



Physical training improves exercise tolerance, cardiac function and promotes changes in neurotrophins levels in chagasic mice

Rafael Leite Alves^a, Bárbara Ramalho Ladeira Cardoso^a, Isalira Peroba Rezende Ramos^b, Bruna da Silva Oliveira^a, Mara Livia dos Santos^a, Aline Silva de Miranda^a, Tatiane Cristine Silva de Almeida^a, Maria Aparecida Ribeiro Vieira^c, Fabiana Simão Machado^d, Anderson José Ferreira^{a,*}, Gleide Fernandes de Avelar^a

^a Federal University of Minas Gerais, Department of Morphology, Av. Pres. Antônio Carlos, 6627 Campus Pampulha, Belo Horizonte, Brazil

^b Federal University of Rio de Janeiro, Center of Health Science, Av. Carlos Chagas Filho, 373 Rio de Janeiro, Brazil

^c Federal University of Minas Gerais, Department of Physiology and Biophysics, Av. Pres. Antônio Carlos, 6627 Campus Pampulha, Belo Horizonte, Brazil

^d Federal University of Minas Gerais, Department of Biochemistry and Immunology, Av. Pres. Antônio Carlos, 6627 Campus Pampulha, Belo Horizonte, Brazil

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ABSTRACT

Aims: To investigate the effects of moderate aerobic physical training on cardiac function and morphology as well as on the levels of glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) of animals infected with the Y strain of *Trypanosoma cruzi*.

Main methods: Twenty-eight male C57BL/6 mice were distributed into 4 groups: sedentary control (SC), trained control (TC), sedentary infected (CHC) and trained infected (CHT). The infection was performed by intraperitoneal injection of trypomastigote forms and the animals were adapted to treadmill in the week before the beginning of the training protocol, initiated 45 days post infection. Maximal exercise test (TEM) was performed at the baseline as well as at the end of the 4th, 8th and 12th weeks of training. At the end of the 12th week, all animals were evaluated for cardiac morphology and function by echocardiography.

Key findings: CHC group showed a larger area of right ventricle (RVA), increased end-systolic volume and reduction in ejection fraction (EF), stroke volume (SV), cardiac output (CO) and fractional area change (FAC). The training reduced the RVA and improved the FAC of chagasic animals. GDNF level was higher in TC and CHC groups compared to SC in heart and BDNF levels were higher in CHC compared to SC in heart and serum.

Significance: Physical training ameliorated the cardiac function of infected animals and promoted adjusts in BDNF and GDNF levels. These findings evidenced these neurotrophins as possible biomarkers of cardiac function responsive to exercise stimulus.

1. Introduction

Chagas' disease (CD) is caused by infection with *Trypanosoma cruzi* trypomastigotes forms [1] and remains as an endemic illness in Latin America. The prevalence of CD is around 8 million worldwide [2] and progressively more number of cases was found in countries previously considered free of this disease [3–5]. It has been considered a major cause of cardiac infectious disease in endemic countries [2] and represents one of the most important health problems with high costs of treatment.

The clinical course of CD is composed by an acute phase, which is in general asymptomatic and characterized by high level of parasitemia [6]. Frequently, people who survive to this phase proceed to the chronic

phase in its indeterminate form and can stay on it for several years without clinical manifestations [7]. From this point, infected subjects may develop one of the known clinical forms of CD characterized by cardiac and/or digestive manifestations [6,8–10].

Previous studies show that 20% to 30% of infected individuals from endemic areas develop some cardiac damage during the infection [6,11,12]. The most important clinical finding of CD is the chronic chagasic cardiomyopathy [10,13] with progressive impairment of cardiac function [6].

The cardiac function impairment is a result of multifactorial events which change organs and tissues homeostasis, besides affecting the functional capacity and exercise tolerance. However, it remains unclear how *T. cruzi* infection leads to this scenario [14]. Although the

* Corresponding author at: Federal University of Minas Gerais, Av. Pres. Antônio Carlos, 6627 - Pampulha, Belo Horizonte, Brazil.

E-mail address: anderson@icb.ufmg.br (A.J. Ferreira).

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reduction in exercise tolerance observed in CD, physical exercise is recognized as a powerful and beneficial stimulus [15,16]. Exercise effects as cardiovascular remodeling [15], adjustment in blood pressure [16] and cardiac function improvement may help to poise or handle the progress of the disease.

