups ($P < 0.05$), which showed similar results between them ($P > 0.05$). The levels of INF-γ, IL-4 and IL-10 were increased ($P < 0.05$) and MCP-1 was reduced ($P < 0.05$) in TI compared to SI animals (Fig. 3).

Both SI and TI animals presented myocardial connective tissue expansion, tissue necrosis and myocarditis, together with evident pericellular infiltration of polymorphonuclear and mainly mononuclear leucocytes. CT, SIT and TIT rats presented a similar and more organized heart microstructure, with the cardiomyocytes surrounded by scarce connective tissue and low interstitial cellularity. Necrosis and inflammatory foci were not observed in these groups (Fig. 4).

The mapping of myocardial cellularity indicated elevated inflammatory infiltrate in TI and especially SI animals (Fig. 5A). These findings are supported by the quantitative analyses, which show a higher number of MN and PMN cells in the SI group compared to the other groups ($P < 0.05$). The number of MN cells was reduced in CT, SIT and TIT compared to TI ($P < 0.05$). The number of MN and PMN cells in the CT, TI, SIT and TIT groups were similar ($P > 0.05$; Fig. 5B).

Stereological analysis demonstrated that SI and TI groups presented more extensive heart microstructural remodeling than SIT and TIT animals, which were similar to CT rats in all parameters analyzed ($P < 0.05$; Fig. 6). Rats in the SI and TI groups presented reduced parenchyma distribution and an increased proportion of connective tissue, parenchyma/stroma ratio and total tissue cellularity compared to CT, SIT and TIT animals ($P < 0.05$). Blood vessel distribution and myocardial vascularization index were similar in all groups ($P > 0.05$; Fig. 6).

Levels of H_2O_2, NO and MDA were significantly higher in the hearts of SI animals compared to the other groups ($P < 0.05$). SI and TI animals presented similar heart PCN levels ($P > 0.05$).

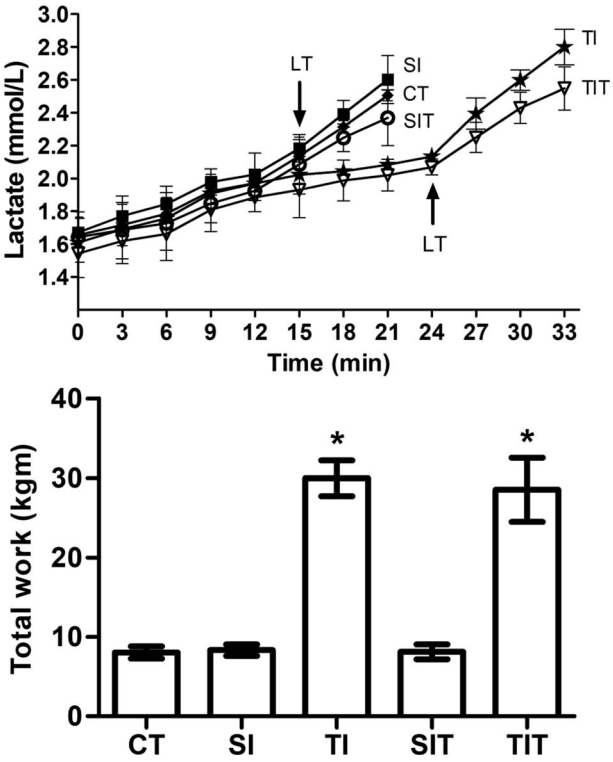

Fig. 2. Lactate threshold (LT) and total physical work until fatigue in sedentary and trained rats. CT, control sedentary, uninfected and untreated; SI, sedentary infected; TI, trained infected; SIT, sedentary infected treated with benznidazole; TIT, trained infected treated with benznidazole. Data are expressed as mean and standard deviation (mean ± S.D.). *Statistical differences ($P < 0.05$) compared to *CT, SI and TI.

These parameters were similar in CT, SIT and TIT animals ($P > 0.05$), but were reduced when compared to the SI and TI groups ($P < 0.05$; Fig. 7).

