

## Transplantation of Adipose-Derived Stem Cells in Experimental Chronic Chagasic Cardiopathy

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### Abstract

**Background:** Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is a major cause of heart failure in Latin America. Tissue therapy has been investigated as a possible therapeutic option for patients with cardiovascular disease.

**Objective:** This study evaluated the effects of mesenchymal stem cells therapy in an experimental model of chronic Chagasic cardiomyopathy.

**Methods:** C57BL/6 mice were infected with 1000 trypomastigotes from the *T. cruzi* Colombian strain and, after six months of infection, were treated with mesenchymal human adipose-derived stem cells (ADSC) or with Dulbecco/Vogt modified Eagle's minimal essential medium – DMEM (control). The treated group received two intraperitoneal injections of ADSC (1x10<sup>6</sup> cells/dose), with a month interval between the two doses. Before and after the first and second months of treatment, the chagasic and normal control animals underwent cardiopulmonary exercise testing and electrocardiography. All animals were sacrificed under anesthesia after two months of treatment for histopathological analysis of the heart.

**Results:** No improvement was observed in arrhythmias or cardiovascular function in animals treated with ADSC; however, ADSC-treated mice heart sections revealed a significant reduction in the number of inflammatory cells ( $p < 0.0001$ ) and areas of fibrosis ( $p < 0.01$ ) in comparison with chagasic animals treated with DMEM.

**Conclusion:** Thus, it was concluded that administration of intraperitoneal ADSC can reduce inflammation and fibrosis in the heart of mice chronically infected with *T. cruzi*; however, there were no effects on cardiac function two months after transplantation (Arq Bras Cardiol. 2013; 100(5):460-468).

**Keywords:** Chagas Cardiomyopathy / therapy; Stem Cells; Tissue Therapy; Adipose Tissue.

### Introduction

Chagas disease, triggered after infection with the flagellated protozoa *Trypanosoma cruzi*, represents a serious public health problem, affecting about 18 million people in Latin America, with 200 thousand new cases per year<sup>1</sup>. It is estimated that in endemic countries, about 20,000 patients die each year from complications associated with chronic Chagas cardiomyopathy, for which there is still no sufficiently effective therapy. For these reasons, the study of new therapeutic options for patients with chronic Chagas cardiomyopathy is of fundamental importance, considering its high prevalence, morbidity and mortality, in addition to the great socioeconomic impact caused by this disease.

Several studies regarding the therapeutic potential of stem cell transplantation have been performed in recent years, especially in the area of cardiovascular diseases. Bocchi et al<sup>2</sup> studied the effect of bone marrow mononuclear cells in patients with refractory

nonischemic heart failure, resulting in improved ejection fraction, functional class and quality of life. It has also been previously demonstrated that transplantation of syngeneic bone marrow cells caused the improvement of chagasic myocarditis in mice chronically infected with *T. cruzi*<sup>3</sup>, with the possible mechanism of action being apoptosis induction of mononuclear cells from the inflammatory infiltrate, accompanied by reduction in inflammation and percentage of fibrosis. Despite the pilot clinical study using mononuclear cells in patients with chronic chagasic cardiomyopathy demonstrating suggested benefits<sup>4</sup>, these data were not confirmed by randomized clinical testing<sup>5</sup>. Thus, studies in animal models must be developed in order to investigate new therapeutic protocols utilizing stem cells.

Mesenchymal stem cells (MSC), found in the stroma of various organs including bone marrow, have been intensively studied as to their characteristics and therapeutic potential in several experimental models due to the ease with which they can be obtained and grown *in vitro*. In the work of Guarita-Souza et al<sup>6</sup>, Wistar rats with dilated chagasic cardiomyopathy and left ventricular systolic dysfunction were transplanted with a MSC co-culture of skeletal myoblasts, demonstrating significant improvement in ventricular function and diameters observed one month after transplantation.

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In this context, the present study tested the hypothesis that therapy with mesenchymal stem cells derived from human adipose tissue is capable of reducing inflammation and fibrosis and improves cardiorespiratory fitness in an experimental model of chronic chagasic cardiomyopathy in mice.

## Methods

### Animals

Thirty C57BL/6 mice were housed in the animal facility of the Center for Biotechnology and Tissue Therapy and provided with food and water *ad libitum*, under ideal temperature and light conditions. The protocol was approved by the Ethics Committee on Animal Use of São Rafael Hospital, on January 1<sup>st</sup>, 2010 (protocol number 05/10). Manipulations were performed according to the animal manipulation standards established in the "Guide for the Care and Use of Laboratory Animals" (Institute of Laboratory Animal Resources, National Academy of Sciences, Washington, D.C. 1996), abiding by the Ethical Principles in Animal Experimentation of Colégio Brasileiro de Experimentação Animal (Cobea).