Moreover, neurotrophins have been associated with many health problems such as coronary artery disease [17], myocardial infarction [18], metabolic syndrome, obesity and diabetes [19] and as peripheral markers of metabolic indexes and cardiovascular conditions [20]. Previous investigations reported that atrial and ventricular levels of nerve growth factor (NGF) significantly increase at the day 20 post inoculation with trypomastigotes forms in rats. In contrast, at the same time point, glial cell line-derived neurotrophic factor (GDNF) did not change, suggesting that NGF plays an important role in the regenerative phenomenon subsequent to a myocarditis and GDNF could have a minor or a more transient participation [21].

Brain derived neurotrophic factor (BDNF) is also investigated as possible marker of cardiac health in clinical studies of CD. These studies suggested that BDNF levels increase in subjects with CD compared with healthy ones [22] and the improvement of functional capacity after twelve weeks of physical exercise is related to lower levels of BDNF at the baseline [23].

However, it remains unclear how exercise could affect the expression and/or concentration of neurotrophins in CD, as well as how it could be associated to cardiac function in animal models. Thus, in order to better understand these gaps in the literature, here we evaluated the cardiac function and neurotrophins levels in hearts, spleens and serum of infected mice after twelve weeks of moderate physical exercise.

2. Materials and methods

2.1. Ethics statement

This study was performed in strict accordance with recommendations of the guide for care and use of laboratory animals of the Brazilian National Council of Animal Experimentation and all experiments and procedures were approved by the ethic committee at Federal University of Minas Gerais CEUA/UFMG (protocol number 300/2015). Animals were obtained from CEBIO/UFMG, maintained under pathogen-free conditions, housed in standard clear plastic cages, and kept at 23 °C with free access to food and water and a 12 h light/dark cycle.

2.2. Animal groups and infection

Twenty-eight C57BL/6 male mice were randomized into four groups: sedentary control (SC; $n = 7$); trained control (TC; $n = 7$); chagasic sedentary control (CHC; $n = 7$); and chagasic trained (CHT; $n = 7$). The Y strain of *T. cruzi* was maintained *in vivo* and used to infect animals with 7–8 weeks of age by the intraperitoneal route with 1×10^3 trypomastigote forms. Control groups received the same final volume of phosphate-buffered saline (PBS, pH 7.2). The parasitemia was determined from the seventh to the fifteenth day post inoculation on alternate days using 5 μ L of blood collected in a tail vein.

2.3. Measurement of exercise tolerance

Forty-five days after inoculation and at the end of 4th, 8th and 12th weeks of training, all animals were evaluated for exercise tolerance using a maximal exercise test (TEM). Thus, TEM was performed 4 times during the experimental protocol. The mice were familiarized with a motor-driven treadmill (Gousteq®) one week before the first TEM by running at the speed of 8 m/min at 5% inclination, 10 min/day for 5 consecutive days. The TEM was performed at a constant slope of 5% with the starting speed at 3 m/min, and adjusting the velocity in 3 m/min every 3 min until the mice fatigue, defined as the point on which the animals were no longer able to keep the rhythm with treadmill. The

maximum speed reached was used to measure the velocity of training for the following four weeks using 70% of it until the new TEM. The workload (W, in Joules) was calculated multiplying the mice body weight (kg) by the time (minutes) and speed (m/min) for each step reached by the sine θ of treadmill slope [24]. At the end of twelve weeks of exercise training and before euthanasia, the cardiac function was evaluated by echocardiography. Training sessions were taken five times per week with 1 h duration per day.

2.4. Cardiac function measurements

Cardiac function was evaluated by echocardiography. Animals were anesthetized with isoflurane (2%) in a flow of 1 L/min of oxygen and the precordium area was trichotomized. The equipment Vevo 770 (Visual Sonics®, Canada) and a 30 MHz transducer were used to record cardiac function data. This equipment was designed for small rodents and shows high quality to assess minor structures. Cardiac geometry was assessed in 2-dimensional mode. The area of the left and right ventricle at the end of diastole was measured in cross-section at the papillary muscles level. The systolic function of the left ventricle was evaluated by ejection fraction calculated according to the Simpson's method, which is able to determine the volume of the left ventricle. Automatically, the software offers values of end-diastolic and end-systolic volumes, as well as the stroke volume and fractional area changes. Animals were evaluated 48 h after the last TEM.

