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Concomitant exercise training attenuates the cardioprotective effects of pharmacological therapy in a murine model of acute infectious myocarditis

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ABSTRACT

When administered alone, preinfection exercise training and benznidazole-based chemotherapy induce cardioprotection in Chagas disease. However, the effect of concomitant exercise and benznidazole treatment is unknown. We investigated whether exercise and specific chemotherapy could interact to modulate parasitemia, inflammation, redox status and heart damage in a murine model of *T. cruzi* infection. Wistar rats were randomized into an uninfected control group (CNT) and four groups infected with *T. cruzi*: sedentary untreated (SUN) and treated (STR), and trained untreated (TUN) and treated (TTR). Running training was administered 5 days/week for 4 weeks. Treated animals concomitantly received 100 mg/kg/day benznidazole. Heart inflammation and reactive damage were not detected in CNT animals. Compared to SUN, TUN animals presented increased levels of parasitemia, myocarditis, nitric oxide, hydrogen peroxide, protein carbonyl, malondialdehyde, cytokines (IFN-γ, TNF-α, IL-4, IL-6, IL-10 and IL-17), catalase, superoxide dismutase and glutathione reductase activity, as well as reduced heart non-protein antioxidant levels ($P < 0.05$). TTR animals exhibited higher levels of parasitemia, myocarditis, hydrogen peroxide, malondialdehyde, IFN-γ, TNF-α and IL-6 than STR animals ($P < 0.05$), which showed the lowest levels of all analyzed parameters compared to the other groups ($P < 0.05$). Our findings indicate that exercise aggravates acute infection. When concomitantly administered with benznidazole, exercise training impaired parasitic control and chemotherapy-induced cardioprotection in *T. cruzi*-infected rats. Considering that exercise training and *T. cruzi* infection constitute independent metabolic challenges, the negative effects of concomitant treatment are potentially related to the overlapping oxidative and immunoinflammatory demands of exercise and the infection itself.

1. Introduction

American trypanosomiasis or Chagas disease is a neglected tropical infection caused by the protozoan parasite *Trypanosoma cruzi*, which is endemic in Central and South America [1]. At least 8 million people are currently infected by *T. cruzi*, and 25 million people are at risk of infection worldwide, especially in areas with low socioeconomic development [2]. Chagas disease is responsible for > 10,000 deaths every year, primarily due to heart failure associated with chronic Chagas cardiomyopathy (CCC) [1,2]. At least 181,181 cases of Chagas disease were recently reported in Europe and 350,000 cases in North America [3,4]. However, the prevalence of Chagas disease in non-endemic areas may be much higher considering diagnostic limitations, underreporting of positive cases, and the increasing migratory flux of infected people [2,5].

The current etiological treatment for Chagas disease is based on benznidazole (Bz) and nifurtimox (NFx), two nitroheterocyclic drugs with high toxicity and limited efficacy in chronic infections [6–8]. About 30% of Chagasic patients develop CCC, the most severe and disabling manifestation of *T. cruzi* infection, which is associated with poor prognostic and a 2.48-times higher risk of death than non-infectious cardiomyopathies [5,9]. Complex and multifactorial processes are associated with the development of CCC, including parasite persistence, autonomic denervation, microvascular insufficiency, oxidative

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damage, cardiomyocytolysis, autoimmunity and progressive myocardial fibrosis [2,10]. Difficulties in controlling infection rates have made Chagas disease the leading cause of nonischemic cardiomyopathy and the third most common indication for heart transplantation in Latin America [5].

As a complementary strategy in the treatment of Chagasic patients, exercise training has emerged over the past decades with the aim to improve host resistance against *T. cruzi* infection [11–14]. Although the evidence for this intervention is limited, immunomodulatory effects and improvements in parasitological control, redox balance and cardiorespiratory function are among the exercise-induced cardioprotective effects observed in *T. cruzi*-infected animals and humans [11–13]. Considering that the immunological system is the first line of host defense against *T. cruzi*, and that exacerbated inflammatory processes lead to intense oxidative stress and heart damage, metabolic adaptations such as an improved Th1/Th2 immunological balance and upregulation of antioxidant defenses support the indication of exercise training for the treatment of Chagas disease [13,15].

Although exercise training may induce cardioprotective effects, there is no evidence of a parasitological cure. In this sense, the combination of exercise and antiparasitic chemotherapy may be a relevant strategy to achieve more comprehensive therapeutic outcomes when compared to the administration of these treatments in isolation. There is evidence that exercise training and drug therapy can interact to enhance their benefits in cardiovascular diseases [16–18]; however, the relevance of this combination for the treatment of *T. cruzi* infection has not yet been investigated. Thus, we compared the isolated and concomitant effects of exercise and benznidazole-based therapy on parasitological control, heart inflammation and oxidative heart damage in a murine model of Chagas disease.

2. Materials and methods

2.1. Experimental groups

Eight-week old male Wistar rats were randomized into five groups, each containing nine uninfected or *T. cruzi*-infected animals, as follows: CNT, control sedentary uninfected and untreated; SUN, infected sedentary untreated; STR, infected sedentary treated with 100 mg/kg benznidazole, TUN: infected trained untreated; TTR: infected trained treated with 100 mg/kg benznidazole. The experiments were conducted in an animal facility with a controlled environment (temperature 22 ± 2 °C, humidity 60–70%, 12/12 h dark/light cycle). The animals had free access to water and food. The Institutional Ethics Committee approved the study (protocol 30/2009).

2.2. Model of Trypanosoma cruzi infection

To induce *T. cruzi* infection, each animal was intraperitoneally inoculated with 300,000 trypomastigotes (Y strain)/50 g body mass [13]. Trypomastigotes were obtained from blood samples of mice previously infected exhibiting the peak of parasitemia [19]. Blood samples (5 μL) were collected by tail puncture, and infection was confirmed by the microscopic observation of blood trypomastigotes in inoculated animals [20,21].

