

Cell Transplantation After The Coculture of Skeletal Myoblasts and Mesenchymal Stem Cells in the Regeneration of the Myocardium Scar: An Experimental Study in Rats

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ABSTRACT

In myocardial infarction and Chagas's disease, some physiopathological aspects are common: cardiomyocyte loss due to ischemia leads to a reduction of contractility and heart function. Different cells have been proposed for cellular cardiomyoplasty.

Objective. Our goal was to evaluate the method of co-culture of skeletal muscle (SM) and mesenchymal stem cells (MSC) for cell therapy of heart failure in Chagas's disease (CD) and myocardium postinfarction (MI).

Methods. For MI, 39 rats completed the study at 1 month. Seventeen rats received cell therapy into the scar and 22 rats only medium. For CD, 15 rats completed the study at 1 month including 7 that received cell therapy and eight followed the natural evolution. All animals underwent ecocardiographic analysis at baseline and 1 month. Left ventricular, ejection fraction, end systolic, and end diastolic volume were registered and analyzed by ANOVA. The co-culture method of SM and MSC was performed at 14 days (DMEM, with 15% FCS, 1% antibiotic, IGF-I, dexamethasone). Standard stain analysis was performed.

Results. For MI ejection fraction in the animals that received the co-cultured cells increased from 23.52 ± 8.67 to 31.45 ± 8.87 ($P = .006$) versus the results in the control group: 26.68 ± 6.92 to 22.32 ± 6.94 ($P = .004$). For CD, ejection fraction in animals that received the co-cultured cells increased from 31.10 ± 5.78 to 53.37 ± 5.84 ($P < .001$) versus the control group values of 36.21 ± 3.70 to 38.19 ± 7.03 ($P = 0.426$). Histopathological analysis of the animals receiving co-cultured cells demonstrated the presence of myogenesis and angiogenesis.

Conclusion. The results validated the product of SM and MSC co-cultures for treatment of diseases.

Cardiomyocyte death may result from myocardial infarction or other cardiomyopathy, causing fibrosis in remodeling of the left ventricle due to the fact that adult cardiomyocytes have only limited capacity to regenerate and are insufficient to resolve heart tissue injury.

In myocardial infarction and Chagas's disease, some physiopathological aspects are common, resulting in a reduction of contractility and heart function.

In myocardial infarction there is a loss of cardiac vascular supply, accompanied by pro-inflammatory events with increased production of interleukin 6 and tumor necrosis factor leading to cellular necrosis, loss of cardiomyocytes

and heart dysfunction.¹ In Chagas's disease, infection by *Trypanosoma cruzi* causes a generalized inflammatory vas-