2.5. Morphometric analysis

The euthanasia was performed using intraperitoneal injection of 150 mg/kg BW of thiopental and verified by the absence of the tail reflex. The heart was removed and weighed. The left ventricle was dissected and a fragment was used for histological analysis. The tissue was fixed by immersion in 4% (v/v) glutaraldehyde and 0.05 M phosphate buffer. After fixation, cardiac tissue was processed and embedded in plastic (glycolmethacrylate), as described elsewhere [25]. Sections with 4 μ m thickness were obtained and the distance between these sections was superior to 12 μ m. The tissue was stained by Toluidine blue 1% sodium borate and mounted on histological slides. The slides were visualized in a light microscope (Olympus BX-53®; Olympus Tokyo, Japan) and images captured using an objective of 40 \times by a digital camera (Olympus QColor - 5®, U-CMAD3; Olympus, Tokyo, Japan) connected to it. Fifteen fields from each slide were randomly chosen and used to analyze the diameter of cardiomyocytes nuclei and volume densities of cells of the left ventricle. All these measures were done by using the ImageJ software. The diameter of cardiomyocytes was obtained by the average length of the longer and shorter axis in the nuclei of thirty cells of each animal. The volumetric proportions of cardiac tissue cellular components were determined by a 520 intersections grid placed over each field. Artifacts were not considered in the total number of points used to obtain the volumetric proportion. The components considered were fibroblasts and cardiomyocytes nucleus and its cytoplasm. We used these data to calculate the ratio between cardiomyocytes and fibroblasts, as well as to analyze the cardiomyocytes volume nuclei by using the volume formula of the sphere: $V = 4/3\pi r^3$.

2.6. Neurotrophins levels

Neurotrophins levels were assessed by enzyme-linked immunosorbent assay (ELISA). GDNF, NGF and BDNF levels were measured in hearts and spleens. BDNF was also measured in serum. All analyses were done according to manufacturer's protocols (R&D Systems, Minneapolis, MN, USA). The concentrations were determined in a spectrophotometer (Spectramax 190) at a 450 nm wavelength and the detection limit was in the range of 5–10 pg/mL.

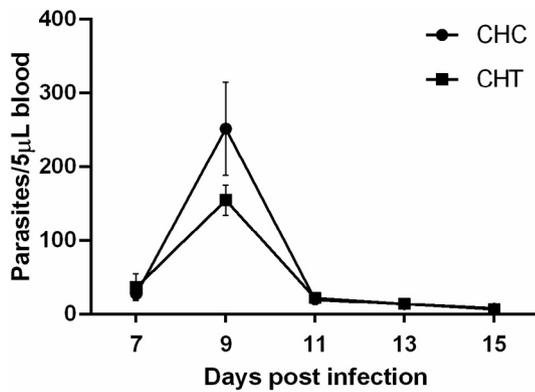


Fig. 1. Parasitemia of mice infected with Y strain of *T. cruzi*. Natural course of *T. cruzi* infection, as measured by parasitemia, of chagasic sedentary animals (CHC, $n = 6$) and chagasic trained mice (CHT, $n = 7$) infected with 1000 trypomastigotes of *T. cruzi*. The data is shown as mean \pm SEM of the number of parasites per microliter of blood. Two-way ANOVA followed by the Bonferroni post-test.

2.7. Statistics analysis

Values are expressed as mean \pm SEM and the data were analyzed using one-way or two-way ANOVA followed by the Bonferroni post-test using the software GraphPad Prism (version 5; Graph-Pad Software Inc., San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

3. Results

There were no significant changes in parasitemia between Y strain infected groups (Fig. 1) comparing the same period from 7th to 15th day post-infection.

The exercise tolerance was assessed by TEM at baseline (TEM 0) and at the end of weeks 4 (TEM 1), 8 (TEM 2) and 12 (TEM 3) and for each period we calculated the Work (W; Joules; Fig. 2). The W was higher in all TEM of trained groups compared with sedentary mice ($p < 0.0001$). No significant alterations were found when comparing the groups SC and CHC or TC and CHT, except on TEM 2 when the TEM of CHT was lower compared with TC ($p = 0.0416$). In addition, comparing the groups through the all tests, trained groups improved their performance from TEM 0 to TEM 1 in both groups (1.762 J vs. 2.792 J in TC and 1.614 J vs. 2.372 J in CHT; $p < 0.0001$); from TEM 1 to TEM 2 in TC

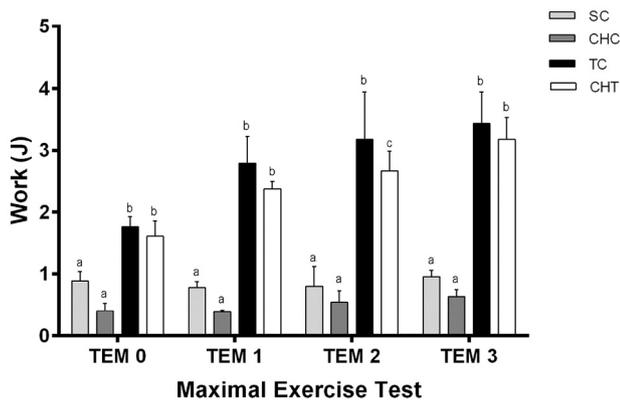


Fig. 2. Maximal exercise tests (TEM) from the baseline (TEM 0) to the end of the 12th week (TEM 3) of training. W (Joules); TC (trained control, $n = 7$); CHT (chagasic trained, $n = 7$); CHC (chagasic control, $n = 6$); and SC (sedentary control, $n = 7$). Data are expressed as mean \pm SEM ($p < 0.05$). Different letters denote statistical differences between groups. One-way (to compare the groups in the same test) and two-way (to compare the groups through the time in different tests) ANOVA followed by the Bonferroni post-test.