### Mice infection by *T. cruzi*

Twenty C57BL/6 mice, six to eight weeks old, were inoculated intraperitoneally with 1000 *T. cruzi* Colombian strain trypomastigotes<sup>7</sup>, obtained from the supernatant of cell cultures infected with the LCC-MK2 strain. The assessment of acute infection was carried out by periodic parasitemia.

### Mesenchymal adiposed-derived stem cells

The strain of human adiposed-derived stem cells (ADSC) was obtained from the disposal of liposuction material. After incubation with collagenase (Blendzyme1, Roche), the preparation was centrifuged and the cells were cultured in DMEM media, supplemented with L-glutamine (2  $\mu$ mol/L), gentamicin (50  $\mu$ g/mL), HEPES (10  $\mu$ mol) and sodium bicarbonate (2 g/L), enriched with 10% bovine fetal serum and maintained at 37°C and 5% CO<sub>2</sub>. The ADSC were isolated from other mononuclear cells by their capacity to adhere to the plastic wells and due to their expansion, then subsequently evaluated for the expression of surface markers by flow cytometry, osteogenic and adipogenic differentiation potential and stability of chromosomes, confirming mesenchymal cells characteristics.

### Treatment of chronic chagasic animals

Each C57BL/6 mouse was transplanted intraperitoneally with  $1 \times 10^6$  human ADSC, six months after the infection with *T. cruzi*. The transplant was repeated after thirty days. The control group of infected animals was treated with DMEM, also intraperitoneally.

### Electrocardiographic evaluation

After induction of anesthesia using isoflurane (0.5 to 2%), the acquisition of electrocardiographic recordings was started. Electrocardiograms recordings were acquired using Bio Amp

PowerLab system equipment (PowerLab 2/20, ADInstruments, Castle Hill, Australia), which allows the recording of biological signals in animals with complete electrical isolation. Data were acquired and stored in a computer, and were then analyzed using the program Chart 5 for Windows (PowerLab; ADInstruments, Castle Hill, Australia). The ECG analysis included measurements of heart rate, adjusted PR and QT intervals and evaluation of the presence of arrhythmias and conduction disturbances. To minimize interference, a filter of 0.1 to 1 Hz was used.

### Functional evaluation by ergometry

For ergometric studies, LE 8700 - CO equipment (Panlab, Barcelona, Spain) was used, with chamber air flow controlled by a gas exchanger (LE 400, Panlab). A gas sample was taken by a closed circuit with the gas analyzer (OXYLET 00, Panlab), and the data was sent to the computer through an amplifier containing an analog-digital board (ML 820, PowerLab, ADInstruments, Australia). Data were stored on computer and analysed using Chart 5 for Windows - Metabolism for PowerLab System. The animals were placed on the treadmill for 20 minutes before exercise testing started. The initial speed was 12 cm/s, increasing the speed 6 cm/sec every 5 minutes. After 5 minutes, the animal entered into the second stage with a speed of 18 cm/s, and so on. Tests were conducted until the animals reached exhaustion remaining for at least 5 seconds on the shock area. To minimize interference, a 0.1 to 1 Hz filter was used. The parameters evaluated were exercise time, distance covered, final speed, maximum stage reached, oxygen consumption, and carbon dioxide production.

### Histological and morphometric evaluations

After euthanasia of the animals, the hearts and fragments of the skeletal muscle were removed and fixed in 4% formalin for histological processing. Sections of hearts and muscles were stained with hematoxylin and eosin and analyzed by bright field microscopy to count inflammatory cells, or by Masson's Trichrome to evaluate the percentage of fibrosis. The measurements were performed on four sections of 5 micrometers of whole heart, with 20 to 30 micrometers between each section, after scanned with the Aperio ScanScope system (Aperio Technologies, Vista, CA). The images were analyzed by Image Pro Plus (release 7.0, Media Cybernetics, San Diego, CA).

### Statistical Analysis

The data obtained were evaluated for parametric distribution, using Graphpad Prism 5 (2007) and BioCalc software. For PR interval, QRS duration and heart rate comparisons, one-way ANOVA with Tukey post-test was used. Fisher's test was used to compare the percentage of animals with arrhythmias. Unpaired *t* test was used for exercise testing and histopathology to compare the chronically infected animals with uninfected from the same age, and to compare chronic chagasic animals in the two groups. Results were considered significant at  $p < 0.05$ .

## Results

### Mortality

The study started with 30 C57BL/6 mice, divided into three groups: uninfected controls (n = 10); chronic chagasic animals treated with DMEM (n = 10), and chronic chagasic animals treated with ADSC (n = 10). There were no deaths among the uninfected animals and those treated with DMEM. Two deaths were observed in the group of animals treated with ADSC, and one of them was considered to still be in the pretreatment phase, with death being caused by abdominal hemorrhagic accident during intraperitoneal stem cells infusion. The second death in this group occurred approximately one month after transplanting CTTA due to a non-identified cause. There was no statistical significance in survival rate between the groups.