2.3. Exercise training and concomitant benznidazole-based chemotherapy

After the confirmation of infection (day 5 post-inoculation), TUN and TRT animals were submitted to a running protocol on a motor-driven treadmill (Insight Instruments, Ribeirão Preto, Brazil) for 5 days/week for 4 weeks. Each exercise session was performed for 40 min at a 5% incline, following an adapted protocol [15]. The exercise intensity (running velocity) was determined as 80% of the lactate threshold for each animal, which was established using a standardized progressive running protocol until fatigue. Lactate levels were measured in 5-μL peripheral blood samples collected by tail puncture every 3 min during the performance test (Accutrend Lactate, Roche, Basel, Switzerland) [15].

In addition to exercise, TRT animals were concomitantly treated with 100 mg/kg/day benznidazole, administered by gavage (LAFEPE, Pernambuco, Brazil). The Bz dose was chosen considering the reference dose used in preclinical models of Chagas disease, as supported by the Drugs for Neglected Disease initiative (DNDi, Geneva, Switzerland) [22]. CNT, SUN and STR animals remained sedentary throughout the experimental period. While CNT and SUN animals received no treatment, STR rats were treated with the same chemotherapy protocol applied in the TRT group.

2.4. Patent period and parasitemia

After *T. cruzi* inoculation, the patent period, mean and peak parasitemia were determined following Brener's protocol [23]. Briefly, 5 μL of fresh blood was collected by tail puncture and distributed on to 22 × 22 mm glass slides. Parasitemia was determined by counting the number of trypomastigotes in 50 microscopic fields at 400× magnification with bright field microscopy. The patent period was established as the total time in which circulating trypomastigotes were microscopically observed from fresh blood examination [24].

2.5. Parasite detection by hemoculture

Twenty-four hours after the last treatment, animals were euthanized by cardiac puncture under anesthesia (150 mg/kg ketamine and 16 mg/kg xylazine, administered intraperitoneally). Blood and heart samples were collected and analyzed. Hemoculture was used to estimate the efficiency of exercise training and specific chemotherapy to control parasitemia recrudescence. In this method, 400 μL of blood was divided equally into two tubes containing 3 mL of sterile LIT culture medium. The tubes were incubated at 28 °C for 90 days, then microscopically examined every month for parasite detection [25].

2.6. Heart cytokine assays

Heart cytokine levels were determined by enzyme-linked immunosorbent assay (ELISA). Heart fragments were homogenized in ice-cold sodium phosphate buffer (pH 7.2) and centrifuged at 3500 ×*g* for 10 min at 4 °C. The homogenate was collected and used to determine the concentrations of the cytokines IFN-γ, TNF-α, IL-4, IL-10, IL-17 and CCL2/MCP1 according the manufacturer's instructions (Promega, Madison, WI, USA). The reactions were revealed using a peroxidase-conjugated streptavidin colorimetric method (Vector Lab., CA, USA) and a substrate based on 3,3′,5,5″-tetramethylbenzidine (Promega, WI, USA). The reactions were stopped with 50 μL of 1 N hydrochloric acid and read in a spectrophotometer at 450 nm. Cytokine levels were measured by comparing the optical densities obtained to a standard curve constructed from different concentrations of recombinant cytokines [13].

2.7. Heart processing and histopathology

Heart fragments were fixed for 48 h in 4% paraformaldehyde prepared in sodium phosphate buffer (pH 7.2). After dehydration in ethanol, the specimens were embedded in glycol methacrylate resin and cut into 3-μm thick sections using glass knives coupled to a rotary microtome (Leica Biosystems, Wetzlar, Germany). For each animal, six histological sections were collected in semi-series, using one out of every 50 sections to avoid reanalyzing the same tissue area. After toluidine blue and basic fuchsin staining, the heart histoarchitecture was analyzed by bright field microscopy [26].

Heart inflammation was estimated by comparing the myocardial cellularity of all groups [27], achieved by quantifying the number of

interstitial cells in a standardized test area (At = 25 × 10^3 μm^2). For each animal, cells were quantified in 30 random non-coincident microscopic fields obtained using a bright field microscope with a 40× objective lens (Axioscope A1, Carl Zeiss, Germany). A total tissue area of 6.75 × 10^6 μm^2 was analyzed for each group. Interstitial cellularity was analyzed using the image analysis software Image-Pro Plus (Media Cybernetics Inc., Silver Spring, MA, USA) [28].

2.8. Heart microstructural remodeling

The same histological images were analyzed using a stereological method to estimate the distribution of cardiomyocytes, connective tissue and blood vessels [29]. For this, a test system with 42 points (P_T) and a 3.25 × 10^3 μm^2 test area (A_T) was superimposed over all microscopic images. The volume density (Vv, %) of cardiomyocytes (cmy), connective tissue (cnt) and blood vessels (bvs) was estimated as $Vv_{cmy/cnt/bvs} = P / P_T$, where P is the number of test points on the structure of interest and P_T is the total number of test points. The ratio between heart stroma and parenchyma was estimated as $Vv[_{cnt}]/Vv[_{cmy}]$. The ratio between blood vessels and cardiomyocytes ($Vv[_{bvs}]/Vv[_{cmy}]$) was adopted as a structural index of heart microvascularization [27].

2.9. Nitric oxide and hydrogen peroxide assays

The same heart homogenate used to quantify cytokines was used for all biochemical analyses. Nitric oxide (NO) was estimated as heart nitrite/nitrate levels, which were measured with a microplate spectophotometer (Anthos Zenyth 200, Biochrom, Cambridge, UK) using a 96-well commercial kit according to the manufacturer's instructions (ThermoFisher Scientific, Waltham, MA, USA). This method is based on a reaction that converts nitrate to nitrite from the catalitic activity of the nitrate reductase enzyme. Once this conversion was complete, the nitrite was detected at 58 nm as a colored product produced by the Griess reaction. Hydrogen peroxide (H_2O_2) levels were analyzed in heart homogenate using a 96-well commercial colorimetric kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MI, USA). The 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 colored product whose optical density at 585 nm is proportional to tissue H_2O_2 levels.

