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# Simultaneous Autologous Transplantation of Cocultured Mesenchymal Stem Cells and Skeletal Myoblasts Improves Ventricular Function in a Murine Model of Chagas Disease

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**Background**—Cellular transplantation is emerging as a promising strategy for the treatment of postinfarction ventricular dysfunction. Whether its beneficial effects can be extended to other cardiomyopathies remains an unexplored question. We evaluated the histological and functional effects of simultaneous autologous transplantation of co-cultured stem cells and skeletal myoblasts in an experimental model of dilated cardiomyopathy caused by Chagas disease, characterized by diffuse fibrosis and impairment of microcirculation.

**Methods and Results**—Wistar rats weighing 200 grams were infected intraperitoneally with  $15 \times 10^4$  trypomastigotes. After 8 months, 2-dimensional echocardiographic study was performed for baseline assessment of left ventricle (LV) ejection fraction (EF) (%), left ventricle end-diastolic volume (LVEDV) (mL), and left ventricle end-systolic volume (LVESV) (mL). Animals with LV dysfunction (EF <37%) were selected for the study. Autologous skeletal myoblasts were isolated from muscle biopsy and mesenchymal stem cells from bone marrow aspirates were co-cultured in vitro for 14 days, yielding a cell viability of >90%. Eleven animals received autologous transplant of  $5.4 \times 10^6 \pm 8.0 \times 10^6$  cells (300  $\mu$ L) into the LV wall. The control group (n=10) received culture medium (300  $\mu$ L). Cell types were identified with vimentin and fast myosin. After 4 weeks, ventricular function was reassessed by echo. For histological analysis, heart tissue was stained with hematoxylin and eosin and immunostained for fast myosin. After 4 weeks, cell transplantation significantly improved EF and reduced LVEDV and LVESV. No change was observed in the control group.

**Conclusion**—The co-transplant of stem cells and skeletal myoblasts is functionally effective in the Chagas disease ventricular dysfunction. (*Circulation*. 2006;114[suppl I]:I-120–I-124.)

**Key Words:** cells ■ Chagas disease ■ heart failure ■ transplantation

Although some authors have shown that cardiomyocytes potentially are able to undergo mitotic division,<sup>1</sup> scar formation after myocardial infarction leads to remodeling and permanent loss of ventricular contractile capacity. Recently, restoration of ventricular contraction in ischemic cardiomyopathy by cell transplantation has recently emerged as a novel and feasible therapeutic option,<sup>2–3</sup> but the beneficial effects of this strategy in the full range of cardiomyopathies remains to be investigated.

The most appropriate cell for this therapy is still a matter of discussion; skeletal myoblasts and bone marrow cells have been widely used with this aim. Skeletal myoblasts transplantation has been shown effective in experimental<sup>4–6</sup> and clinical<sup>2,7</sup> studies. Myoblasts differentiate into viable muscle fibers within the scarred tissue but they lack morphological differentiation into cardiomyocytes. However, adult stem cells are pluripotent and have the ability to differentiate into specific cells depending on surrounding tissue and factors.<sup>8</sup> Stem cell transplant studies suggested a cardiac functional improvement<sup>9</sup> and a differentiation into cardiomyocytes,<sup>10</sup> but others only suggested an angiogenic potential.<sup>11</sup>

Chagasic cardiomyopathy caused by the hemoflagellate protozoa *Trypanosoma cruzi* infection<sup>12</sup> has been one of the leading causes of heart disease in Latin America for decades, where  $\approx 11$  million people are affected.<sup>13</sup> Myocarditis can be seen in the acute phase of Chagas disease, but the chronic phase is characterized by more pronounced structural abnormalities. A diffuse inflammatory response occurs, with focal fibrosis, damage of microcirculation, and consequent myocardial ischemia.<sup>14</sup> This altered inflammatory response leads to decreased contractile function, heart enlargement, and symptoms of heart failure. Despite infection and positive serology, some patients will remain asymptomatic and will not develop any structural abnormality, which characterizes the indeterminate form of the disease. To date, only a single study has evaluated cell transplantation in a model of Chagasic cardiomyopathy.<sup>15</sup>

We have previously demonstrated in a model of ischemic cardiomyopathy that skeletal myoblasts transplantation resulted in myogenesis and improvement of ventricular function. In

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contrast, treatment with mesenchymal stem cells resulted in neoangiogenesis and no functional effect.<sup>16</sup> In the same model, when both cells were transplanted together after co-cultivation, they were able to provide new contractile cells and vessels.<sup>17</sup>

Because physiopathology in Chagas disease resembles the findings in chronic ischemic cardiomyopathy, with fibrosis and ischemia, we hypothesized that simultaneous transplantation of co-cultured mesenchymal stem cells and skeletal myoblasts may be an effective approach in this disease.

