Transplantation of Adipose Tissue Mesenchymal 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 therapy with mesenchymal stem cells in an experimental model of chronic Chagasic cardiomyopathy.

Methods: C57BL/6 mice were infected with 1000 trypomastigotes from the Colombian strain of T. cruzi and, after six months of infection, were treated with mesenchymal human stem cells from adipose tissue (STAT) or with Dulbecco/Vogt modified Eagle’s minimal essential medium – DMEM (control). The treated group received two intraperitoneal injections of STAT (1x10^6 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 and cardiovascular function in the group of animals treated with STAT; however, sections of mice hearts in this group 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 is concluded that administration of intraperitoneal STAT can reduce inflammation and fibrosis in the heart of mice chronically infected with T. cruzi; however, there were no effects on the cardiac function two months after transplantation (Arq Bras Cardiol. 2013; [online].ahead print, PP.0-0).

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

Introduction

Chagas disease, triggered after infection with the flagellated protozoon Trypanosoma cruzi, represents a serious public health problem, affecting about 18 million people in Latin America, with 200 thousand new cases per year1. 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 and high 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 al2 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 causes the improvement of chagasic myocarditis in mice chronically infected with T. cruzi3, with the possible mechanism of action being the induction of apoptosis of the mononuclear cells of the inflammatory infiltrate, with reduction of inflammation and percentage of fibrosis. Despite the pilot clinical study with the use of mononuclear cells in patients with chronic chagasic myocardiopathy having suggested benefits4, these data were not confirmed by randomized clinical testing4. 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 al6, Wistar rats with dilated chagasic cardiomyopathy and left ventricular systolic dysfunction were transplanted with an MSC coculture of skeletal myoblasts, with 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 mice of the C57BL/6 strain were kept in the animal house of the Center for Biotechnology and Tissue Therapy and provided with food and water ad libitum, under ideal conditions of temperature and luminosity. The protocol was approved by the Ethics Committee on Animal Use of São Rafael Hospital, on January 1st, 2010 under 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 mice of the C57BL/6 strain, six to eight weeks old, were inoculated intraperitoneally with 1000 trypomastigotes of the Colombian strain of T. cruzi, 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 stem cells from adipose tissue

The strain of human stem cells from adipose tissue (SCAT) was obtained from the disposal of liposuction material. After incubation with collagenase (Blendingyme1, Roche), the preparation was centrifuged and the cells were cultured in DMEM media, supplemented with L-glutamine (2 μmol/L), gentamicin (50 μg/mL), Hepes (10 μmol) and sodium bicarbonate (2 g/L), enriched with 10% bovine fetal serum and kept in the oven at 37°C and 5% CO2. The SCAT were isolated from other mononuclear cells by their capacity to adhere to plastic and due to their expansion, being subsequently evaluated as to the expression of surface markers by flow cytometry, osteogenic and adipogenic differentiation potential and stability of chromosomes, confirming the characteristics of mesenchymal cells.

Treatment of chronic chagasic animals

Each C57BL/6 mouse was transplanted intraperitoneally with 1 x 10⁶ human SCAT, 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. Recordings of electrocardiograms 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 (Power Lab; ADInstruments, Castle Hill, Australia). The ECG analysis included measurements of heart rate, adjusted PR and QT intervals and evaluation as to 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 air flow in the chamber controlled by a gas exchanger (LE 400, Panlab), and the data was sent to the computer through an amplifier containing an analog-digital board (ML 820, PowerLab, ADInstruments, Australia). The data were stored on computer for analysis using the program 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. The first stage was established by the starting speed of 12 cm/s. 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 lasting for 5 seconds or showed signs of shock. 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 of the animals 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 whole heart, with 20 to 30 micrometers between each section, after scanning with the Aperio ScanScope system (Aperio Technologies, Vista, CA). The images were analyzed with the program Image Pro Plus (release 7.0, Media Cybernetics, San Diego, CA).

Statistical Analysis

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

Mortality

The study started with 30 mice of the C57BL/6 strain, divided into three groups: uninfected controls (n = 10); chronic chagasic animals treated with DMEM (n = 10), and chronic chagasic animals treated with SCAT (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 SCAT, and one of them was considered to still be in the pretreatment phase, with death being caused by abdominal hemorrhagic accident during intraperitoneal infusion of the stem cells. 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 between the two chagasic groups of animals when the two groups were compared. 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 STAT, which did not occur with the QTc interval. There was no STAT therapy influence in terms of prolongation of the PR interval when this group was assessed at 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).