2.10. Lipid and protein oxidation assays

Malondialdehyde (MDA) was used as a molecular indicator of lipid oxidation in cardiac tissue. For MDA quantification, heart homogenate was reacted with thiobarbituric acid solution (0.25 N HCl, thiobarbituric acid 0.375%, and trichloroacetic acid 15%) for 15 min at 25 °C. Heart levels of MDA were monitored on a spectrophotometer at 535 nm, as described previously [30]. After homogenate removal, pellets were used to analyse the heart protein carbonyl (PCN) level, a general marker of protein oxidation. With this method, 0.5 mL of 10 mM dinitrophenylhydrazine (DNPH) was added to the pellets. The detection of PCN involves a reaction that generates a 2,4-dinitrophenyl (DNP) hydrazone product from the derivatization of the carbonyl group with 2,4-dinitrophenylhydrazine. Cardiac PCN levels were measured spectrophotometrically (Anthos Zenyth 200, Biochrom, Cambridge, UK) at 370 nm [31].

2.11. Antioxidant enzyme assays

The activities of catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) were measured spectrophotometrically in heart samples that had been homogenized in ice-cold sodium phosphate buffer (pH 7.0) then centrifuged at 3500 ×g for 15 min at 5 °C. The catalase activity was monitored at 240 nm using a biochemical kinetic assay, in which the velocity of H_2O_2 decomposition is proportional to the enzyme activity in tissue samples [32]. The GR activity was estimated from the rate of NADPH oxidation, which was analyzed by spectrophotometry at 340 nm in the presence of oxidized glutathione [33]. This assay is based on the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) by GR, using NADPH as the substrate. Superoxide dismutase activity was monitored at 560 nm using a xanthine oxidase assay, in which superoxide radicals are generated by xanthine and xanthine oxidase, which react with 2-(4-iodophe-nyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye [34].

2.12. Assay for non-protein antioxidant defenses

Non-protein antioxidant defenses in the heart homogenate were quantified using a total antioxidant capacity colorimetric kit according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MI, USA). This assay is based on the inhibition of endogenous antioxidant enzymes and Cu^{2+} oxidation by small, soluble non-protein antioxidant molecules, which were detected by spectrophotometry at 570 nm. The antioxidant capacity was estimated from a standard curve using trolox as the antioxidant reference [35].

2.13. Statistical method

Data were reported as the mean and standard deviation (mean ± SD) or the median and interquartile range. The Kolmogorov-Smirnov test was applied to verify data distribution. Parametric data were analyzed with a one-way analysis of variance (ANOVA) followed by a Student-Newman-Keuls (SNK) post-hoc test. Kruskal-Wallis test was applied to compare non-parametric data. Statistical results with $P < 0.05$ were considered significant.

3. Results

The absence of infection was confirmed in all CNT animals. All untreated groups (SUN and TUN) presented an increased prepatent period, parasitemia peak and mean parasitemia compared to Bz-treated animals (STR and TTR; $P < 0.05$). These parameters were significantly higher in TUN than STR animals ($P < 0.05$). At the end of the study, trypomastigote forms of *T. cruzi* were detected in all SUN and TUN

Table 1
Parasitological parameters in sedentary and trained rats infected with *Trypanosoma cruzi*, untreated or concomitantly treated with benznidazole.

	Patent period (days)	Peak of parasitemia (parasites/0.1 mL × 10^3)	Mean parasitemia (parasites/0.1 mL × 10^3)	Positive blood culture (n/%)
CNT	ND	ND	ND	0/0
SUN	9.25 ± 1.04	9.18 ± 3.66	3.08 ± 0.70	9/100
TUN	20.00 ± 1.25*	17.60 ± 5.32*	8.26 ± 2.44*	9/100
STR	3.50 ± 0.93 †	1.08 ± 0.46†	0.32 ± 0.12†	5/55.56
TTR	4.25 ± 0.89†	3.50 ± 0.52‡	1.54 ± 0.28‡	7/77.78

ND: not detected, CNT: control sedentary uninfected untreated, SUN: infected sedentary untreated, STR: infected sedentary treated with 100 mg/kg/day benznidazole, TUN: infected trained untreated, TTR: infected trained treated with 100 mg/kg/day benznidazole. *†‡ Statistical difference ($P < 0.05$) compared to *SUN; †SUN and TUN; ‡ SUN, TUN and STR.