(2.372 J vs. 3.174 J; $p = 0.0081$) and from TEM 2 to TEM 3 in CHT (2.661 J vs. 3.175 J; $p = 0.0002$). We did not find any significant difference comparing sedentary groups through the time.

The cardiac geometry was measured by echocardiography (Fig. 3). The right ventricular area (RVA) was larger in CHC group compared to CHT ($p = 0.0004$) and SC ($p < 0.0001$). No significant differences in left ventricular area (LVA) were found (Fig. 3A and B).

The cardiac function measured by echocardiography showed higher ejection fraction in CHT and SC groups compared to CHC, as well as it was reduced when the CHT group was compared to TC (Fig. 4A). The stroke volume and cardiac output also were lower in CHC group compared to SC (Fig. 4B and C). No significant differences among the groups were observed in terms of end-diastolic volume (Fig. 4D). However, higher end-systolic volume was found in CHC when compared to SC (Fig. 4E). Fractional area change was higher in CHT and SC compared to CHC (Fig. 4F) and TC group showed lower left ventricular internal diameter and volume compared to SC (Fig. 4G and H).

GDNF level was higher in TC and CHC group compared to SC in heart tissue while lower values for this neurotrophin were found in CHT and SC compared to CHC, and higher value in TC compared to CHT and SC in spleen samples (Fig. 5A). Regarding to NGF levels, in both tissues, TC group showed higher values compared to SC. It was also higher in spleen of CHC mice when compared to SC group (Fig. 5B).

Moreover, BDNF levels were higher in CHC compared to SC in heart (Fig. 6A) and serum (Fig. 6B).

Morphometric analyses did not show significant changes among the groups in terms of cardiomyocytes nucleus and ratio between nucleus proportions of cardiomyocytes and fibroblasts (Fig. 7).

4. Discussion

These results confirmed that *T. cruzi* infection is able to impair the exercise tolerance and cardiac function, since twelve weeks of moderate exercise training was able to retard the progress of disease. It may have occurred by keeping the RVA closer to those of non-infected animals and greater FAC in CHT. Furthermore, neurotrophins levels, particularly in terms of BDNF in serum and heart, were higher in CHC.

The parasitemia showed an expected behavior for Y strain similar to other studies [26]. No significant differences in parasitemia between infected groups were viewed, showing that changes in parasitemia were not the reason of the findings observed in other parameters.

Exercise tolerance was measured by TEM and it showed a decrease of the performance in CHC compared to trained mice, as well as between SC and trained animals in all evaluations. These data show that the training protocol was able to promote gain in exercise tolerance in trained groups, as corroborated by previous studies in rats infected with Y strain [14,27,28]. Despite of differences between trained groups in TEM 2, we did not find significant differences between these groups in TEM 3, indicating that CHT was able to reach similar exercise tolerance of TC. The absence of differences in work between sedentary groups can be explained based on systolic function, which although presenting lower values in CHC, was sufficient to answer to exercise stress similarly to SC. Despite of it, heart rate assessment could provide interesting data on exercise adaptations and since we did not evaluate this parameter, we accept this as a limitation of our study.

The larger RVA found in CHC group is in accordance with previous studies using different experimental models [29,30] suggesting the primary involvement of the right ventricle in CD. Many reports observed changes in the physiology of the right ventricle and support the idea that it plays an important role in the development of chagasic cardiomyopathy [29]. Some studies reported right branch block, dilated right ventricular chamber, compromised right ventricular myocyte contractility, and thinning of the right ventricular wall even with the absence of damage in the left ventricle [31]. Others suggested that the right ventricular systolic dysfunction is more commonly associated with the left ventricular systolic dysfunction, although isolated and