### Electrocardiographic results

In the analysis of electrocardiographic intervals, no statistically significant difference was found when comparing the two chagasic groups. There was a statistically significant difference, with ( $p < 0.001$ ), when comparing the PR interval of uninfected animals with chagasic animals treated with DMEM or with ADSC, which did not occur with the QTc interval. There was no ADSC therapy influence in terms of prolongation of the PR interval when this group was assessed in the pretreatment and post-treatment phases. The PR and QTc intervals remained stable in both groups of chagasic animals throughout the study period (Table 1).

Evaluating the presence of cardiac arrhythmias among the chagasic animals treated with DMEM revealed two animals with complete atrioventricular block (CAVB). Three animals in this group already had CAVB in the pre-treatment phase, and two developed CAVB concurrently with frequent ventricular extrasystoles in the post-treatment phase.

Among mice treated with ADSC, three animals had CAVB in the pre-treatment phase, and one of them showed arrhythmia reversal, with periods of sinus rhythm, which did not occur in any of the animals treated with DMEM. Of the four animals in the ADSC group with normal ECGs, one developed 2nd degree type II atrioventricular block (AVB) and three animals developed CAVB.

The differences between the percentages of animal arrhythmias in general, and CAVB in particular, did not reach statistical significance when comparing the groups treated with DMEM or ADSC. Also, there was no difference between groups during the two times of infection, although a trend

towards increased arrhythmias was observed in both groups. For arrhythmias in general, the percentages were 57% and 71% in animals treated with DMEM, and 33% and 78% in animals treated with ADSC, in pre-and post-treatment phases respectively. For CAVB, the percentages were 43% and 71% in animals treated with DMEM and 33% and 56% in animals treated with ADSC, in pre-and post-treatment phases respectively (Figure 1).

### Results from ergoespirometry functional evaluation

Regarding exercise time parameters, distance traveled, final speed and maximum stage reached, there was no statistically significant difference between the two groups of chagasic animals, when compared with each other or when considered separately in the pre-and post-treatment phases. All parameters were significantly different between uninfected animals and chagasic animals. The exercise time, in seconds, was  $2577 \pm 371$  in uninfected animals;  $1840 \pm 342$  and  $1620 \pm 690$  in the DMEM group at pretreatment phase and after 2 months of treatment, respectively; and  $1570 \pm 436$  and  $1278 \pm 454$  in the ADSC group in pre- and post-treatment phases, respectively. The distance run, in meters, was  $730 \pm 187$  in uninfected animals;  $396 \pm 127$  and  $342 \pm 171$  in the DMEM group at pretreatment phase and after 2 months of treatment, respectively; and  $358 \pm 131$  and  $221 \pm 125$  in the ADSC group in pre- and post-treatment phases, respectively (Figure 2).

After the first month, the DMEM group developed an increase in  $VO_2$ , a value that was maintained through the second month of observation. The ADSC group showed a tendency to increase  $VO_2$  after treatment; however, there was no statistical significance.

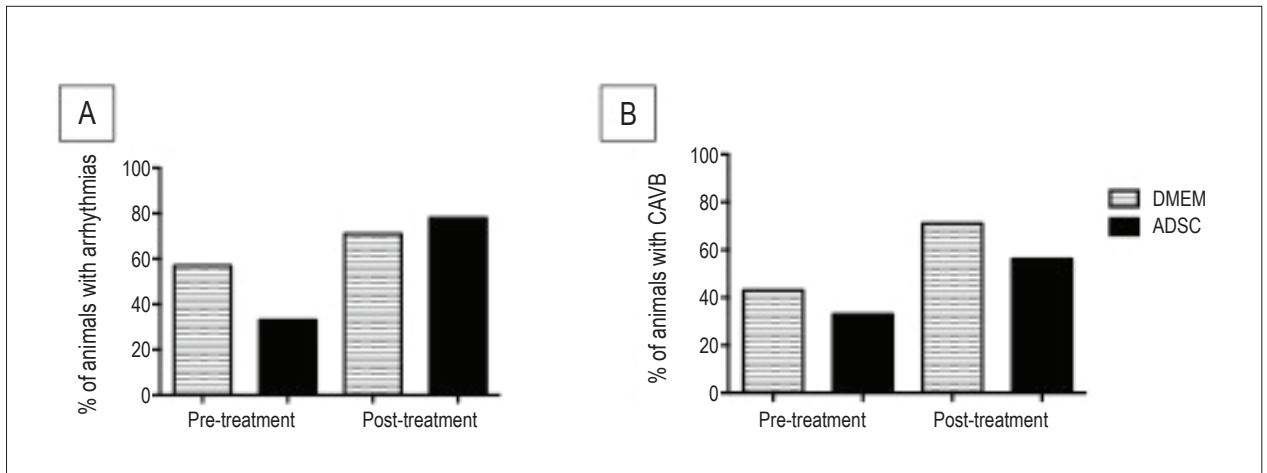
As for the production of carbon dioxide,  $VCO_2$  at rest increased in the DMEM group after the first month, but not in the ADSC group. There was a statistically significant drop in  $VCO_2$  at rest and peak effort after the second month in the DMEM and ADSC groups, when compared with measurements following the first month of treatment.