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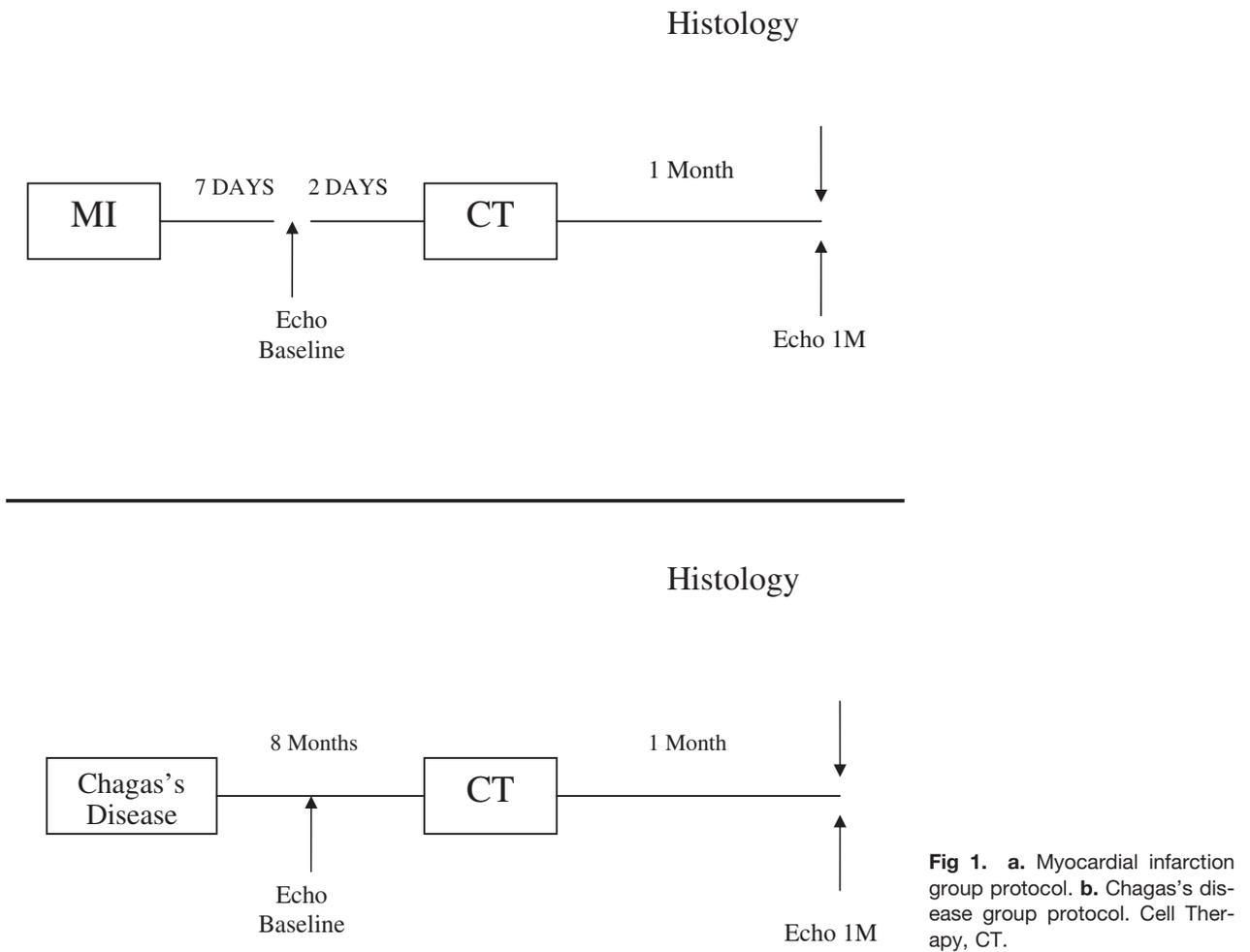


Fig 1. a. Myocardial infarction group protocol. **b.** Chagas's disease group protocol. Cell Therapy, CT.

cular disease characterized by the presence of vasospasm, reduction of blood flow, focal ischemia, thrombosis, increased platelet aggregation, and higher levels of thrombox-

ane A₂ and endothelin-1.² Endothelial cell infection by the parasite increases with the synthesis of endothelin-1, which participates in vasospasm of the coronary microcirculation.³

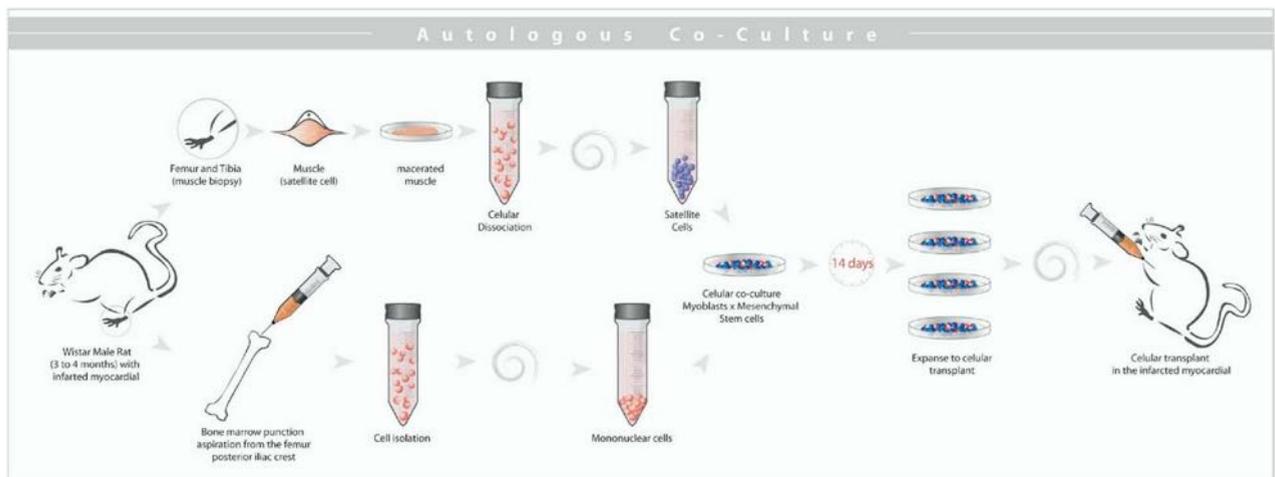


Fig 2. Autologous co-culture process.