The aim of this study is to evaluate the function and the pathology analysis of combined cellular transplantation with myoblasts and mesenchymal stem cells in a murine model of Chagasic cardiomyopathy.

## Materials and Methods

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

### Experimental Model: Chagas Disease

All experiments were performed in accordance with the *Guiding Principles in Care and Use of Animals* approved by the American Physiological Society.

The model of Chagas disease is described elsewhere.<sup>18</sup> Briefly, Wistar rats weighing  $\approx 200$  grams (TECPAR, Curitiba-Pr-Brazil) were infected intraperitoneally with  $15 \times 10^4$  trypomastigotes, and infection was confirmed by Machado-Guerreiro reaction in all animals.

After 8 months, ventricular function was assessed by bidimensional transthoracic echocardiography. Twenty-one animals with left ventricle ejection fraction (LVEF)  $< 37\%$  were included in the study.

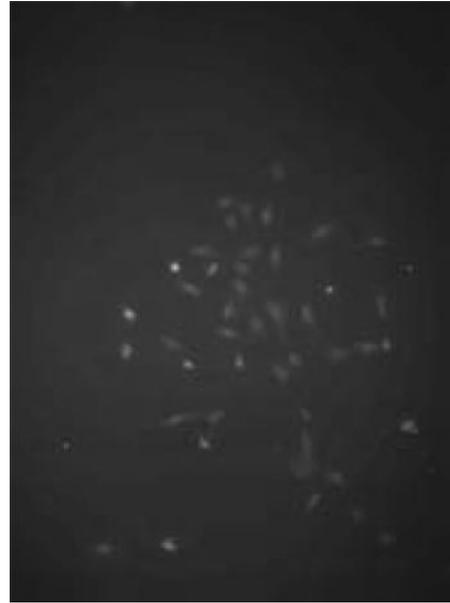
### Cell Isolation Procedures

Autologous cells were obtained as follow: skeletal myoblasts were obtained under anesthesia with xylazine (50 mg/kg) and ketamine (10 mg/kg), by biopsy of the posterior tibial muscle, followed by enzymatic dissociation technique, as described by Delaporte;<sup>19</sup> bone marrow cells were obtained by iliac crest aspiration, and mononuclear cells were separated by Ficoll-Hypaque ( $d=1.077$ ) density gradient centrifugation according to Boyüm.<sup>20</sup> Cells were seeded in a 2:1 ratio (2 skeletal myoblasts to 1 mononuclear cell); after 48 hours myoblasts and mesenchymal cells from bone marrow were adhered to flask surface, whereas cells of hematopoietic origin were not. The culture medium was the Dulbecco's modified Eagle medium supplemented with 15% fetal calf serum, 1% antibiotic (streptomycin-penicillin), 10  $\mu\text{g}/\text{mL}$  insulin growth factor type I, and dexamethasone at  $10^{-7}$  M (all products from Gibco BRL, Life Technologies, Inc, Rockville, Md). The flasks were maintained in an incubator with 5%  $\text{CO}_2$  at 37°C for 14 days and medium was exchanged every other day. Cell viability was evaluated by Trypan Blue.

The product of the culture was submitted to an immunocytochemistry assay for identification of skeletal myoblast and mesenchymal cells. Muscle cells were identified by immunofluorescence with monoclonal anti-desmin antibody (1:400; BD PharMingen, Mass) (Figure 1), and mesenchymal cell origin was cytologically confirmed with anti-vimentin antibody (Santa Cruz Biotechnology, Calif) (Figure 2). Flow cytometric analysis was performed to identify bone marrow origin stem cells (CD45+, 3.53%; CD34+, 1.94%) and skeletal origin cells (CDMyoD+, 4.36%) before transplantation.