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

Among mice treated with STAT, 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 four animals in the STAT 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 STAT. Also, there was no difference when the groups were compared during the two times of infection, although a trend towards increased arrhythmias had been 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 STAT, 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 STAT, in pre-and post-treatment phases respectively (Figure 1).

Results from the functional evaluation of ergoespirometry

Regarding the parameters of exercise time, 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 in general. The exercise time, in seconds, was 2577 ± 371 in uninfected animals; 1840 ± 342 and 1620 ± 690 in the group of DMEM animals at pretreatment phase and after 2 months of treatment, respectively; and 1570 ± 436 and 1278 ± 454 in animals in the STAT group in the pre- and post-treatment phases, respectively. The distance run, in meters, was 730 ± 187 in uninfected animals; 396 ± 127 and 342 ± 171 in the group of DMEM animals at pretreatment phase and after 2 months of treatment, respectively; and 358 ± 131 and 221 ± 125 in animals of the STAT group in the pre- and post-treatment phases, respectively (Figure 2).

After the first month, the DMEM group developed an increase of VO2, with this value maintained after the second month of observation. The STAT group showed a tendency to increased VO2 after treatment; however, the standard error was of statistical significance.

As for the production of carbon dioxide, it was observed there was an increase of VCO2 at rest in the DMEM group after the first month, but not in the STAT group. There was a sharp drop in VCO2 at rest and at peak effort after the second month in the DMEM and STAT groups, with statistical significance when compared with the first month after treatment.

The VO2 at rest, in mL/Kg/min, was 3959 ± 830 in uninfected animals; 2779 ± 1004 and 3925 ± 1158 in animals of group DMEM in the pre-phase and after two months of treatment, respectively; and 3442 ± 770 and 4094 ± 1203 in the animals of the STAT group in the pre- and post-treatment phases, respectively. At peak stress, these values were 6107 ± 983 in uninfected animals; 4213 ± 1438 and 5540 ± 1088 in animals of the DMEM group in the pre-phase and after two months of

<p>| Table 1 - Values of PR interval and QTc in ms in the control, DMEM and STAT groups |
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<th>Controls</th>
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<th>Pre-treatment DMEM</th>
<th>STAT</th>
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<td>PR (ms)</td>
<td>53.3 ± 5.8 *</td>
<td>82.5 ± 2.6</td>
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<tr>
<td>QTc (ms)</td>
<td>32.4 ± 8.1</td>
<td>30.6 ± 4.7</td>
<td>33.2 ± 7.4</td>
<td>29.3 ± 3.6</td>
<td>30.0 ± 5.9</td>
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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 stem cells derived from adipose tissue (STAT) at different stages of treatment. Percentage of arrhythmias (A) and CAVB (B) in chagasic mice in the 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 STAT group.

Figure 2 - Evaluation of ergometric data in uninfected and chronic chagasic animals treated with medium (DMEM) or with stem cells from adipose tissue (STAT) 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 ± standard error of 10 uninfected animals, 7 of the animals of group DMEM and 8 animals of group STAT. *** p < 0.0001.
treatment, respectively; and 5479 ± 1061 and 5000 ± 1475 in the animals of group STAT in the pre- and post-treatment phases, respectively. The VCO, at rest, in mL/kg/min, was 3716 ± 1113 in uninfected animals; 3544 ± 472 and 1779 ± 1222 in animals of group DMEM in the pre-phase and after two months of treatment, respectively; and 3988 ± 366 and 1993 ± 1784 in the animals of the STAT group in the pre- and post-treatment phases, respectively. At peak stress, these values were 5171 ± 1454 in uninfected animals; 4894 ± 880 and 2393 ± 1610 in animals of the DMEM group in the pre-phase and after two months of treatment, respectively; and 5261 ± 688 and 2425 ± 1802 in the animals of the STAT group in the pre- and post-treatment phases, respectively. (Figure 3).

Histological and morphological evaluations

Sections of hearts from chronic chagasic mice showed histological characteristics of chronic chagasic cardiomyopathy (Figure 4). Note the presence of focal inflammatory infiltrates and disseminated compounds, predominantly mononuclear cells, myocytolysis, myonecrosis and fibrosis. Both groups (treated with DMEM or STAT) showed a similar pattern, but the degree of inflammation and fibrosis of the hearts of the animals treated with STAT 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 absence of inflammatory infiltrates. Figure 4B shows chronic chagasic heart sections, stained with Masson's trichrome, showing intense multifocal inflammatory infiltrates produced by mononuclear cells often adhered to cardiac fibers, producing myocytolitic lesions, with the inflamed areas interspersed with intense fibrosis (stained in blue). In Figure 4C, there is a sectioning of a chronic chagasic heart treated with STAT, stained with Masson's trichrome, with discrete focal infiltrates consisting of mononuclear cells and the inflamed areas interspersed with mild fibrosis (stained in blue).