Research in the area of cell therapy has been proposed for the treatment of heart failure through transplant of various cell types, such as mesenchymal stem cells (MSC) and skeletal myoblasts (SM).⁴⁻⁸ MSC and SM have been utilized due to their capacity to differentiate, making collection easier and autogenous cell transplantation possible without the need for immunosuppressors.

The objective of this study was to evaluate a method of co-culture of skeletal myoblasts and mesenchymal stem cells for transplantation treatment of heart failure in two diseases that show similar physiopathological mechanisms: myocardial Chagas's disease and myocardial postinfarction.

METHODS

All experiments were performed in accordance with the principles of treatment of laboratory animals of the Brazilian College of Experiments in Animals (Cobea).

Experimental Model: The Protocol of Myocardial Infarction Group

In the myocardial infarction group, 80 Wistar male rats weighing 250 to 300 g, were anesthetized with xylazine (50 mg/kg) and ketamine (10 mg/kg), ventilated with the small animal respirator (683 Havard model. Apparatus, Inc. USA) and occlusion of the left coronary artery. One week after artery occlusion, left ventricular function was registered by the first echocardiography (baseline) (Fig 1a).

Of the 80 animals, the 60 that demonstrated an ejection fraction less than 40% were randomly treated. Thirty-nine rats completed the study at 1 month. In the co-culture group, 17 rats received 7.5×10^6 cells with 200 μ L of Dulbecco's Modified Eagle Medium (DMEM; Gibcco, USA) in the scar. Since the cell transplants were allografts, they received intraperitoneal injections of cyclosporine (15 mg/weight/per day). In the control group, 22 animals were injected with 200 μ L of DMEM (without cells) into the scar.

Experimental Model: The Protocol of Chagas's Disease Group

In the Chagas's disease group, 80 Wistar male rats weighing 200 to 250 g were inoculated with a single intraperitoneal injection of 150,000 trypomastigotes of *Trypanosoma cruzi*. In some animals an

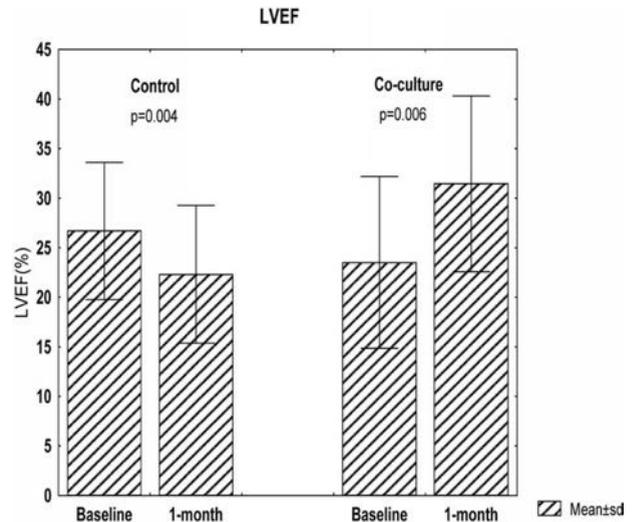


Fig 3. Myocardial infarction.

ELISA test for Chagas's disease was performed. After 8 months of inoculation, they underwent transthoracic echocardiography for baseline evaluation of heart function. The 15 animals that developed ventricular dysfunction with ejection fraction less than 35% were randomly submitted to treatment. Seven animals underwent autogenous co-culture transplantation with a mean of $5.4 \times 10^6 \pm 8.0 \times 10^6$ cells with 200 μ L of DMEM. The eight animals in the control group did not receive culture medium and followed a natural evolution. One month after treatment all animals underwent another transthoracic echocardiography (Fig 1).

Cell Isolation Procedures

Collection of SM was obtained by biopsy of the posterior tibial muscle. Isolations used enzymatic dissociation in accordance with the method of Delaporte.⁹ Collection of mononuclear cells was obtained by puncture aspiration of bone marrow from the iliac crest, and isolation performed using a density gradient, using Ficoll-Hypaque (d = 1.077) in accordance with Boyum.¹⁰

Table 1. Myocardial Infarction: Baseline and After 1-Month LVEDV, LVESV and LVEF (%)

Variable	Control (n = 22) Mean ± SD	Co-culture (n = 17) Mean ± SD	P
LVEDV baseline	0.63 ± 0.15	0.51 ± 0.09	.005
LVEDV 1-month	0.76 ± 0.17	0.68 ± 0.12	.138
P	.001	<.001	
LVESV baseline	0.47 ± 0.14	0.39 ± 0.08	.033
LVESV 1-month	0.60 ± 0.17	0.47 ± 0.12	.017
P	<.001	.012	
LVEF (%) baseline	26.84 ± 7.05	23.52 ± 8.67	.200
LVEF (%) 1-month	22.32 ± 6.94	31.45 ± 8.87	.001
P	.004	.006	

LVEDV, left ventricular-end diastolic volume; LVESV, left ventricular-end systolic volume; and LVEF, left ventricular ejection fraction.