Non-protein antioxidant (NPA) levels were higher in CT animals compared to the other groups ($P < 0.05$). These molecules were reduced in TI and especially in SI animals compared to the SIT and TIT groups ($P < 0.05$), which showed similar levels ($P > 0.05$). Heart NPA levels and GST activity were higher in TI than SI animals ($P > 0.05$). Animals in the CT, SIT and TIT groups showed similar CAT, GST and SOD activities ($P > 0.05$), but these activities were reduced when compared to SI and TI rats ($P < 0.05$; Fig. 8).

Discussion

In this study, the impact of sequential administration of non-pharmacological and pharmacological strategies in a rodent model of *T. cruzi* infection was investigated for the first time. When administered alone, pre-infection exercise training was effective for improving physical performance and attenuating the patent period, peak and mean parasitemia, heart inflammation, oxidative stress and pathological myocardial remodeling. These effects were similar but even more pronounced when Bz-based chemotherapy was administered alone or after pre-infection exercise training. Pre-infection exercise training-induced metabolic adaptations had no impact on parasite persistence in the peripheral blood, which was markedly reduced in animals receiving Bz alone or after exercise training.

Although a beneficial exercise-drug interaction is an objective reality in metabolic and cardiovascular diseases (Lenz *et al.*, 2004; Quindry and Franklin, 2018), it remains unclear whether synergistic or additive effects between these therapeutic modalities exist in Chagas disease. Consistent with our findings, the

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Table 1. Parasitological parameters in sedentary and trained rats infected with *Trypanosoma cruzi*, sequentially treated or untreated with specific antiparasitic chemotherapy

	Prepatent period (days)	Patent period (days)	Peak of parasitemia (parasites/0.1 mL × 10^3)	Mean parasitemia (parasites/0.1 mL × 10^3)	Positive blood culture (%)
CT	ND	ND	ND	ND	0
SI	4.1 ± 0.31	11.4 ± 1.08	39.45 ± 3.95	6.34 ± 1.20	100
TI	4.9 ± 0.74	8.2 ± 1.14*	19.66 ± 1.50*	3.93 ± 0.99*	100
SIT	4.3 ± 0.48	4.3 ± 0.95†	7.91 ± 1.10†	0.87 ± 0.23†	50
TIT	4.7 ± 0.68	4.6 ± 0.97†	8.28 ± 1.06†	1.00 ± 0.21†	60

CT, control sedentary, uninfected and untreated; SI, sedentary infected; TI, trained infected; SIT, sedentary infected treated with benznidazole; TIT, trained infected treated with benznidazole. Data are expressed as mean and standard deviation (mean ± S.D.).
*†Statistical differences ($P < 0.05$) compared to *SI, †SI and TI.