The  $VO_2$  at rest, in mL/Kg/min, was  $3959 \pm 830$  in uninfected animals;  $2779 \pm 1004$  and  $3925 \pm 1158$  in the DMEM group in pre-phase and after two months of treatment, respectively; and  $3442 \pm 770$  and  $4094 \pm 1203$  in the ADSC group in pre- and post-treatment phases, respectively. At peak stress, these values were  $6107 \pm 983$  in uninfected animals;  $4213 \pm 1438$  and  $5540 \pm 1088$  in the DMEM group in pre-phase and after two months of treatment, respectively; and  $5479 \pm 1061$  and  $5000 \pm 1475$  in the ADSC group in pre- and post-treatment phases, respectively.

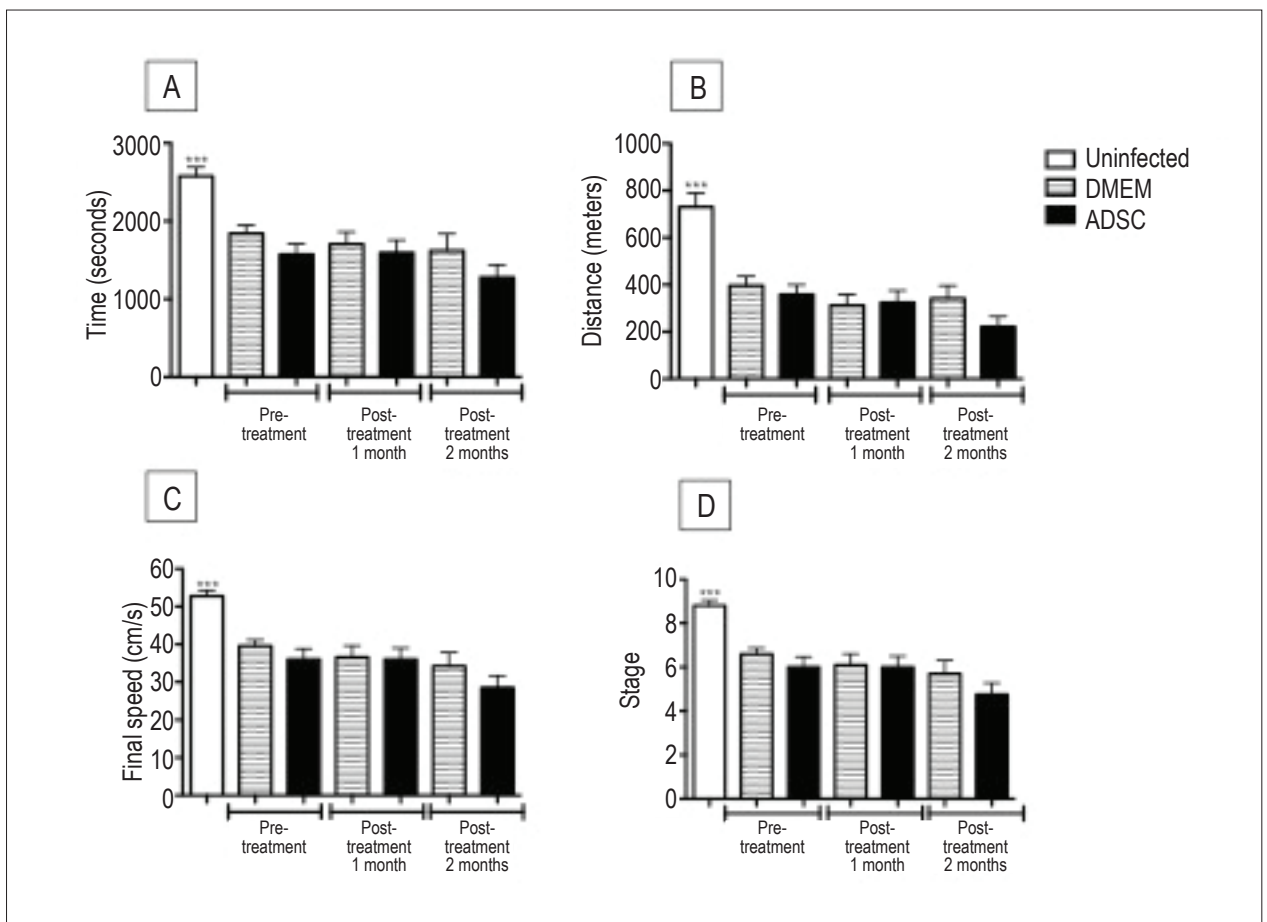
**Table 1 - Values of PR interval and QTc in ms in the control, DMEM and ADSC groups**