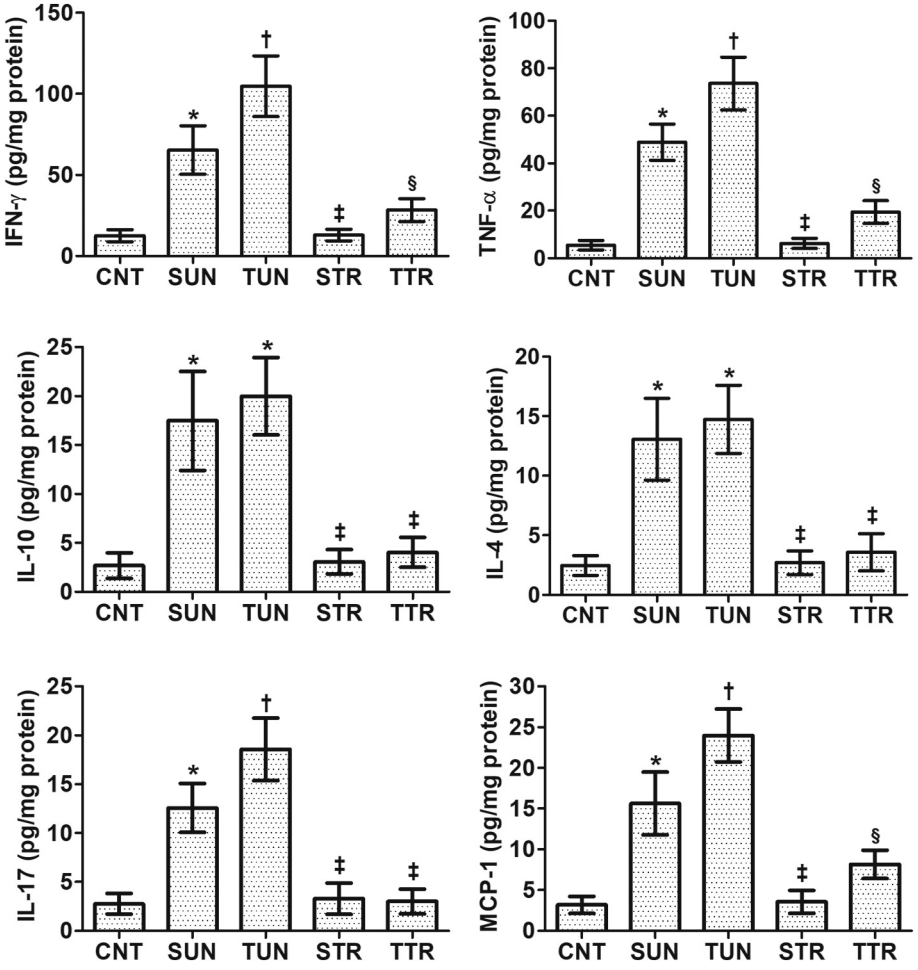

Fig. 1. Cytokine heart levels in sedentary and trained *Trypanosoma cruzi*-infected rats untreated or concomitantly treated with benznidazole. CNT: control sedentary uninfected untreated, SUN: infected sedentary untreated, STR: infected sedentary treated with 100 mg/kg/day benznidazole, TUN: infected trained untreated, TTR: infected trained treated with 100 mg/kg/day benznidazole. *†‡ Statistical difference ($P < 0.05$) compared to *SUN; †SUN and TUN; ‡ SUN, TUN and STR.

animals (100%) by hemoculture. STR and TTR animals presented 55.56% and 77.78% positive hemoculture tests, respectively (Table 1).

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 SUN and TUN animals compared to the STR, TTR and CNT groups ($P < 0.05$). The levels of INF-γ, TNF-α, IL-17 and MCP-1 were increased in TUN animals compared to SUN animals ($P < 0.05$). INF-γ, TNF-α and MCP-1 were increased in TTR compared to STR and CNT animals ($P < 0.05$; Fig. 1).

Control animals exhibited a preserved myocardial microstructure, with well-defined and organized cardiomyocytes, evident intercalated discs, scarce connective tissue and interstitial cellularity. On the other hand, SUN and especially TUN animals presented marked myocarditis associated with diffuse inflammatory infiltrate, tissue necrosis, extensive connective tissue expansion and disorganization of cardiomyocytes. STR animals exhibited a well-organized heart microstructure, with parallel cardiomyocytes surrounded by scarce connective tissue with low cellularity. TTR animals presented pericellular inflammatory infiltrate and mild pericellular and perivascular connective tissue expansion (Fig. 2).

Stereological analysis indicated that SUN and especially TUN animals presented extensive heart microstructural pathological remodeling when compared to the CNT, STR and TTR groups. While cardiomyocyte distribution was reduced, myocardial accumulation of inflammatory cells, connective tissue and the stroma/parenchyma ratio were higher in SUN and TUN than CNT, STR and TTR animals ($P < 0.05$). These changes were most pronounced in TUN animals compared to the SUN group ($P < 0.05$). Blood vessel distribution and microvascular myocardial ratio was similarly higher in CNT, STR and TTR animals ($P > 0.05$) compared to those in the SUN and TUN groups ($P < 0.05$, Fig. 3).

Nitric oxide, H_2O_2, MDA and PCN levels were higher in the heart samples of SUN and especially TUN animals compared to the other groups ($P < 0.05$). These parameters were higher in TUN than SUN animals ($P < 0.05$). Nitric oxide, H_2O_2 and MDA levels were higher in TTR than CNT and STR animals ($P < 0.05$), while PCN was similar among these groups ($P > 0.05$; Fig. 4).

Heart catalase, SOD and GR activities were increased in SUN and TUN animals compared to the STR and TTR groups ($P < 0.05$). Catalase and SOD activities were higher in TUN than SUN animals ($P > 0.05$), while GR was similar in these groups ($P > 0.05$). Catalase and GR levels were higher in TTR than CNT and STR animals ($P < 0.05$). Non-enzymatic antioxidants were similarly reduced in SUN and TUN animals compared to the CNT, STR and TTR groups ($P > 0.05$; Fig. 5).

4. Discussion

This study investigated the impact of concomitant administration of exercise training and benznidazole, both of which have been associated with antiparasitic and cardioprotective effects in *T. cruzi*-infected hosts when applied alone [11,13–15,24,36]. Surprisingly, we found that when overlapped with the acute stage of *T. cruzi* infection, running