### Cell Transplantation

Cell transplantation was performed 14 days after the cell culture was initiated. Under the same anesthesia regimen, rats were ventilated with a small animal respirator (683 Harvard model; Apparatus Inc) and a median sternotomy was performed. Rats were randomly assigned to receive subepicardic injection of co-culture of skeletal myoblast and mesenchymal stem cells,  $5.4 \times 10^6 \pm 8.0 \times 10^6$  cells



**Figure 1.** Skeletal muscle cells identified in the co-culture (anti-desmin, immunofluorescence, 40 $\times$ ).

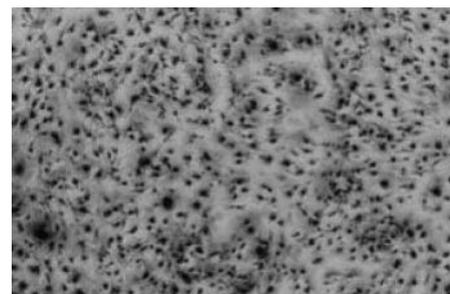
(from  $1.8 \times 10^5$  to  $2.1 \times 10^7$  cells) diluted in 0.30 mL of culture medium ( $n=11$ ) or 0.30 mL of culture medium as control ( $n=10$ ). Cells were injected in anterior and lateral left ventricle wall.

### Functional Assessment

One month after cell transplantation, left ventricular function was assessed by bidimensional transthoracic echocardiography (5500 Sonos model; Hewlett Packard Co), frame rate 21 Hz to 64 Hz, with S12 (5 mHz to 12 mHz) sectorial and 15L6 (7 mHz to 15 mHz) linear transducers, specifically designed for ultrasound studies of small animals. Under anesthesia, transducer was positioned in the left antero-lateral portion of the thorax, and the heart was visualized by using 2-dimensional mode with the axial view of the left ventricle, including the mitral and aortic valves and the apex in the same image. Digital conversion of the image was obtained by delimiting the interventricular septum and the left ventricular posterior wall. Left ventricle end-systolic, left ventricle end-diastolic volumes, and left ventricle ejection fraction (LVEF) were calculated using the following formulas: ventricular volume (V) was  $8 \times (S)^2 / (3 \times 3.1415926 \times C)$ , where V=volume, S=area, and C=weight. The formula for calculating the LVEF was:  $EF = LEDV - LESV / LEDV$ . All measurements were performed 3 times by the same technician, unaware of received treatment, and mean values were recorded. Animals were euthanized after the procedure.

### Histological Assessment

Hearts were removed and washed quickly in phosphate-buffered saline (Gibco) and cryopreserved in liquid nitrogen. Serial transversal sections



**Figure 2.** Mesenchymal cells identified in the co-culture (anti-vimentin, immunoperoxidase, 40 $\times$ ).

### Values of LVEDV (mL), LVESV (mL), and EF (%) of Both Groups in Before and After Cell Transplantation

Group	Control	Co-culture	Difference Between Groups ( <i>P</i> )
LVEDV, mL, before	0.69±0.11	0.82±0.07	0.0060
LVEDV, mL, after	0.73±0.14	0.65±0.14	0.0166
<i>P</i>	0.6311	0.0004	
LVESV, mL, before	0.44±0.08	0.56±0.05	0.0001
LVESV, mL after	0.46±0.12	0.32±0.09	0.0001
<i>P</i>	0.6523	<0.0001	
EF % before	36.7±3.6	30.1±5.7	0.0051
EF% after	37.4±6.7	51.8±6.6	<0.0001
<i>P</i>	0.7684	<0.0001	

(8 μm) were obtained in Leica cryostat (model 1850). Slides were stained with hematoxylin and eosin (H&E) and modified Gomori's trichrome for morphological assessment. The immunohistochemistry assay of the specimens was performed with anti-fast myosin antibody for immunofluorescence (Sigma, Saint Louis, Mo) at a dilution of 1:400. Slides were then incubated with secondary biotin-labeled, affinity-isolated anti-rabbit and anti-mouse immunoglobulins (LSAB®+ Kit, Peroxidase; DAKO Corp, Carpinteria, Calif).

### Statistics

All data are presented as mean and standard deviation. Intra-group comparison of baseline versus 1-month follow-up was performed with a paired *t* test. Differences between treated and control group was assessed by unpaired *t* test. Significance was defined as a value of *P*<0.05.