*Student's t test; paired student's t test; P < .05.

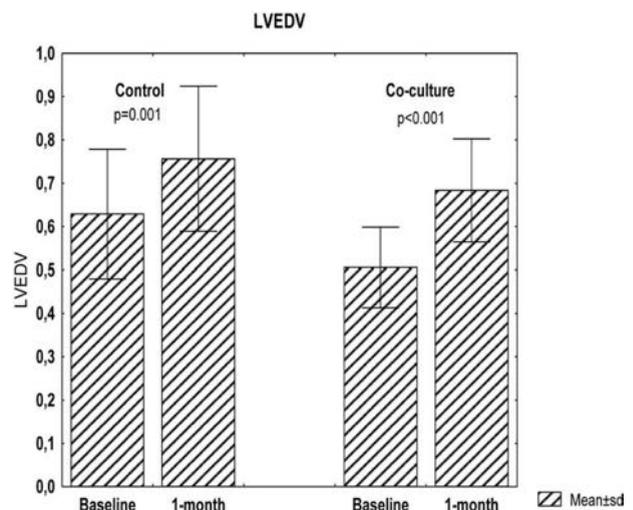


Fig 4. Myocardial Infarction.

The cells were then plated in a proportion of 2:1 (satellite cells: mononuclear cells), nearly 5×10^5 /mL in accordance with Carvalho¹¹ (Fig 2). The medium was changed after 48 hours, and the resting adherent elements were skeletal myoblasts, fibroblasts, and mesenchymal cells, the latter having a population of mesenchymal stem cells known as bone marrow mesodermal progenitor cells. The culture medium was DMEM supplemented with 15% fetal calf serum (Gibcco, USA), 1% antibiotic (streptomycin-penicillin), 10 μ g/mL insulin growth factor type I (Sigma, USA), and dexamethasone (10^{-7} M). The cultures were maintained in an incubator with 5% CO₂ at 37°C for 14 days. The viability of the cultivated cells was 95% to 98% by trypan blue exclusion staining. The product of the culture was submitted to an immunocytochemistry assay for identification of SM and mesenchymal cells. The SM was identified with fast-myosin (Sigma; monoclonal antiskeletal myosin clone MY-32) by immunofluorescence. The mesenchymal cell origin was cytologically confirmed by the presence of vimentin and identified by immunofluorescence and immunoperoxidase with the antibody anti-vimentin (Peroxidase; Sigma, IMM-10 – Vimentin S-20; Santa Cruz Biotechnology).

Functional Analysis

The functional analysis was carried out by a two-dimensional device for transthoracic echocardiography (5500 Sonos, Hewlett Packard) with S12 (5–12 MHz) sectors conductor and 15L6 (7–15 MHz), which allowed for an analysis of up to 160 MHz specifically for ultrasonic study of small animals. The conductor was placed on the left anterior-lateral region of the thorax. The hearts were visualized in two-dimensional form with an axial view of the left ventricle, including the mitral and aortic valves and the apex in the same image. The digital conversion image was performed by delimitation of the ventricular septum and the posterior wall of the left ventricle. The measures were performed in blinded fashion by a single observer. The measures of left ventricular ejection fraction (LVEF), left ventricular-end systolic volume and left ventricular-end diastolic volume were registered in three to five consecutive cardiac cycles. The observer variability was registered for two measures in a random sample of rats for correlation coefficient, and standardization of the estimated error was calculated in accordance with the Bland and Altman method.¹²

Table 2. Chagas's Disease: Before and After 1 Month of Transplantation (Comparison Into Two Groups)

Variable	Control (n = 8) Mean ± SD	Co-culture (n = 7) Mean ± SD
LVEDV baseline	0.68 ± 0.12	0.83 ± 0.08
LVEDV 1-month	0.72 ± 0.16	0.64 ± 0.16
P	.642	.005
LVESV baseline	0.43 ± 0.08	0.56 ± 0.06
LVESV 1-month	0.45 ± 0.14	0.30 ± 0.10
P	.777	.001
LVEF% baseline	36.21 ± 3.70	31.10 ± 5.78
LVEF% 1-month	38.19 ± 7.03	53.37 ± 5.84
P	.426	<.001

LVEDV, left ventricular-end diastolic volume; LVESV, left ventricular-end systolic volume; and LVEF, left ventricular ejection fraction.
*paired Student's t test; P < .05.