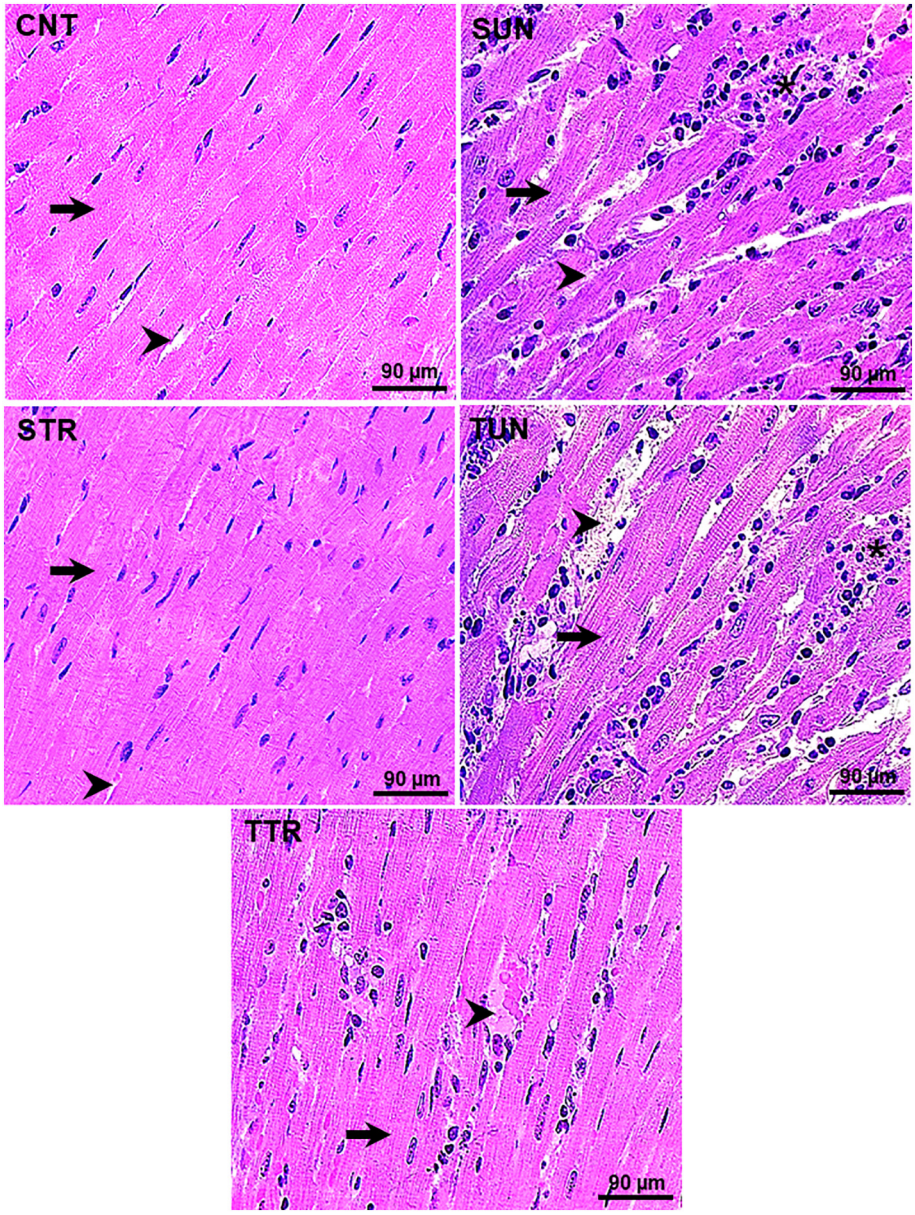

Fig. 2. Representative photomicrography's of the cardiac tissue from sedentary and trained *Trypanosoma cruzi*-infected rats untreated or concomitantly treated with benznidazole. CNT: control sedentary uninfected untreated, SUN: infected sedentary untreated, STR: infected sedentary treated with 100 mg/kg/day benznidazole, TUN: infected trained untreated, TTR: infected trained treated with 100 mg/kg/day benznidazole. Asterisk: necrotic tissue with intense inflammatory infiltrate. Arrowheads: connective stroma. Arrows: cardiomyocytes.

training hampered parasitic control by prolonging the patent period and increasing peak and mean parasitemia, heart inflammation, oxidative and nitrosative stress and myocardial pathological remodeling, with no impact on parasite recrudescence in blood cultures of *T. cruzi*-infected rats. The concomitant exercise training also impaired the effect of benznidazole-based chemotherapy, which was found to be more effective in inhibiting parasitemia, parasite recrudescence, heart inflammation, reactive tissue damage and myocardial remodeling when administered alone.

Interestingly, by identifying that exercise training may increase host susceptibility to infection, our findings diverge from the available experimental evidence. Our outcomes are potentially linked to the timing of the intervention, as exercise training was administered during the acute stage of *T. cruzi* infection. In our model, exercise training and the infection itself constituted two sources of metabolic overload, the overlap of which impaired the host's adaptive responses to the *T. cruzi* challenge. There is no doubt that the metabolic overload triggered by exercise is associated with the upregulation of respiratory chain complexes, increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [37,38], and transient immunological activation [36,39,40]. These processes are caused by different mechanisms and are more pronounced in *T. cruzi* infection, playing a central role in the pathophysiology of Chagas disease [41–45]. As exercise training and infection had never been simultaneously overlapped in experimental studies [15,24,36], our findings indicate that the stage of *T. cruzi* infection should be carefully considered when exercise-based strategies are planned for the treatment of Chagas disease.

The relationship between the moment at which exercise training is administered and the stage of infection is potentially related to the beneficial outcomes previously reported in animal [15,24,36,46] and human [11,12] studies. In this sense, antiparasitic, cardioprotective and neuroprotective effects are mainly reported in animal models of pre-infection exercise training [14,15,24,36], while exercise-induced enhancements in cardiovascular function have been described in humans