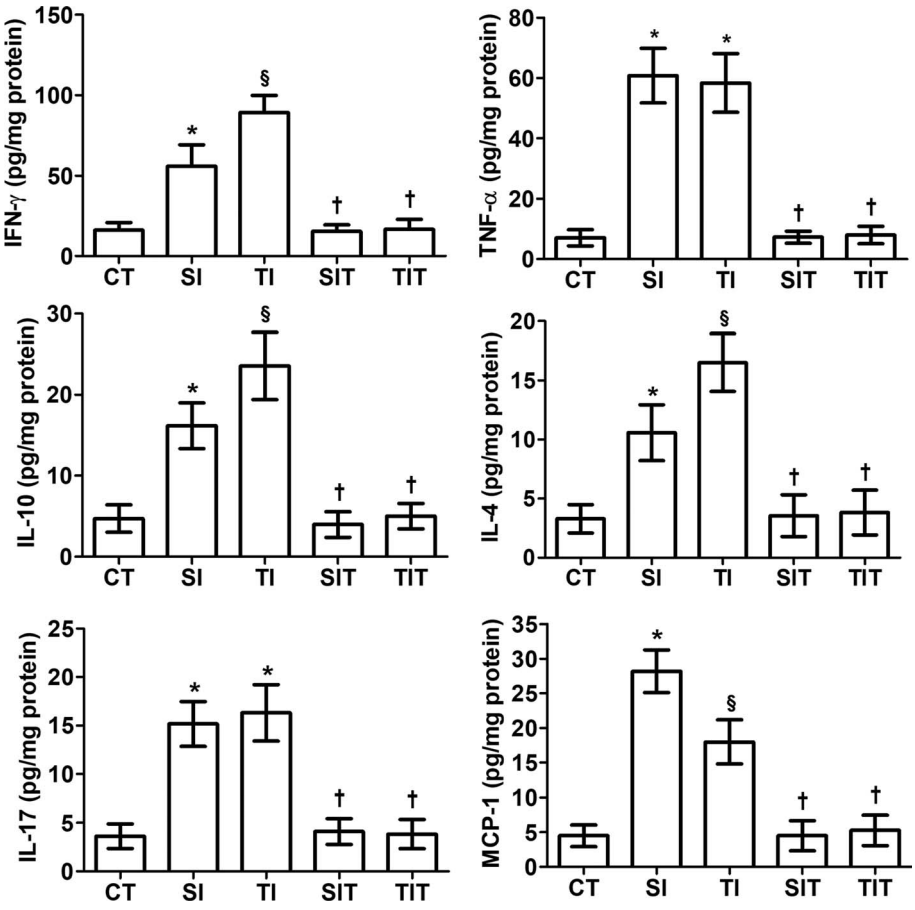

Fig. 3. Heart cytokine levels in sedentary and trained rats infected with *Trypanosoma cruzi*, sequentially treated or untreated with specific antiparasitic chemotherapy. CT, control sedentary, uninfected and untreated; SI, sedentary infected; SIT, sedentary infected treated with benznidazole; TI, trained infected; TIT, trained infected treated with benznidazole. Data are expressed as mean and standard deviation (mean ± S.D.). *§†Statistical difference ($P < 0.05$) compared to *CT, §CT and SI, †SI and TI.

antiparasitic and cardioprotective effects induced by exercise training have been observed in previous studies using different protocols of physical training in preclinical models of Chagas disease (Novaes *et al.*, 2016*a*, 2016*b*; Lucchetti *et al.*, 2017). In contrast to our hypothesis, exercise-induced metabolic adaptations and subsequent treatment with Bz did not interact to potentiate host resistance against *T. cruzi* infection. Animals treated with Bz alone or exercise plus Bz displayed similar beneficial results, therefore, it is clear that the high efficacy of the etiological treatment exceeded the effect of effect of physical conditioning when exercise training was administered alone.

As expected, the therapeutic efficacy of Bz in reducing heart inflammation was undeniably higher than exercise-induced metabolic adaptations. The toxic effect of Bz against *T. cruzi* is broadly recognized, and is triggered by dialdehyde glyoxal produced from Bz enzymatic activation by the parasite trypanosomal type I nitroreductases (NTRs) (Wilkinson and Kelly, 2009). This highly reactive product forms molecular complexes with guanosine and thiols, inhibiting antioxidant defenses, DNA and protein biosynthesis in *T. cruzi* (Wilkinson and Kelly, 2009; Hall and Wilkinson, 2012). In addition to controlling the parasitic load (Santos *et al.*, 2015; Caldas *et al.*, 2019), Bz exerts direct anti-inflammatory effects by inhibiting NF_KB signaling and modulating cytokine biosynthesis (Manarin *et al.*, 2010; Campos-Estrada *et al.*, 2015). Thus, by attenuating cardiomyocyte parasitism and the intensity of infectious myocarditis, Bz confers potent cardioprotective effects in Chagas disease (Caldas *et al.*, 2019; Camara *et al.*, 2019). As no additional anti-inflammatory effect was observed when Bz was administered in addition to exercise, the etiological treatment was responsible for modulating the host immune response against *T. cruzi*.

The effect of exercise in inducing immunological, biochemical and morphological beneficial adaptations cannot be completely disregarded. Exercise increased IFN-γ and TNF-α production