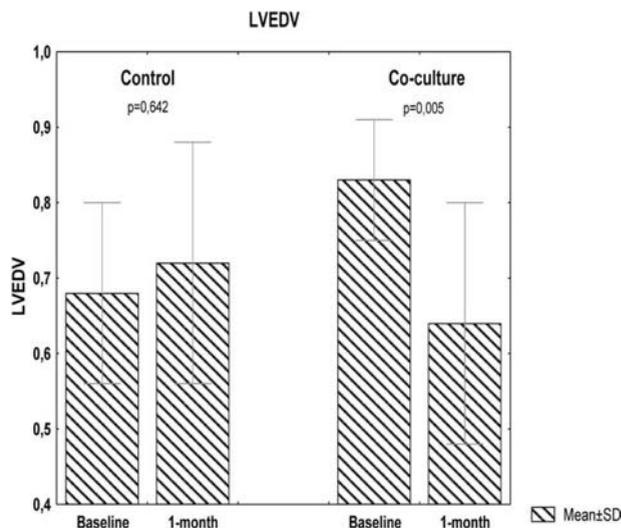


Fig 5. Chagas's group. Baseline and 1-month after transplantation LVEDV. LVEDV (Left ventricular end diastolic volume).

Data Analysis

Analysis of the data was performed by the ANOVA method. In each group the echocardiographic data were compared before and 1 month after cell therapy for Wilcoxon tests. The data were described for the shunting line average (shunting line standard), and P < .05 was considered significant.

Histological Assessment of Animals Receiving Transplants Cells and Controls

Before the histological assessment, we reviewed the literature regarding heart tissue scar. In postinfarct evolution, from day 7 to day 10, collagen replaces necrotic tissue, and a dense scar begins to form. At day 10, most of the necrotic myocardium has been removed, but fibrous scar tissue has not yet been substantially

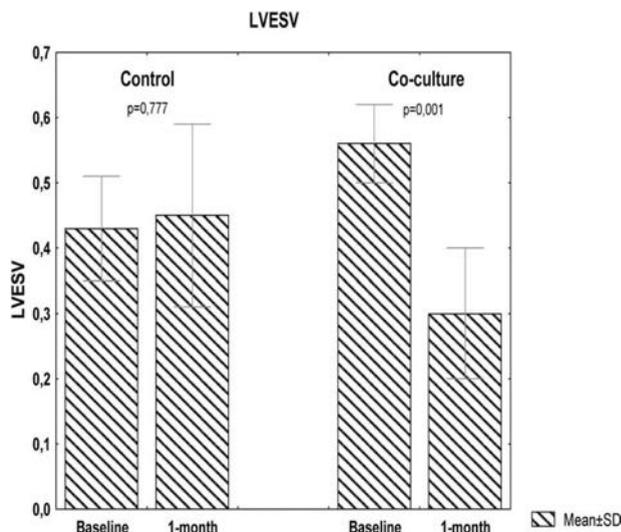


Fig 6. Chagas's group. Baseline and 1-month after transplantation LVESV. LVESV (left ventricular end systolic volume).

formed; at this time rupture of the heart is common. At this point the scar is mature.¹³ To interpret the implanted cells in standard stained tissue, we considered new vessels and skeletal muscle as those that were isolated in the scar, or, in other words, surrounded by collagen fibers in heart tissue. Also, the skeletal muscle showed morphological differences from the cardiomyocytes.

The histological assessment of animals receiving transplanted cells and controls was performed at 1 month after cell transplantation. The rats were then sacrificed. Their hearts were removed and washed quickly in phosphate buffered saline (Gibco, USA) before cryopreservation in liquid nitrogen. Serial transverse 8 μ m cuts cryostat (Leica-model cm 1850) were subsequently stained with: hematoxylin and eosin and modified Gomori's trichrome.

Immunohistochemistry Assay

The immunohistochemistry assay of the specimens was performed with anti-desmin (Dako; anti-human desmin – clone D33) and fast-myosin (Sigma; monoclonal antiskeletal myosin-clone MY-32)

for immunofluorescence; and anti-VIII factor (Dako; antihuman Von Willebrand Factor) for immunoperoxidase.