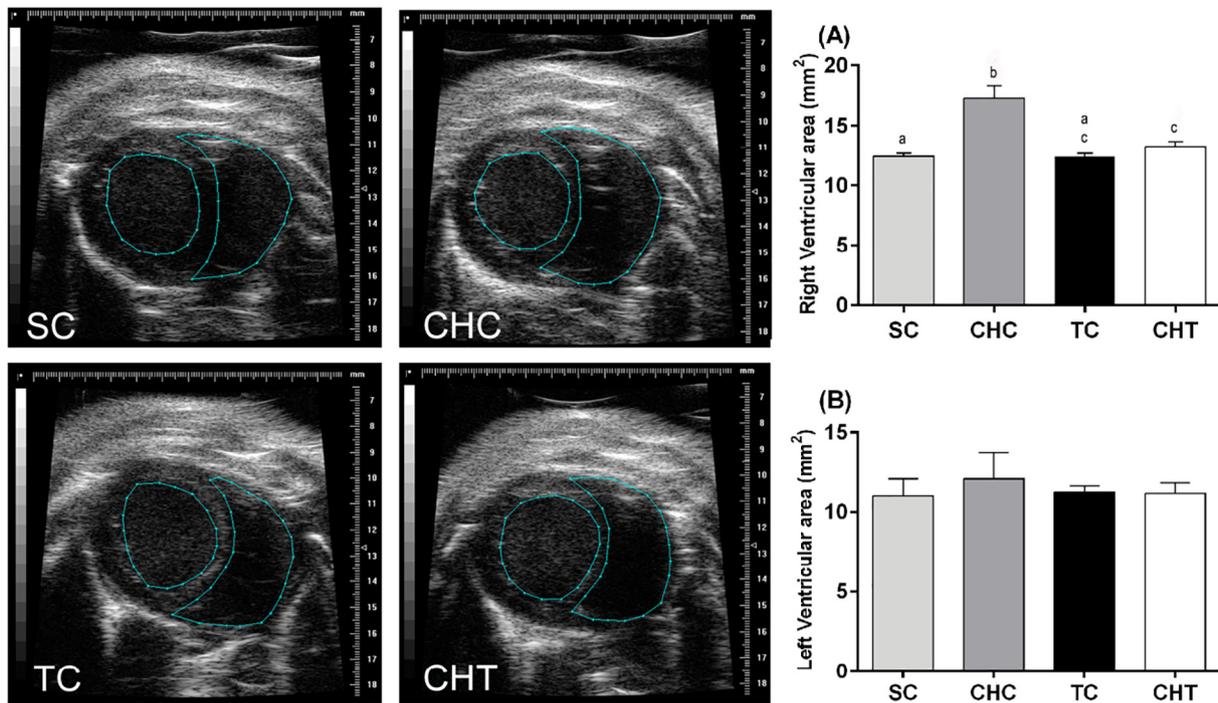


Fig. 3. – Representative echocardiographic images of cardiac area of (A) right and (B) left ventricles. TC (trained control, $n = 7$); CHT (chagasic trained, $n = 7$); CHC (chagasic control, $n = 6$); and SC (sedentary control, $n = 7$). Data are expressed as mean \pm SEM ($p < 0.05$). Different letters denote statistical differences between groups. One-way ANOVA followed by the Bonferroni post-test.

early right ventricular dysfunction can also be identified [32]. Moreover, the cardiomyopathy progression of CD may depend on the genetic background of the host and/or strain [33].

We did not find significant differences in the LVA but the systolic function of the left ventricle was affected in infected animals. Our findings suggest that at the end of twelve weeks, besides training, the impairment occurs in both ventricles. However, since we only assessed the cardiac function at the end of twelve weeks, it is possible that RVA was separately affected at preceding periods. Future studies should consider performing cardiac function analysis at baseline as well as at the end of periods of 4 weeks of training.

FAC is a measurement that provides an estimate of the global systolic function using chamber area variation within the left ventricle between diastole and systole. The impairment of myocardial contractility evidenced by lower values of FAC in CHC compared to SC and CHT, may explain the lower CO and SV in CHC when compared to SC, as well as the EF, which showed better values for CHT compared to CHC. It is also suggested by the higher values of the end-systolic volume in CHC compared to SC. Others interesting results were the absence of differences between CHC and CHT in the end-systolic volume and the impairment of EF in CHT compared to TC. Together, this could indicate an initial damage in systolic function of CHT group not enough to reduce the exercise tolerance once we did not observe significant differences between CHT and TC in the TEM 3.

A large amount of clinical studies have been reported the phenotype of cardiac function impairment which shows higher prevalence of only LV or biventricular involvement, not evidencing the presence of isolated right ventricular involvement [31]. Our data showed that CHC presented a larger RVA and systolic deficit, as well as lower EF, while no differences in RVA were observed in CHT group. However, greater dilation of the RV was associated with larger diastolic and systolic diameters of the LV [31], but, in our study, we did not find differences in end-diastolic diameter of LV between infected animals. On the other hand, end-diastolic diameter of LV was different in SC and TC groups, as well as the end-diastolic volume. These differences between non-chagasic groups could indicate a consequence of the physical training

on cardiac remodeling and maybe they were more evident in these animals that only faced the adaptive stress of exercise without pathological challenges. It is important to note that one limitation of our study is that the echocardiography was performed only after the training. Analyses before and after training would be more precise.