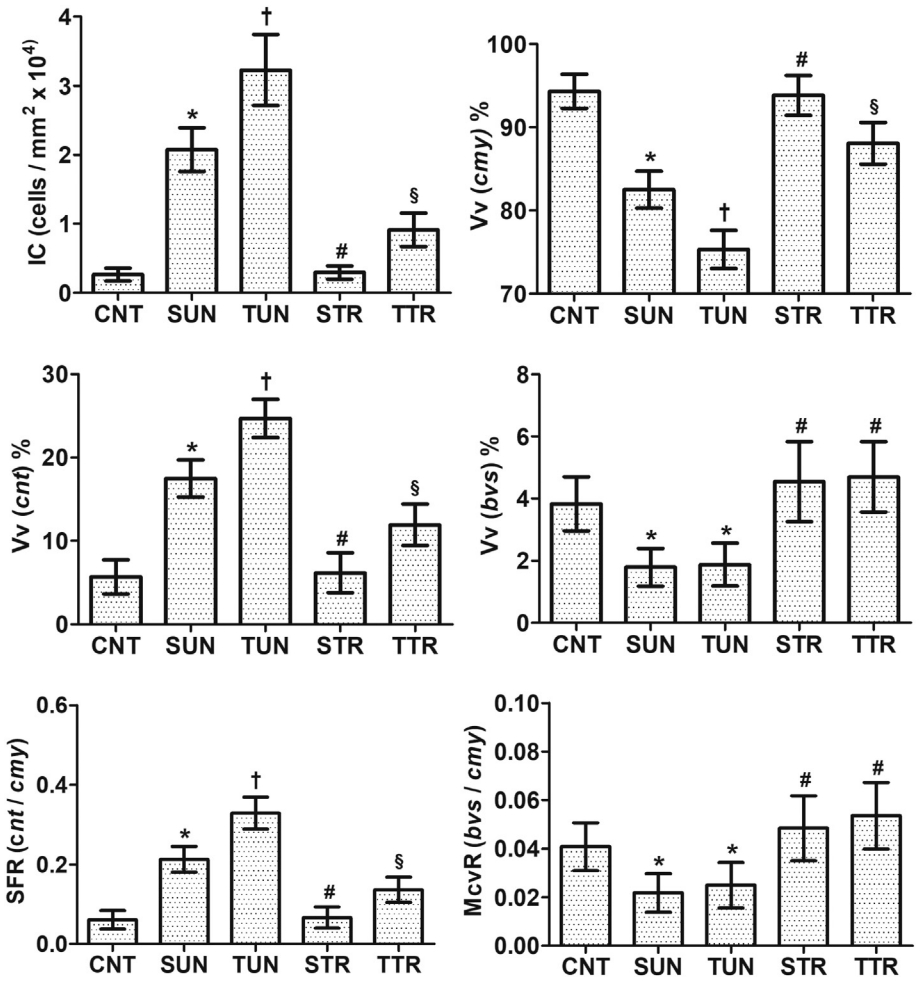

Fig. 3. Inflammatory infiltrate and cardiac microstructural remodeling in sedentary and trained *Trypanosoma cruzi*-infected rats untreated or concomitantly treated with benznidazole. CNT: control sedentary uninfected untreated, SUN: infected sedentary untreated, STR: infected sedentary treated with 100 mg/kg/day benznidazole, TUN: infected trained untreated, TTR: infected trained treated with 100 mg/kg/day benznidazole. IC: Inflammatory cells; Vv: Volume density, Cmy: Cardiomyocytes, Cnt: Connective tissue, Bvs: Blood vessels, SFR: Structural/functional ratio, McvR: microvascularization ratio. Data are expressed as mean and standard deviation. *†‡ Statistical difference ($P < 0.05$) compared to *SUN; †SUN and TUN; ‡ SUN, TUN and STR.

who are chronically infected with *T. cruzi* [11,12]. Although training protocols were not applied at the same time as the acute infection, undetectable parasitemia and/or stable low grade inflammation were common characteristics associated with the training period in both cases. Therefore, the current evidence ignores the impact of exercise administered during the acute phase of infection. In this phase, exercise-induced metabolic overload could potentially be dangerous to the host as it contributes to the severe systemic infection, with

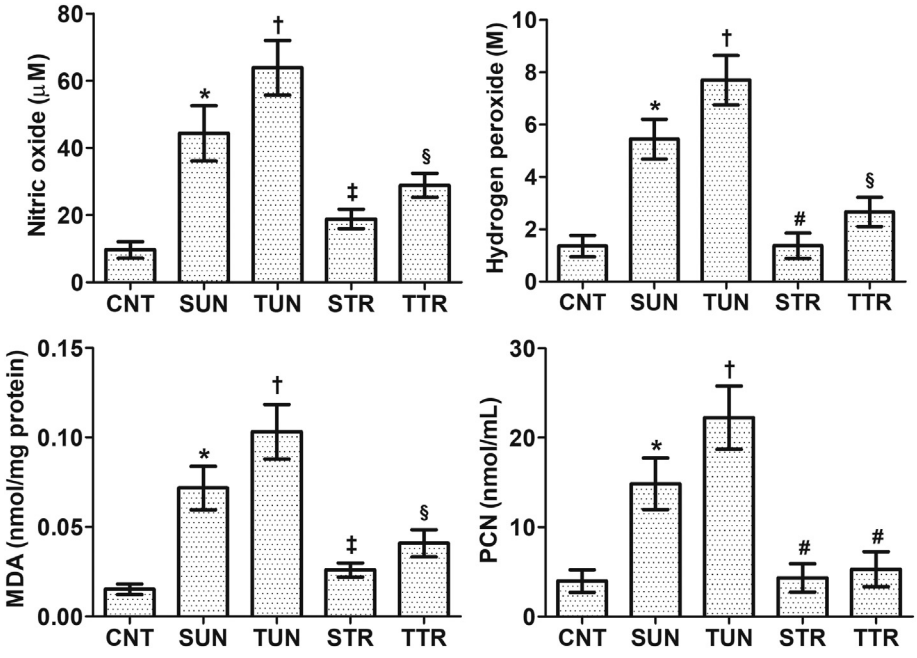

Fig. 4. Reactive species, lipid and protein oxidation in hearts from sedentary and trained *Trypanosoma cruzi*-infected rats untreated or concomitantly treated with benznidazole. CNT: control sedentary uninfected untreated, SUN: infected sedentary untreated, STR: infected sedentary treated with 100 mg/kg/day benznidazole, TUN: infected trained untreated, TTR: infected trained treated with 100 mg/kg/day benznidazole. H_2O_2: Hydrogen peroxide, MDA: Malondialdehyde, PCN: Protein carbonyl. Data are expressed as mean and standard deviation. *†‡ Statistical difference ($P < 0.05$) compared to *SUN; †SUN and TUN; ‡ SUN, TUN and STR.