	Controls	Pre-treatment		Post-treatment	
		DMEM	ADSC	DMEM	ADSC
PR (ms)	$53.3 \pm 5.8^*$	$82.5 \pm 2.6$	$81.5 \pm 20.5$	$81.7 \pm 12.6$	$95.0 \pm 7.1$
QTc (ms)	$32.4 \pm 8.1$	$30.6 \pm 4.7$	$33.2 \pm 7.4$	$29.3 \pm 3.6$	$30.0 \pm 5.9$

*p < 0.001 for the comparison of the PR interval values of the uninfected chagasic animals of both groups. Other results without statistical significance.*



**Figure 1** - Evaluation of arrhythmias in chronic chagasic animals treated with medium (DMEM) or with adipose-derived stem cells (ADSC) at different stages of treatment. Percentage of arrhythmias (A) and CAVB (B) in chagasic mice in pre- (6 mpi) and post-treatment (8 mpi) phases. Results expressed in percentage of seven of the animals of the DMEM group and 8 of the animals of the ADSC group.



**Figure 2** - Evaluation of ergometric data in uninfected and chronic chagasic animals treated with medium (DMEM) or with adipose-derived stem cells (ADSC) at different stages of treatment. (A) Time of exercise. (B) Distance run. (C) Final speed achieved. (D) Maximum stage reached. Results are expressed as mean  $\pm$  standard error of 10 uninfected animals, 7 animals of the DMEM group and 8 animals of the ADSC group. \*\*\*  $p < 0.0001$ .



The  $\text{VCO}_2$  at rest, in mL/Kg/min, was  $3716 \pm 1113$  in uninfected animals;  $3544 \pm 472$  and  $1779 \pm 1222$  in the DMEM group in pre-phase and after two months of treatment, respectively; and  $3988 \pm 366$  and  $1993 \pm 1784$  in the ADSC group in pre- and post-treatment phases, respectively. At peak stress, these values were  $5171 \pm 1454$  in uninfected animals;  $4894 \pm 880$  and  $2393 \pm 1610$  in the DMEM group in pre-phase and after two months of treatment, respectively; and  $5261 \pm 688$  and  $2425 \pm 1802$  in the ADSC group in pre- and post-treatment phases, respectively. (Figure 3).

### Histological and morphological evaluations

Hearts sections from chronic chagasic mice showed histological characteristics of chronic chagasic cardiomyopathy (Figure 4). Noted was the presence of focal inflammatory infiltrates and disseminated compounds, predominantly mononuclear cells, myocytolysis, myonecrosis and fibrosis. Both groups (treated with DMEM or ADSC) showed a similar pattern, but the degree of inflammation and fibrosis of the hearts in the animals treated with ADSC was lower than those treated with DMEM.

In Figure 4A, a section of a normal heart with Masson's trichrome staining shows an arteriolar structure, with surrounding collagen (stained in blue), normal cardiac fibers and the absence of inflammatory infiltrates. Figure 4B shows chronic chagasic heart sections treated with DMEM, stained with Masson's trichrome, showing intense multifocal inflammatory infiltrates produced by mononuclear cells often adhered to cardiac fibers, producing myocytolytic lesions, with the inflamed areas interspersed with intense fibrosis (stained in blue).

Figure 4C demonstrates a sectioning of a chronic chagasic heart treated with ADSC, stained with Masson's trichrome, with discrete focal infiltrates consisting of mononuclear cells and inflamed areas interspersed with mild fibrosis (stained in blue).

Evaluation and comparison, by morphometry, of inflammation and fibrosis between the two groups of chagasic animals, revealed a significant reduction in fibrosis and inflammation in animals treated with ADSC. The number of inflammatory cells per  $\text{mm}^2$  was  $228.5 \pm 80.4$  in uninfected animals;  $758.4 \pm 194.7$  in the DMEM group, and  $382.4 \pm 91.9$  in the ADSC group. The percentage of fibrosis in the heart was  $2.60.5 \pm 1.78$  in uninfected animals;  $8.95 \pm 3.31$  in the DMEM group; and  $3.89 \pm 1.14$  in the ADSC group (Figure 5).

In addition to the heart, a histopathological evaluation of skeletal muscle was performed. Both chagasic animals treated with DMEM and those treated with ADSC showed skeletal muscle inflammation, which featured intense myositis observed in the chronic phase of the disease (data not shown).