RESULTS

Functional Analysis of Myocardial Infarction Group

In the myocardial infarction group, 1 month after transplantation the animals that received co-cultured cells demonstrated improved LVEF: 23.52 ± 8.67 to 31.45 ± 8.87 ($P = .006$) versus the control group: 26.68 ± 6.92 to 22.32 ± 6.94 ($P = .004$; Table 1, Fig 3). The increased LVEDV was 0.51 ± 0.09 to 0.68 ± 0.12 ($P < .001$) versus the control group: 0.63 ± 0.15 to 0.76 ± 0.17 ($P = .001$; Table 1, Figs 3, 4).

Functional Analysis of the Chagas's Disease Group

In the Chagas's disease group, 1 month after transplantation the animals that had received co-cultured cells demonstrated improved LVEF: 31.10 ± 5.78 to 53.37 ± 5.84

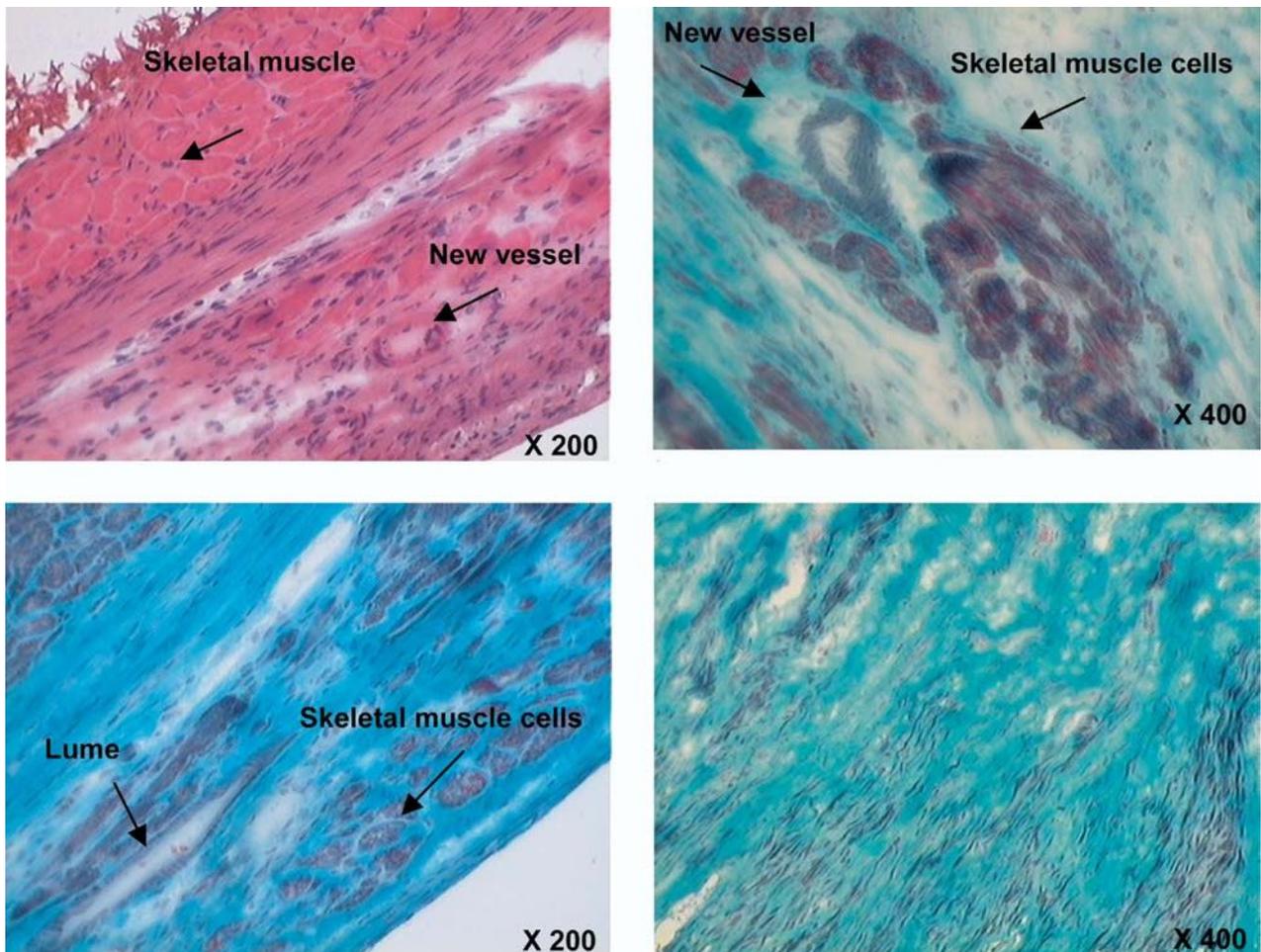


Fig 7. a. Myotube formation of skeletal muscle and presence of angiogenesis in the scar's myocardial infarction at 1-month after cell therapy. Stained with H&E. b. Myotube formation and presence of angiogenesis in the scar's myocardial infarction at one-month after cell therapy and were stained with Gomori's trichrome. c. Angiogenesis: the presence new vessel with endothelial cells and smooth muscle those have formed the vessel lumen and skeletal muscle cells in myocardial infarction's scar. Stained with Gomori's trichrome. d. In control group, the Myocardial infarction's scar demonstrated the absence of both skeletal muscle cells and vessels. Stained with Gomori's trichrome.