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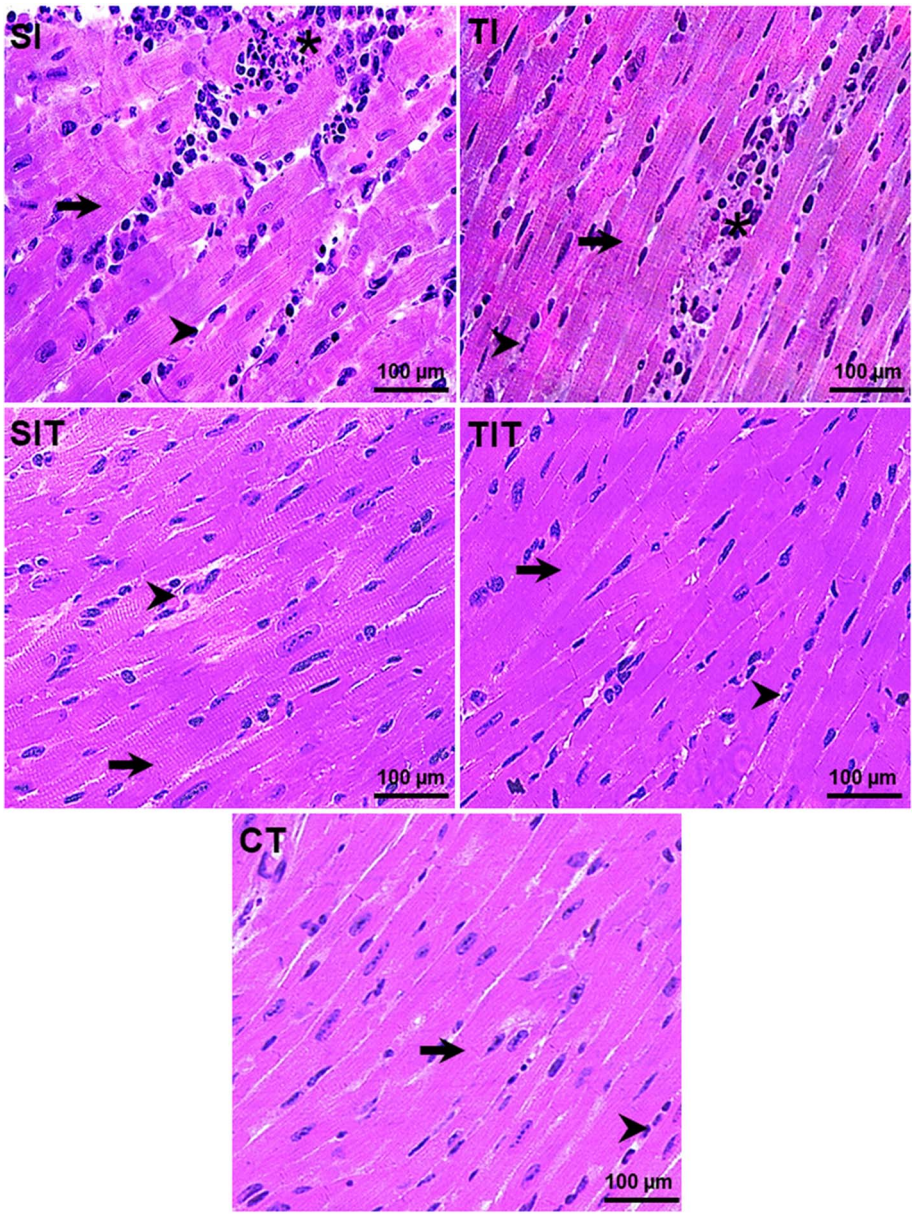

Fig. 4. Heart microstructure in sedentary and trained rats infected with *Trypanosoma cruzi*, sequentially treated or untreated with specific antiparasitic chemotherapy. CT, control sedentary, uninfected and untreated; SI, sedentary infected; SIT, sedentary infected treated with benznidazole; TI, trained infected; TIT, trained infected treated with benznidazole. Arrows: cardiomyocytes (parenchyma); Arrowheads, connective tissue (stroma); Asterisk, necrotic tissue with intense inflammatory infiltrate.