Based on previous studies [22,34], BDNF levels could be changed by infection and may have its levels adjusted by exercise stimulus, so accepting this neurotrophin as a potential protector against cardiac dysfunction [18]. These studies showed that BDNF levels was greater in chagasic subjects compared to non-chagasics, as well as it was lower in subjects with chagasic dilated cardiomyopathy compared to non-dilated and asymptomatic ones [22]. It suggests that the preservation of cardiac autonomic response is responsible for the greater level of this neurotrophin. It was also confirmed by another investigation that showed better correlation between lower BDNF levels before twelve weeks of aerobic training and the improvement of exercise tolerance after this period mainly when autonomic function was better preserved [23].

No differences between pre and post one maximal exercise session were evidenced for BDNF in chagasic dilated cardiomyopathy and most subject in this group reached moderate intensity effort. In contrast, non-dilated subjects showed significant decrease in serum BDNF levels and all of them reached high intensity during the session [34]. Thus, it suggests that BDNF levels could be affected by the intensity of the exercise stimulus and that a single session of moderate exercise may not affect its levels.

The higher values found for BDNF in heart and serum in CHC, supports this neurotrophin as a possible marker and protector of cardiac dysfunction in a condition where exercises were not considered. No difference was found between TC and SC, which suggest that twelve weeks of moderate aerobic exercise training may not be enough to change significantly BDNF levels. Taking together the absence of differences in its levels between CHC and CHT and the greater global systolic function in CHT, we can accept that exercise develops an important role adjusting BDNF levels and leading to a higher cardiac tolerance threshold in trained group.