### Discussion

This study demonstrated reduced inflammation and fibrosis in the hearts of mice with chagasic cardiomyopathy induced by the Colombian strain of *Trypanosoma cruzi*, treated with ADSC. Earlier studies had shown similar data, but with the use of mononuclear cells derived from bone marrow<sup>3</sup>. Despite this, treatment with ADSC did not influence the development

of cardiac arrhythmias and did not result in improvement of ergometric parameters, with a low tolerance to stress observed in keeping with disease progression.

The beneficial effects of therapy with mesenchymal cells, through their regenerative potential, have already been demonstrated in several clinical and experimental studies, such as in diseases affecting bone and cartilage<sup>8</sup>, renal impairment<sup>9</sup>, cardiovascular disease<sup>10</sup> and pulmonary<sup>11</sup> diseases, among others. In addition to the regenerative potential, the immunosuppressive activity of these cells was also identified<sup>12,13</sup>, which can modulate the function of T lymphocytes, which are basic to the development of the adaptive immune response.

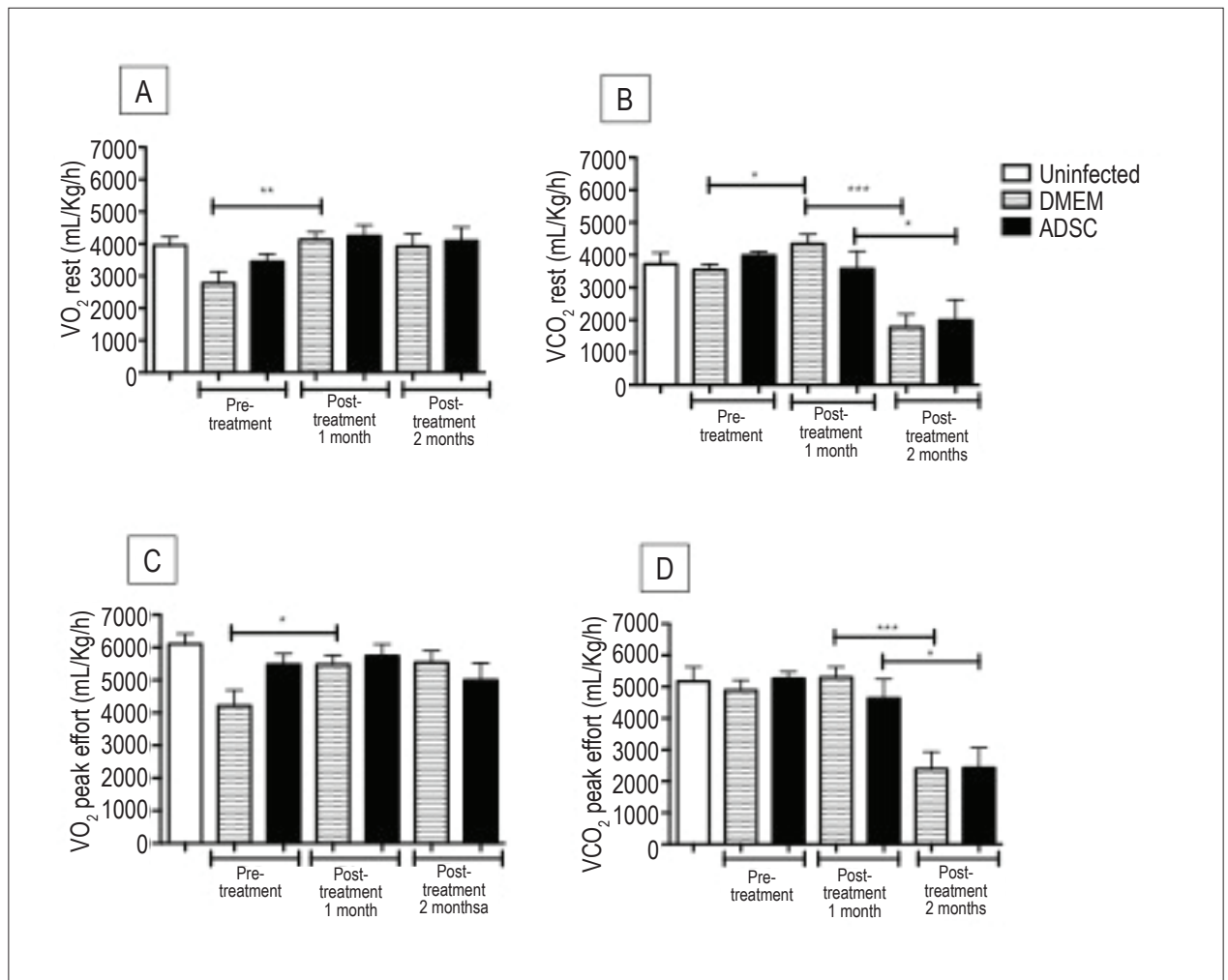
Therefore, it is possible that the effects of ADSC in reducing inflammation and fibrosis, as seen in this study, are due to this immunomodulating property, which has already been previously described. The fact that a reduction in the percentage of arrhythmias was not evident suggests that there may not have been tissue regeneration and/or recovery of the cardiac conduction system after using these therapy schemes, at least in the short post-treatment time assessed.