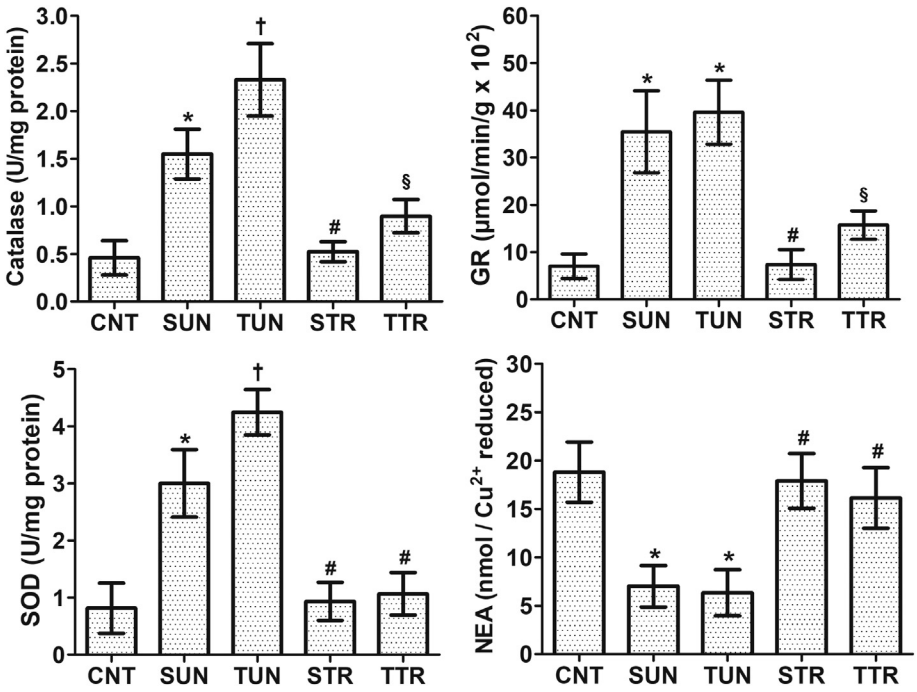

Fig. 5. Enzymatic and non-protein antioxidant effectors in hearts from sedentary and trained *Trypanosoma cruzi*-infected rats untreated or concomitantly treated with benznidazole. CNT: control sedentary uninfected untreated, SUN: infected sedentary untreated, STR: infected sedentary treated with 100 mg/kg/day benznidazole, TUN: infected trained untreated, TTR: infected trained treated with 100 mg/kg/day benznidazole. GR: Glutathione reductase, SOD: Superoxide dismutase, NEA: Non-enzymatic antioxidants. SI: sedentary uninfected, SIT: sedentary infected treated with benznidazole (100 mg/kg/day), TI: trained infected, TIT: trained infected treated with benznidazole (100 mg/kg/day). *†‡ Statistical difference ($P < 0.05$) compared to *SUN; †SUN and TUN; ‡ SUN, TUN and STR.

parasitemia, cell parasitism, inflammatory response, oxidative stress and tissue injury, reaching maximum levels in the patent period [41–43].

Poor parasitological control was clearly observed in trained animals, which exhibited prolonged patent infection with a high load of circulating parasites. A similar effect was observed in animals concomitantly treated with Bz and exercise, which presented worse outcomes than animals receiving chemotherapy alone. Considering the reproductive cycle of *T. cruzi*, early parasitic control is important to attenuate cell parasitism, the inflammatory response directed at infected organs and the severity of tissue damage [19,47]. In this sense, high parasitemia levels have been consistently associated with severe myocarditis in acute infections and heart fibrosis in the chronic phase of Chagas disease [10,48,49]. Therefore, it is not surprising that the impaired parasitic control in trained animals was linked to more severe inflammatory processes, oxidative stress and heart microstructural derangement.

From the cytokine analyses, high IFN-γ, TNF-α, IL-17 and MCP-1 levels indicated a more intense inflammatory profile in animals that underwent training compared to those that were sedentary. In addition to its direct trypanocidal effect, Bz also has anti-inflammatory properties [21,44], which are compatible with better parasite clearance and reduced heart cytokine levels in animals exposed to the etiological treatment [13,20,25]. However, animals that received Bz with concomitant training presented higher IFN-γ, TNF-α and MCP-1 levels than sedentary rats. Therefore, in addition to corroborating the evidence that cytokine expression is aligned with parasitemia levels [45,50], our findings also indicated that exercise potentiated infectious myocarditis and attenuated the inflammatory control conferred by benznidazole-based chemotherapy. As they are typical Th1 cytokines that exert protective effects against *T. cruzi* [51–53], high IFN-γ and TNF-α levels were expected. These cytokines are associated with the activation of classical macrophages and upregulation of NO production, a recognized antitrypanosomal agent [28,54].

Antitrypanosomal effects have also been reported for IL-17 and MCP-1 [55–57]. IL-17 has been associated with increased host resistance to *T. cruzi* infection, mainly by regulating the differentiation of Th1 cells, enhancing the production of IL-6 and TNF-α and the recruitment of neutrophils to infected organs [56]. In addition, IL-17 levels are inversely correlated with the severity of heart damage, suggesting that this cytokine is part of the host's cardioprotective repertoire in Chagas disease [55]. Similarly, MCP-1 exerts protective effects against *T. cruzi* infection as it modulates leucocyte recruitment and activation in infected organs, enhancing parasite uptake and destruction by macrophages through an inducible nitric oxide synthase (iNOS)-dependent pathway [45,57]. Despite these protective mechanisms, exacerbated immunological responses are also responsible for damage to healthy cells and organs, which are mediated by a massive recruitment of leukocytes and enhanced ROS and RNS production [28,43,54]. As reactive molecular damage triggers additional leucocyte recruitment and the synthesis of proinflammatory cytokines and cytotoxins, a self-perpetuating cycle is established, potentiating lipid, protein and DNA oxidation, as well as the risk of cardiac failure and host death [43,58].