GDNF and NGF expression profiles were investigated in the acute

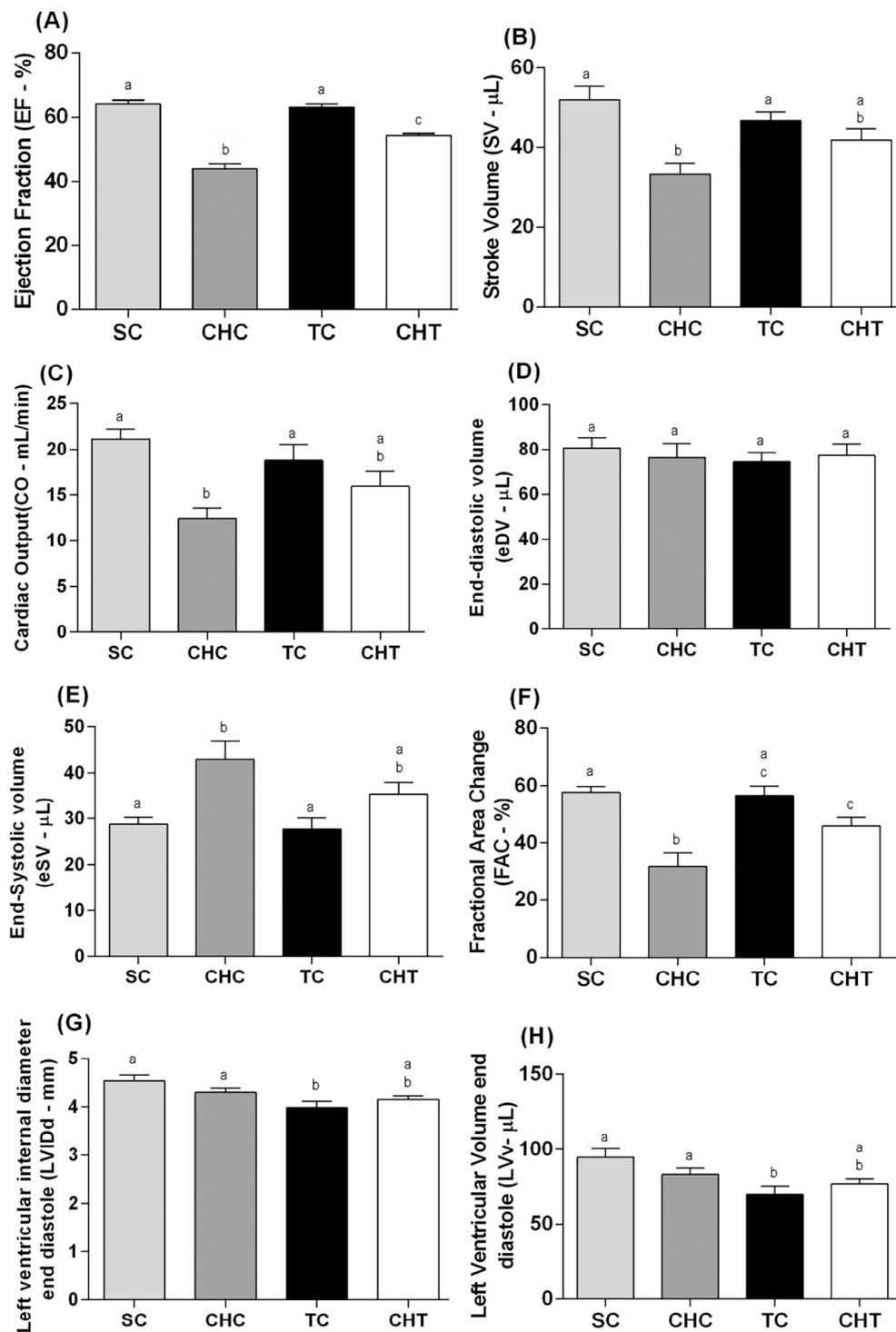


Fig. 4. Cardiac function and morphological measurements. TC (trained control, $n = 7$); CHT (chagasic trained, $n = 7$); CHC (chagasic control, $n = 6$); and SC (sedentary control, $n = 7$). Data are expressed as mean \pm SEM ($p < 0.05$). Different letters denote statistical differences between groups. One-way ANOVA followed by the Bonferroni post-test.

phase of CD. Atrial and ventricular NGF levels increased significantly at the time-point of maximal sympathetic denervation and GDNF levels did not change [21]. These findings suggest an important role for NGF in the regenerative phenomenon subsequent to sympathetic nerve endings damages caused by myocarditis, while GDNF has a minor participation in this phase [21]. In our study, NGF levels were not different between chagasic groups at the end of twelve weeks of training. As suggested by previous studies, NGF could develop an important role in acute phase of CD and, even in the presence of training,

it was not able to change the levels of this neurotrophin in infected animals in the chronic phase. An explanation for that could be the initial damage in autonomic function in acute phase. As showed by the parasitemia, no differences were found between infected groups, suggesting a similar initial impairment, which could have compromised the response to the exercise. However, NGF level was higher in TC compared to SC in heart and spleen, suggesting that exercise can modulate NGF levels in healthy conditions, probably promoting autonomic adjustments.

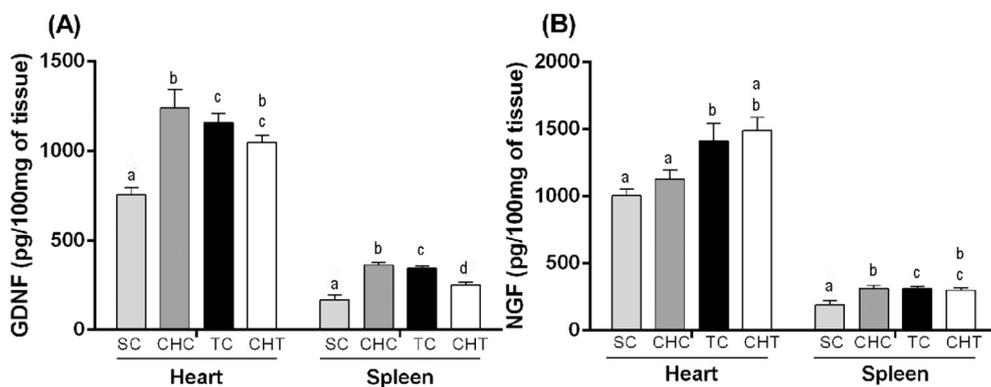


Fig. 5. Glial cell line-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) levels in heart and spleen. TC (trained control, n = 7); CHT (chagasic trained, n = 7); CHC (chagasic control, n = 6); and SC (sedentary control, n = 7). Data are expressed as mean ± SEM (p < 0.05). Different letters denote statistical differences between groups. One-way ANOVA followed by the Bonferroni post-test.