In the work of Guarita-Souza et al<sup>6</sup>, Wistar rats were infected with  $15 \times 10^4$  trypomastigotes, subsequently developing dilated cardiomyopathy with left ventricular systolic dysfunction. These animals were transplanted with MSC coculture of skeletal myoblasts and, within one month of transplantation, an important improvement of ventricular function and diameters was observed. The use of another type of cell along with MSC makes it difficult to evaluate the specific role of MSC in this model. It is possible that skeletal myoblasts act on the recolonization of fibrotic areas, thereby promoting the improvement of cardiac function.

In terms of the cell type used in our study, a few advantages have been previously described with regards to the use of adiposed-derived stem cells for the treatment of cardiac diseases, in comparison to those of the bone marrow which have already been described<sup>14</sup>, as well as their differentiation capacity in cardiomyocytes<sup>15,16</sup>. As to the use of xenogenic cells (human cells in mice), previous studies have already shown the safety and potential effectiveness of these cells, which was demonstrated by Cai et al<sup>17</sup> using a model of myocardial infarction in rats. Similarly, Hwangbo et al<sup>18</sup> evaluated the effect of transplantation of human ADSC in Sprague-Dawley rats with myocardial infarction, evidencing a significant improvement in left ventricular function.

In the present study, the intraperitoneal route was used based on previous studies reporting animals death following intravenous MSC administration. In a study of non-ischemic refractory heart failure, the intracoronary and intravenous routes were used with satisfactory results, but the study utilized mononuclear and not mesenchymal cells<sup>2</sup>.

Also investigating mononuclear cells derived from bone marrow, Nakamuta et al<sup>19</sup> demonstrated greater cardiac cell retention in an experimental model of myocardial infarction when cells were implanted intramuscularly. However, Furlani et al<sup>20</sup> assessed, by means of intravital microscopy, migration kinetics of human MSC after intravascular administration in SCID mice via a catheter inserted into the infrarenal abdominal aorta. In this study, the size of the suspended MSC ranged from 16 to 53  $\mu\text{m}$ , with interference being observed in blood



**Figure 3** - Evaluation of  $VO_2$  and  $VCO_2$  in uninfected animals and chronic chagasic animals treated with medium (DMEM) or with adipose-derived stem cells (ADSC) at different stages of treatment.  $VO_2$  rest (A) and peak effort (C).  $VCO_2$  at rest (B) and peak effort (D). Results are expressed as mean  $\pm$  standard error of 10 uninfected animals, 7 animals of the DMEM group and 8 animals of the ADSC group. \*  $p < 0.05$ . \*\*  $p < 0.001$ . \*\*\*  $p < 0.0001$ .

microcirculation due to cell density, including interruption of blood flow and thrombus formation in arterioles and venules in the animals in which the MSC was injected.

In another study, Gordon et al<sup>21</sup> demonstrated the therapeutic effect of intraperitoneal injection of human mesenchymal stem cells in mice with autoimmune allergic encephalomyelitis. In addition to preventing animal loss due to embolism, these studies indicate that intraperitoneal administration does not compromise the effects of these cells.

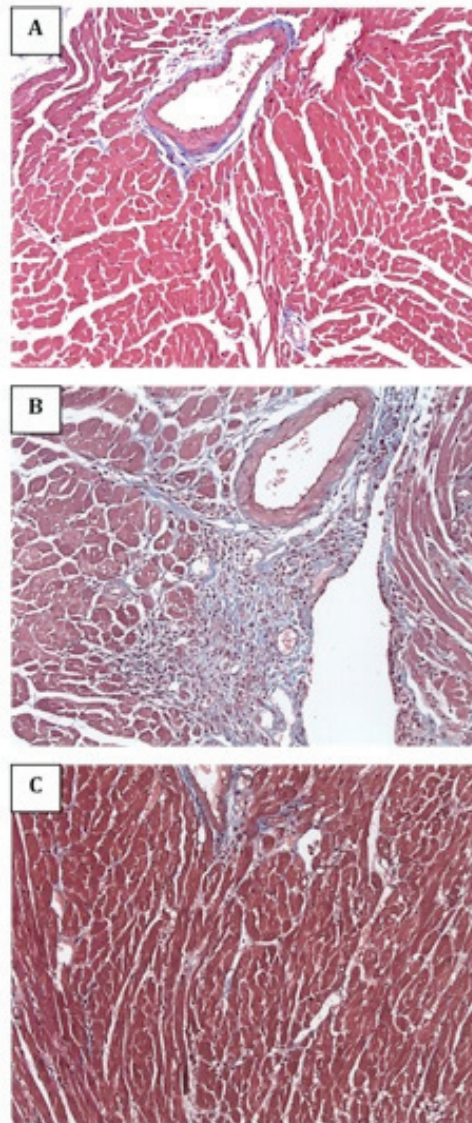
A limitation of this study is a bias in the evaluation of the ergometry, caused by the presence of inflammation in skeletal muscle observed in the chagasic animals. Even in animals that did not have CAVB, there was poor performance in exercise testing, especially in exercise time and distance run, in addition to a limping gait.