As a counter-regulatory inflammatory response, the increased levels of IL-4 and IL-10 observed in the hearts of trained animals were not surprising. IL-4 boosts the Th2 immune phenotype, which does not provide protection against *T. cruzi* [59,60]. However, IL-4 upregulation represents a reactive immunological mechanism that seeks to achieve a balance between the Th1 and Th2 phenotypes, which must be ideally adjusted to combat the parasite while simultaneously minimizing the development of secondary lesions on the host's organs due to an exacerbated Th1 response and secondary prooxidant events [45,52]. Like IL-4, upregulation of IL-10 is associated with increased host susceptibility to *T. cruzi* infection [61]. However, this T regulatory cytokine also helps to modulate the inflammatory process, as it is required to prevent immune hyperactivity and pathologic responses associated with excessive IL-12 production and Th1 hyperpolarization [52,62].

The findings of the heart microstructural analysis were also aligned with the cytokine results, with infected trained animals exhibiting extensive myocardial pathological remodeling evidenced by intense inflammatory infiltrate and connective tissue expansion. In addition to potentiating myocarditis when applied alone, exercise training also reduced Bz-induced cardioprotective effects. As cardiomyocytes are a primary target of *T. cruzi* parasitism, cardiomyocytolysis and reactive connective tissue expansion are expected [13,44,63]. As observed in the present study, these processes contributed to a marked increase in structural/functional ratio in trained animals, which is often associated with the deterioration of cardiac function in Chagas disease [48,63,64].

Conversely, exercise training was not able to prevent a reduction in the myocardial microvascularization ratio, which was prevented by the administration of benznidazole-based chemotherapy either alone or when combined with exercise training. Microvascular damage is recognized as an ancillary factor that potentiates myocytolic necrosis and heart insufficiency in Chagas disease, especially due to ischemic myocardial damage linked to thromboembolic events, endothelial dysfunction and autonomic vasomotor abnormalities [49,63].

Corroborating our findings, microstructural myocardial derangement is closely correlated with redox imbalance and reactive myocardial damage in Chagas disease [13,43,44]. In this sense, we identified that the intensity of heart damage was consistent with increased levels of reactive metabolites (i.e., NO and H_2O_2) and molecular oxidation (i.e., lipid [MDA] and protein [PCN]) in trained rats. Exercise training also potentiated reactive tissue damage in Bz-treated animals. Due to the upregulated oxidative phosphorylation, exercise training [37,38] and *T. cruzi* infection [41,42,65] both stimulate RNS and ROS production. In Chagas disease, leukocyte recruitment, activation of respiratory burst and uncoupling of the respiratory chain in *T. cruzi*-infected cardiomyocytes represent the main sources of ROS and RNS [41,42,66]. Upregulation of these molecules has been associated with cardiac deterioration and a worse prognosis in patients with Chagas cardiomyopathy [67,68]. Although the impact of upregulated reactive species production by the combination of exercise training and infection remains unclear, additional RNS and ROS are potentially dangerous to the host due to their cytotoxic and fibrogenic cardiac effects [69–71].

As expected, the increased reactive tissue damage was accompanied by the upregulation of antioxidant enzyme activity in trained animals. Corroborating the evidence of an inflammation-reactive stress coupling [58], Bz treatment was effective in reducing parasitemia, inflammation, reactive tissue damage, and the activity of antioxidant enzymes. However, these effects were impaired when exercise training and Bz were administered concomitantly. Although these findings indicate a remarkable counter-regulatory cardiac enzymatic response to *T. cruzi* infection [15,58,65], the increased CAT, GR and SOD activities were not enough to block reactive tissue damage. Animals treated with Bz exhibited clear attenuation of reactive tissue damage, therefore parasitic control plays a key role in achieving a better redox balance in Chagas disease. As the administration of exogenous antioxidants is not always successful [58,72], controlling inflammation through etiologic treatment and anti-inflammatory drugs seems to be more effective in attenuating oxidative stress and reactive tissue damage in *T. cruzi* infection [20,25,58]. This effectiveness is reinforced considering that, regardless of exercise training, only the Bz-treated groups showed preservation of non-enzymatic heart antioxidant levels. Although poorly investigated, reduced levels of these low-molecular-weight molecules (i.e., glutathione, polyamines, uric acid, and vitamin C and E) have been reported in animals [35,58,72] and humans [73] infected by *T. cruzi*. Unlike endogenous enzymes, non-enzymatic antioxidants were markedly depleted in infected animals, indicating that the intense consumption of these molecules could partially explain their limited potential to scavenge ROS and RNS, as well as the increased heart levels of oxidized molecules and the myocardial damage observed in *T. cruzi*-infected animals.

Our findings indicate that when administered during the acute phase of *T. cruzi* infection, exercise training aggravates heart inflammation, leading to enhanced RNS and ROS production, marked lipid and protein oxidation, and extensive myocardial microstructural remodeling. When administered alone, benznidazole-based chemotherapy led to the marked attenuation of these pathological events; however, the concomitant administration of exercise training reduced the antiparasitic and cardioprotective effects of the etiological treatment, making the hosts more susceptible to the infection. Considering that exercise training and acute *T. cruzi* infection represented a combined metabolic challenge, the negative effects were potentially related to overlapping of the pro-oxidant and immunoinflammatory demands of the concomitant treatment.

Declaration of Competing Interest

There are no conflicts of interest. All authors contributed to data collection and analysis, article preparation and have approved the final manuscript.

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