In evaluating and comparing, by morphometry, the inflammation and fibrosis between the two groups of chagasic animals, it was noticed there was a reduction of fibrosis and inflammation in animals treated with STAT, with statistical significance. The number of inflammatory cells per mm² was 228.5 ± 80.4 in uninfected animals; 758.4 ± 194.7 for the animals of group DMEM, and 382.4 ± 91.9 in animals from group STAT. The percentage of fibrosis in the heart was 2.60.5 ± 1.78 in uninfected animals; 8.95 ± 3.31 in the animals of group DMEM; and 3.89 ± 1.14 in animals from group STAT (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 STAT showed inflammation in skeletal muscle, featuring an intense myositis observed in the chronic phase of the disease (data not shown).

Discussion

This study demonstrated a reduction of inflammation and fibrosis in the hearts of mice with chagasic cardiomyopathy induced by the Colombian strain of Trypanosoma cruzi, treated with STAT. Earlier studies had shown similar data, but with the use of mononuclear cells derived from bone marrow. Despite this, treatment with STAT 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, renal impairment, cardiovascular disease and pulmonary diseases, among others. In addition to the regenerative potential, the immunosuppressive activity of these cells was also identified, 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 STAT in reducing inflammation and fibrosis, as seen in this study, are due to this immunomodulating property, which has already been described in several articles in the literature. 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, Wistar rats were infected with 15 x 10⁴ trypomastigotes, subsequently developing delayed 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 the MSC makes it difficult to evaluate the actual 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 cellular type used in our study, a few advantages have been described previously with regards to the use of stem cells from adipose tissue for the treatment of cardiac diseases, in comparison to those of the bone marrow which have already been described, as well as their differentiation capacity in cardiomyocytes. As to the use of xenogenic cells (human cells in mice), previous studies have already shown the safety and potential effectiveness of these cells, such as the article published by Cai et al regarding a model of myocardial infarction in rats. Similarly, Hwangbo et al evaluated the effect of transplantation of human STAT in Sprague-Dawley rats with myocardial infarction, with evidence of significant improvement in left ventricular function.

In the present study, the intraperitoneal route was used because previous studies have reported the death of animals following administration of intravenous MSC. In a study of non-ischemic refractory heart failure, the intracoronary and intravenous routes were used with satisfactory results, but the cells studied were mononuclear and not mesenchymal cells. Also using mononuclear cells derived from bone marrow, Nakamuta et al demonstrated greater cardiac cell retention in an experimental model of myocardial infarction when cells were implanted intramuscularly. However, Furlani et al assessed, by means of intravitral microscopy, the kinetics of the migration 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 µm, with interference being observed in blood 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. demonstrated the therapeutic effect of intraperitoneal injection of human mesenchymal stem cells in mice with autoimmune allergic encephalomyelitis. In addition to preventing loss of animals 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, it was observed there was poor performance in exercise testing, especially in parameters of exercise time and distance run, in addition to a limping gait. In evaluating the histology and morphometry of sections of skeletal muscle, a large amount of inflammatory cells were identified, characterizing myositis, considered as a limiting orthopedic factor for the progression of stress in chagasic animals in our work. In another protocol, the effects of the therapy are evaluated with low doses of benznidazole in skeletal myositis in mice chronically infected with T. cruzi who have undergone transplantation of cardiac mesenchymal stem cells.

**Conclusion**

In summary, this study contributed to evaluating the effects of therapy with STAT in the arrhythmic form of Chagas disease and has demonstrated that treatment with STAT did not reduce the incidence of cardiac arrhythmias in mice chronically infected with the Colombian strain of T. cruzi. Treated animals had a reduction of inflammation and fibrosis, with values similar to those found in uninfected animals, when the histological and morphometric evaluation was performed. Further studies may contribute to the development...
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 stem cells derived from adipose tissue (STAT). 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 stem cells from adipose tissue (STAT). (A) Number of inflammatory cells per mm² measured in sections stained with H&E. (B) Percentage of fibrosis quantified in sections stained with Masson’s trichrome. Results are expressed as mean ± standard error for 5 uninfected animals, 6 animals of group DMEM and 8 animals from group STAT. ** p < 0.01. *** p < 0.0001.
of protocols, with adjustment in therapy and experimental model, until a therapeutic approach can be developed that is effective enough to justify further studies in patients with chronic chagasic cardiomyopathy.

Potential Conflict of Interest

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

References


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Study Association

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