Histological and morphometric evaluation of skeletal muscle sections revealed a large amount of inflammatory cells, which is characteristic of myositis and is considered a limiting orthopedic factor for the progression of stress in chagasic

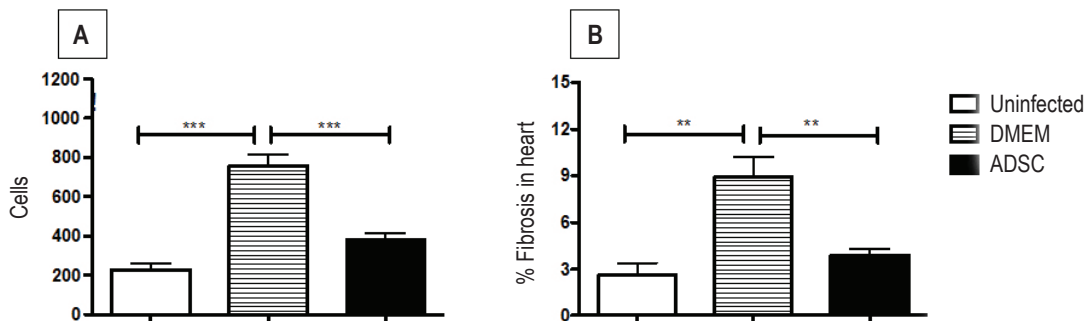
animals in our study. In a different protocol, therapeutic effects are evaluated with low doses of benznidazole in skeletal myositis in mice chronically infected with *T. cruzi* that have undergone transplantation of cardiac mesenchymal stem cells.

## Conclusion

In summary, this study contributed to evaluating the therapeutic effects of ADSC in the arrhythmic form of Chagas disease and has demonstrated that treatment with ADSC did not reduce the incidence of cardiac arrhythmias in mice chronically infected with the *T. cruzi* Colombian strain. Treated animals had reduced inflammation and fibrosis, with values similar to those found in uninfected animals, when histological and morphometric evaluation was performed. Further studies may contribute to the development of protocols, accompanied by adjustments in therapy and experimental model, until a more effective therapeutic approach can be developed to justify further studies in patients with chronic chagasic cardiomyopathy.



**Figure 4** - Histology of sections of hearts from mice euthanized two months after tissue therapy. (A), Uninfected animal. (B) Chronic chagasic animal treated with DMEM. (C), Chronic chagasic animal treated with adipose-derived stem cells (ADSC). Sections stained with Masson's trichrome. Magnification: 200 x.



**Figure 5** - Morphometric evaluation of sections from uninfected animal hearts and the hearts of chagasic animals treated with medium (DMEM) or with adipose-derived stem cells (ADSC). (A) Number of inflammatory cells per mm<sup>2</sup> measured in sections stained with H&E. (B) Percentage of fibrosis quantified in sections stained with Masson's trichrome. Results are expressed as mean  $\pm$  standard error for 5 uninfected animals, 6 animals of the DMEM group and 8 animals of the ADSC group. \*\*  $p < 0.01$ . \*\*\*  $p < 0.0001$ .

## Author contributions

Conception and design of the research: Larocca TF, Souza BSF, Soares MBP, Ribeiro-dos-Santos R; Acquisition of data: Larocca TF, Souza BSF; Analysis and interpretation of the data: Larocca TF, Souza BSF, Macambira SG; statistical analysis: Larocca TF; Obtaining funding: Soares MBP, Ribeiro-dos-Santos R; Writing of the manuscript: Larocca TF, Soares MBP; Critical revision of the manuscript for intellectual content: Macambira SG, Soares MBP, Ribeiro-dos-Santos R; Veterinary follow-up of the animals: Silva CA; Parenchymal cell preparation: Kaneto CM; Cytokine and chemokine profile analysis: Alcântara AC; Analysis by immunofluorescence and parasitemia: Azevedo CM; Complementary tests on the animals: Castro MF.

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## Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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## Study Association

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