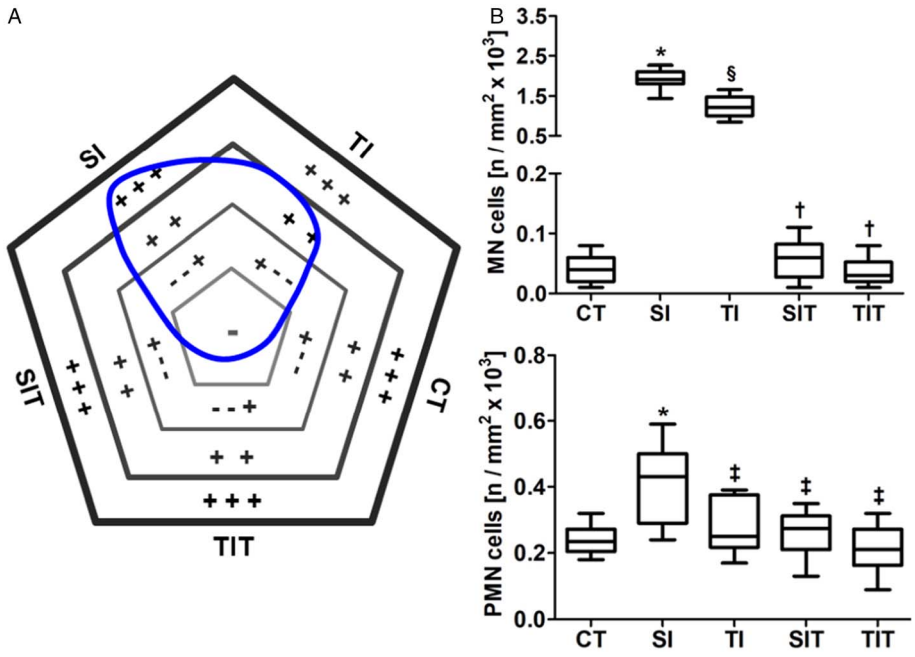

Fig. 5. Heart inflammation in sedentary and trained rats infected with *Trypanosoma cruzi*, sequentially treated or untreated with specific antiparasitic chemotherapy. CT, control sedentary, uninfected and untreated; SI, sedentary infected; SIT, sedentary infected treated with benznidazole; TI, trained infected; TIT, trained infected treated with benznidazole. In the polygonal field diagram (A), the symbols indicate (–) normal, (– – +) mild, (+ +) moderate or (+ + +) intense inflammatory infiltrate. The area delimited by the blue line indicates the status of tissue cellularity determined by all groups, and the line direction indicates the influence of each group in this status. (B) Myocardial distribution of mononuclear (MN) and polymorphonuclear (PMN) interstitial cells are represented in the graphics. Data are expressed as median and interquartile interval. *§†‡Statistical difference ($P < 0.05$) compared to *CT, ‡SI, §CT and SI, †SI and TI.

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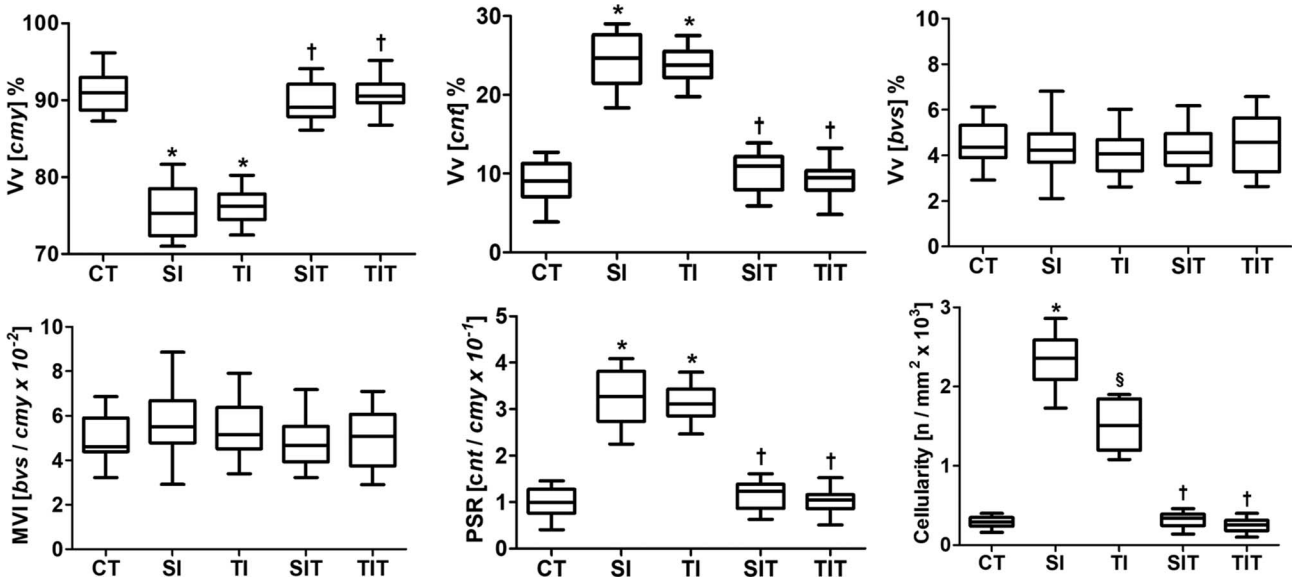