($P < .001$) versus the control group: 36.21 ± 3.70 to 38.19 ± 7.03 ($P = .426$; Table 2). Also, among the cell transplant group we observed left ventricular-end diastolic volume of 0.83 ± 0.08 to 0.64 ± 0.16 ($P = .005$) versus 0.68 ± 0.12 to 0.72 ± 0.16 ($P = .642$) and a left ventricular-end systolic volume: 0.56 ± 0.06 to 0.30 ± 0.10 versus 0.43 ± 0.08 to 0.45 ± 0.14 ($P = .777$; Table 2, Figs 4, 5, 6).

Histological Assessment of Animals Receiving Transplants and Controls

The Myocardial Infarction Group. In this group, the animals that received co-cultured cells showed skeletal muscle cells with myotube formation (centered nuclei) and presence of angiogenesis in the scarred area of myocardial infarction using hematoxylin and eosin and Gomori's trichrome stain. The new vessels and skeletal muscle were isolated in the scar (Fig 7a–c), as confirmed by immunohistochemistry with positive fast-myosin for skeletal. We ob-

served angiogenesis in heart tissue in which endothelial and smooth muscle cells formed vessel lumina as confirmed by immunohistochemistry with positive VIII factor. In the control group, we observed the presence of scar tissue without skeletal muscle cells or vessels (Fig 3d).

The Chagas's Disease Group

In the Chagas's disease therapy group, we observed angiogenesis and the presence of skeletal muscle in the myocardium while the Chagas's disease control group showed only dilated areas in the myocardium without skeletal muscle cells or new vessels (Fig 8).

DISCUSSION

Consistent with the literature, SM transplantation into the myocardium for models of cryoinjury and myocardial infarction improves function and treats heart failure.^{4–8} However, about 80% of the transplanted cells die for