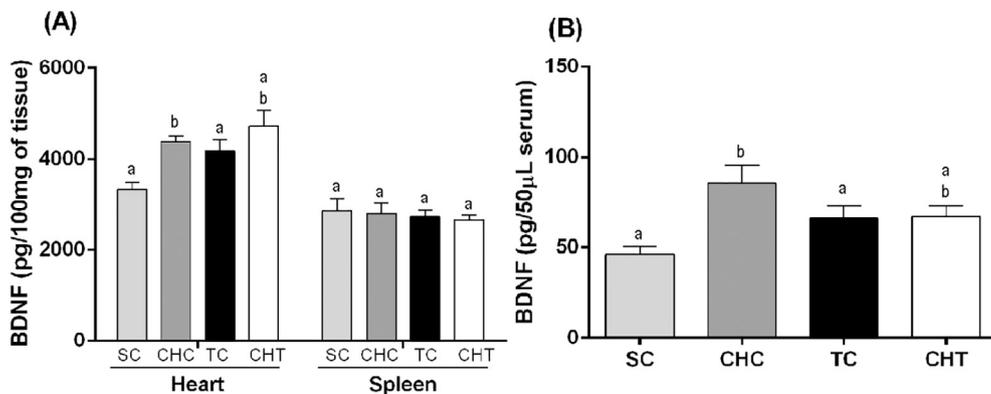


Fig. 6. Brain Derived Neurotrophic Factor (BDNF) levels in heart, spleen and serum. TC (trained control, n = 7); CHT (chagasic trained, n = 7); CHC (chagasic control, n = 6); and SC (sedentary control, n = 7). Data are expressed as mean ± SEM (p < 0.05). Different letters denote statistical differences between groups. One-way ANOVA followed by the Bonferroni post-test.

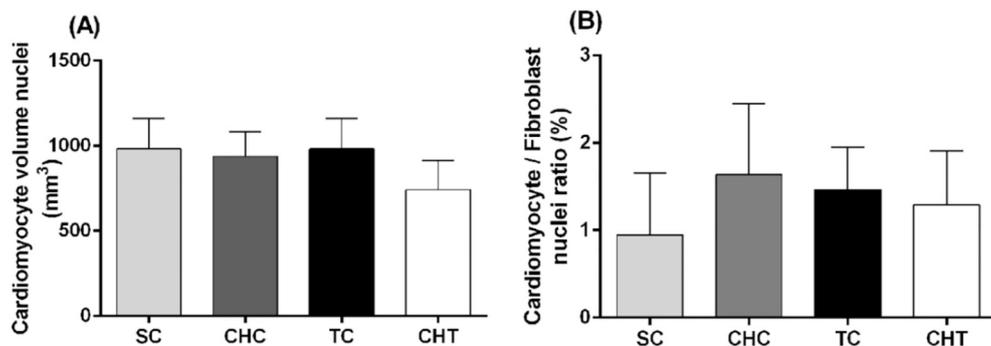


Fig. 7. Morphometric analysis. (A) Cardiomyocyte volume nuclei in mm³ and nuclei ratio between cardiomyocytes and fibroblasts nucleus (B). TC (trained control, n = 7); CHT (chagasic trained, n = 7); CHC (chagasic control, n = 6); and SC (sedentary control, n = 7). Data are expressed as mean ± SEM. One-way ANOVA followed by the Bonferroni post-test.

The greatest values of GDNF observed in heart and spleen of TC and CHC suggests a role of this neurotrophin in chronic phase and its capacity to answer to exercise stimulus. Also, in spleen, GDNF level was lower in CHT compared to TC and CHC which may suggest that exercise plays an important role up regulating GDNF concentration. On the other hand, in healthy conditions, the higher values found in TC may suggest a better capacity to answer to exercise stimulus in absence of CD. Additionally, in a scenery where exercise was not considered, the greater values found in CHC animals may identify a natural response to infection in an attempt to keep cardiac function closely to normal parameters. These results can support GDNF as a possible protector and marker of cardiac function, sensitive to exercise stimulus, placing it by BDNF side.

We did not find any significant changes in morphometric analysis among groups. These results are opposite to previous studies [22] which suggested increases in cardiomyocytes nuclear volume as a possible explanation to higher values of BDNF in CD. We believe that this phenomenon may occurs in the context of an extensive cardiac function impairment, either by the longer time of disease progression or by strains that have a predilection for cardiac tissue.

To the best of our knowledge, this was the first study that investigated the effects of twelve weeks of exercise training on cardiac function and neurotrophins levels in a chronic experimental model of CD. However, future studies should be considered in order to clarify the interaction among infection, neurotrophins levels, cardiac function and exercise tolerance. Adaptations as the time point to beginning the exercise training protocol, the tropism strain, autonomic function evaluation and investigation of neurotrophins pathways in response to exercise stimulus should be considered in future studies.

5. Conclusion

Twelve weeks of moderate aerobic physical training improved the exercise tolerance and reduced the cardiac function impairment of infected animals mainly keeping better values of RVA and FAC and promoting better balance in neurotrophins levels, especially BDNF and GDNF in the chronic phase. Changes in neurotrophins levels confirmed previous data obtained in clinical studies and suggest these molecules as possible markers of cardiac dysfunction, able to change their expression by exercise stimulus.

Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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