Fig. 6. Microstructural quantitative parameters of the cardiac tissue from sedentary and trained rats infected with *Trypanosoma cruzi*, sequentially treated or untreated with specific antiparasitic chemotherapy. CT, control sedentary, uninfected and untreated; SI, sedentary infected; SIT, sedentary infected treated with benznidazole; TI, trained infected; TIT, trained infected treated with benznidazole. Vv, Volume density; Cmy, Cardiomyocytes; Cnt, Connective tissue; Bvs, Blood vessels; MVI, myocardial vascularization index; PSR, Parenchyma/stroma ratio. Data are expressed as median and interquartile interval. *§†Statistical difference ($P < 0.05$) compared to *CT, §CT and SI, †SI and TI.

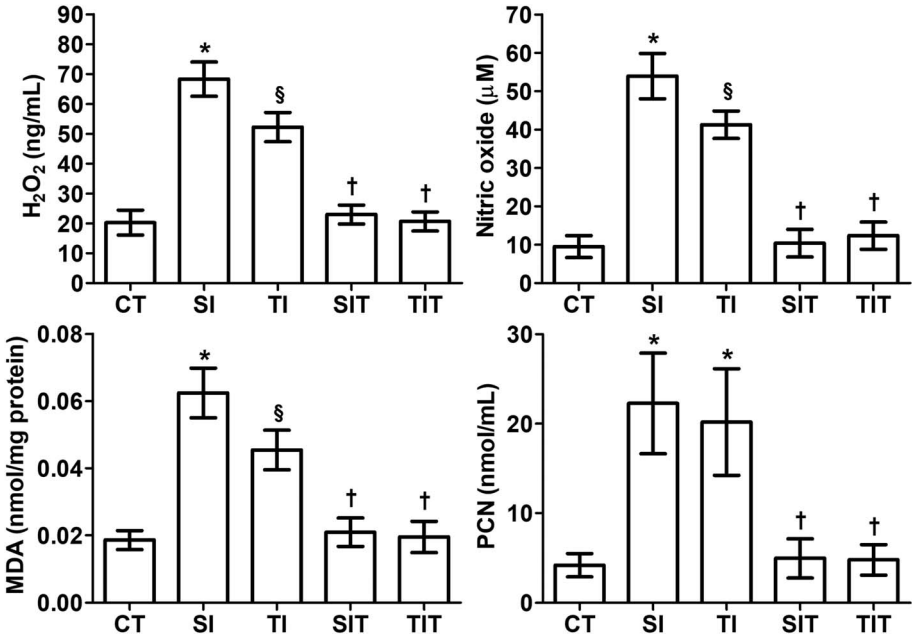

Fig. 7. Reactive species and markers of lipid and protein oxidation in hearts from sedentary and trained rats infected with *Trypanosoma cruzi*, sequentially treated or untreated with specific antiparasitic chemotherapy. H_2O_2, Oxygen peroxide; MDA, Malondialdehyde; PCN, Protein carbonyl. CT, control sedentary, uninfected and untreated; SI, sedentary infected; SIT, sedentary infected treated with benznidazole; TI, trained infected; TIT, trained infected treated with benznidazole. Data are expressed as mean and standard deviation (mean ± S.D.). *§†Statistical difference ($P < 0.05$) compared to *CT, §CT and SI, †SI and TI.