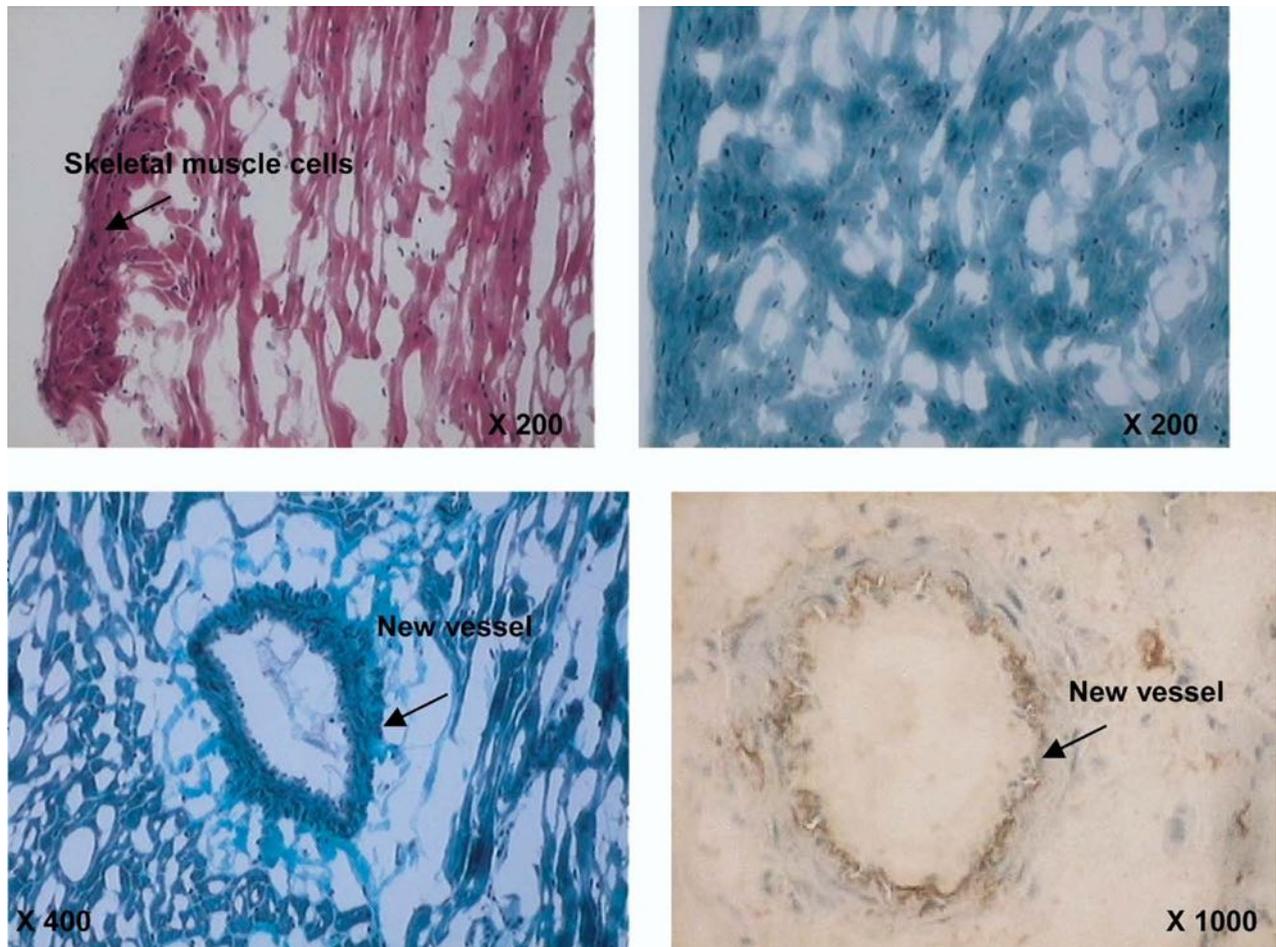


Fig 8. a. In Chagas's disease group: skeletal muscle cells in myocardial tissue at 1 month after cell therapy. Stained with H&E. b. Chagas's disease control group: the dilated aspects Chagas's cardiomyopathy was identified without both: skeletal muscle cells and vessels. Stained with Gomori's trichrome. c. In Chagas's disease group: new vessel was identified in myocardial tissue at 1 month after cell therapy. Stained with Gomori's trichrome. d. In Chagas's disease group: new vessel was identified with monoclonal anti-VIII-factor in the myocardial tissue by peroxidase at 1-month after cell therapy.

various reasons, among these the lack of nutrition.⁶ On the other hand, transplanted MSC seem to favor angiogenesis because they contain endothelial progenitor cells and contractile cells that improve cardiac function.^{4,5} In summary, SM and MSC are attractive therapeutic tools. These results encouraged us to develop a cellular co-culture model for transplantation treatment of myocardial disorders. There is controversy in the literature with respect to the capacity of MSC to differentiate into cardiomyocytes or smooth muscle cells that contribute to angiogenesis together with the endothelial progenitor cells.¹⁴⁻¹⁷

Based on our observed results and the literature,¹⁴ we hypothesized, that it would be possible to improve the effectiveness of cardiac cell therapy with simultaneous transplant of SM and MSC preceded by co-culture to increase the beneficial effect. Based on the principle that MSCs are pluripotent and contain mesodermal progenitors cells, as well as contractile and endothelial progenitors that can differentiate in the environment,¹⁸ we expected that the presence of both SM and MSC would make possible the differentiation and development of contractile and angiogenic cells. The co-culture method of these two cellular types allowed previous interactions in vitro and could be applied simultaneously to increase heart function. Furthermore, it would diminish the operational costs of the culture and expansion of both cellular types, as well as make possible the development of technology.

It is important to point out the difference in the infarction myocardial group and the Chagas's disease group. The latter showed significant remodeling and improvement of LVEF, data could reflect the anti-inflammatory effects of bone marrow cells.¹⁹ Also, it is important to understand that the variability of cell number in the Chagas's disease group was due to individual variability of each animal (autogenous transplant). A flow cytometric analysis is currently in progress to characterize the cellular types involved in this model.

In conclusion, the product of the SM and MSC co-culture process improved the functional outcome of postinfarction and Chagas's myocardiopathy. The histopathological analysis of the postinfarcted heart and Chagas's heart tissue after a co-cultured cell transplant demonstrated angiogenesis and skeletal muscle contractile cells in myocardial tissue. The results validated the product of a SM and MSC co-culture process to treat these diseases.

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