and reduced MCP-1 levels in the heart, all of which are typical Th1 cytokines that represent an important line of defense against *T. cruzi* (Santos *et al.*, 2015; Novaes *et al.*, 2016*b*). By improving leukocyte function and the balance between Th1 and Th2 antagonistic phenotypes, exercise-induced metabolic adaptations reinforce parasitic control and attenuates tissue damage and mortality in *T. cruzi*-infected hosts (Malm, 2004; Gleeson, 2007; Novaes *et al.*, 2016*a*). While Th1 effectors such as IL-12, IFN-γ, TNF-α and MCP-1 exert protective effects, Th2 molecules such as IL-4, IL-10 and TGF-β attenuate NO biosynthesis and increase the host's susceptibility to *T. cruzi* infection (Teixeira *et al.*, 2002; De Andrade *et al.*, 2018). However, concurrent Th2 and T regulatory (Treg) activation are required to avoid Th1 hyperstimulation, which is harmful to the host as it induces exacerbated inflammatory processes, oxidative stress, cardiomyocytolysis, tissue necrosis and reactive fibrosis (Rassi *et al.*, 2010; Soares *et al.*, 2010; Teixeira *et al.*, 2011). Accordingly, the reduced heart MCP-1 levels also indicate a beneficial effect of exercise in attenuating heart inflammation, as the elevated production of MCP-1 is potentially associated with massive leucocyte recruitment and severe inflammatory damage to the heart (Adamopoulos *et al.*, 2002; Paiva *et al.*, 2009). The increased IL-4/IL-10 levels observed also corroborate the exercise-induced cardioprotective effects. This finding supports an improved immunological balance in trained animals, as these Th2/Treg cytokines act as anti-inflammatory mediators to control the intensity of the inflammatory process and the destructive potential of an exacerbated proinflammatory Th1 response directed against the parasite (Teixeira *et al.*, 2002; Savino *et al.*, 2007).

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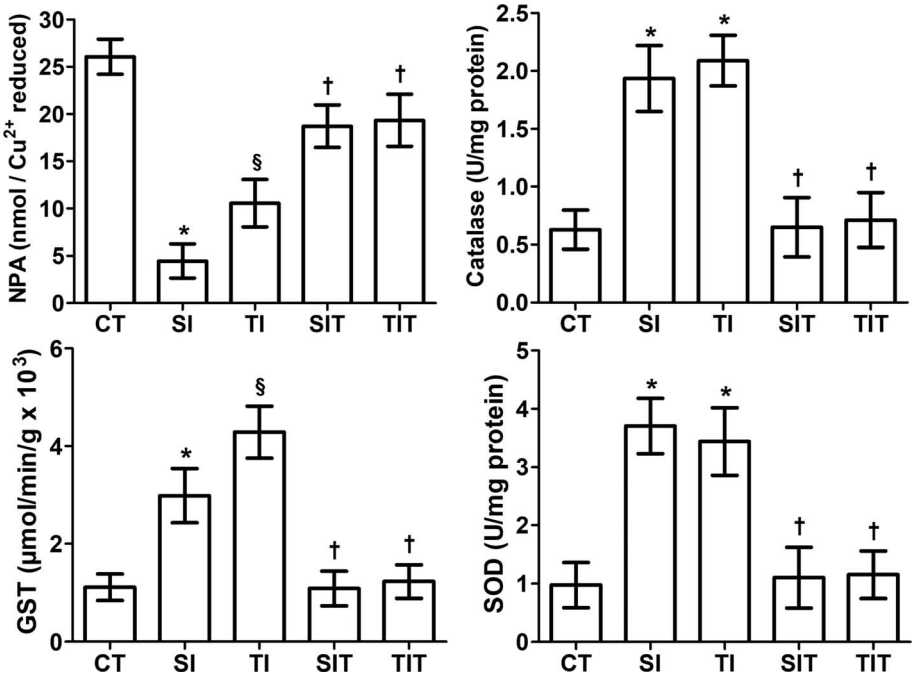

Fig. 8. Enzymatic and non-protein antioxidant defenses in hearts from sedentary and trained rats infected with *Trypanosoma cruzi*, sequentially treated or untreated with specific antiparasitic chemotherapy. NPA, Non-protein antioxidants; GST, Glutathione S-transferase; SOD, Superoxide dismutase. CT, control sedentary, uninfected and untreated; SI, sedentary infected; SIT, sedentary infected treated with benznidazole; TI, trained infected; TIT, trained infected treated with benznidazole. Data are expressed as mean and standard deviation (mean ± s.d.). *§†Statistical difference ($P < 0.05$) compared to *CT, §CT and SI, †SI and TI.

The reduction in heart inflammation observed in trained animals was not enough to prevent pathological myocardial remodeling when compared to sedentary animals. However, the cytokine profile, markedly lower MN and PMN cellularity, as well as microstructural remodeling were similar in both Bz-treated groups, independent of exercise training. There is evidence that the intensity of inflammatory infiltrate is closely correlated with morphological adaptations of the heart parenchyma and stroma (Novaes *et al.*, 2013, 2016*b*). These adaptations were clearly observed in this study, particularly with regard to the parenchyma/stroma ratio, which indicated similar cardiomyocyte loss in the TI and SI groups. As cardiomyocytes are a direct target of *T. cruzi* parasitism, massive parenchymal loss by direct cardiomyocytolysis and myonecrosis is often observed in acute Chagas disease (Manque *et al.*, 2011; Novaes *et al.*, 2016*a*, 2016*b*). At the same time, fibroblast activation and extensive stromal expansion occurs as a reactive myocardial response to parenchymal damage (Teixeira *et al.*, 2011; Machado *et al.*, 2012). In untreated infections, this process progresses quickly and may culminate in heart failure and host death (Manque *et al.*, 2011; Teixeira *et al.*, 2011). Although pathological cardiac remodeling was not prevented by exercise-induced metabolic adaptations, the potent parasitic and inflammatory control induced by Bz supports its cardioprotective effects (Novaes *et al.*, 2016*a*, 2017), evidenced by reduced tissue cellularity, preserved cardiomyocyte organization and parenchyma/stroma ratio.

The severity of heart inflammation and microstructural remodeling was consistent with our findings of reactive tissue damage in all groups investigated. Given the extensive heart inflammation observed in sedentary infected animals, the elevated production of reactive metabolites (i.e. H_2O_2 and NO) and extensive lipid peroxidation were not surprising (Santos *et al.*, 2015; Novaes *et al.*, 2017). The respiratory burst in activated leucocytes and mitochondrial dysfunction in parasitized cardiomyocytes are the main sources of reactive metabolites in *T. cruzi* infection, which contribute to accelerated cardiac deterioration (Gupta *et al.*, 2009*a*, 2009*b*; Machado *et al.*, 2013). As the upregulation of antioxidant defenses is a typical adaptive mechanism induced by exercise training (Novaes *et al.*, 2016*a*, 2017), reduced production of reactive metabolites was expected in trained rats compared to sedentary animals (Lucchetti *et al.*, 2017). However, the increased GST activity and NPA levels were unable to prevent lipid oxidation in the cardiac tissue of these animals. Conversely, H_2O_2, NO and MDA levels were similar and markedly lower in both Bz-treated groups independent of exercise training, an effect which is potentially related to the role of Bz in controlling heart parasitism and inflammation (Santos *et al.*, 2015; Camara *et al.*, 2019). As tissue parasitism, inflammation and reactive tissue damage are coupled processes that are directly related to heart morphofunctional damage and cardiomyopathy progression (Santos *et al.*, 2015; Novaes *et al.*, 2016*b*), controlling these processes is a primary goal in the treatment of Chagas disease (Rassi *et al.*, 2017; Caldas *et al.*, 2019).

Taken together, our findings indicate that pre-infection exercise training is potentially associated with exercise-induced metabolic adaptations that improves host resistance against *T. cruzi*. However, this non-pharmacological modality of treatment is not able to potentiate the antiparasitic and cardioprotective effects of Bz-based chemotherapy, which remains the most effective treatment for controlling the key pathological outcomes involved in the pathophysiology of Chagas cardiomyopathy. As the type and amplitude of exercise-induced metabolic adaptations are closely correlated to exercise characteristics, it is possible that different training protocols are effective to induce variable responses to *T. cruzi* infection, an issue poorly understood that requires further investigation.

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Conflict of interest. None to declare.

Ethical standards. The Institutional Ethics Committee for Animal Care approved the study (protocol 30/2009).

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