## Results

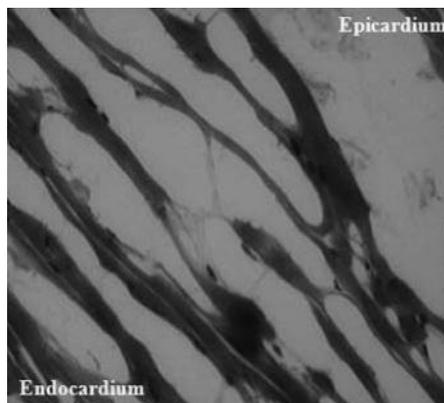
### Cell Viability

Cell viability was 95% to 98%.

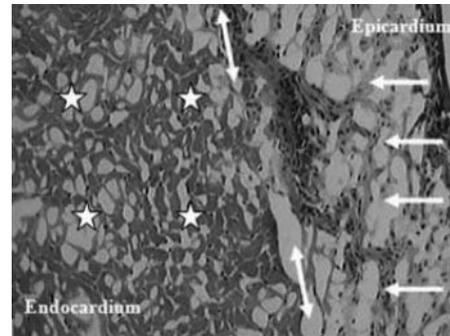
### Echocardiographic Study

One month after procedure, ejection fraction remained unchanged in the control group (36.7±3.6% to 37.4±6.7%; *P*=0.7684) but was enhanced in treated group (30.1±5.7% to 51.8±6.6%; *P*<0.0001). The resulting EF in animals that received cell transplantation was significantly higher compared with control group (*P*<0.0001).

Comparing left ventricle volumes, we also found better results in treated group. Left ventricle end-systolic remained



**Figure 3.** Damaged myocardium in control group, with high degree of fibrosis (hematoxylin and eosin [H&E], 400×).



**Figure 4.** Damaged myocardium (stars) and cell transplantation area (arrows) (H&E, 200×). Identification of transition zone (double arrows) (H&E, 200×).

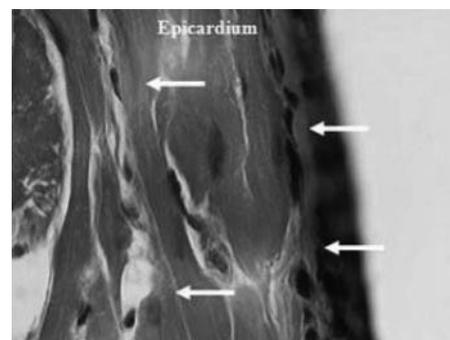
unchanged in control (0.44±0.08 mL to 0.46±0.12 mL, *P*=0.6523) and decreased in treated group (0.56±0.05 mL to 0.32±0.09 mL; *P*<0.0001). The resulting left ventricle end-systolic in treated group was also significantly lower compared with control group (*P*=0.0001). Similar results were observed regarding left ventricle end-diastolic volume, which remained unchanged in control (0.69±0.11 mL to 0.73±0.14 mL; *P*=0.6311) and decreased in treated group (0.82±0.07 mL to 0.65±0.14 mL; *P*=0.0004). The resulting left ventricle end-diastolic volume in treated group was significantly lower compared with control group also (*P*=0.0166) (Table 1).

### Histology

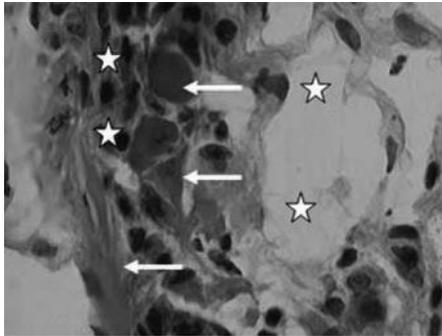
Histological analysis of control group demonstrated a high degree of fibrosis, a feature of Chagas disease (Figure 3). In treated group, skeletal muscles cells, with myotubular characteristics, endothelial cells, and new vessels in formation were identified in the epicardium where cells were transplanted (Figures 4, 5, 6, and 7). Musculoskeletal origin was confirmed by positive fast myosin immunostaining in treated group and negative in control group (Figures 8A and 8B).

### Discussion

Mechanisms that cause chronic damage in Chagas disease leading to extensive fibrosis are not completely understood, but recent evidence strongly suggests an autoimmune response against heart antigens, because the protozoa is rarely found in heart tissue at this stage.<sup>21</sup> Although anti-parasitic therapy is effective in acute phase, no specific therapy is effective at that point, when contractile function may be highly compromised.



**Figure 5.** Skeletal muscles cells—myotubular feature (arrows)—in the epicardium, where cells were transplanted (H&E, 1000×).

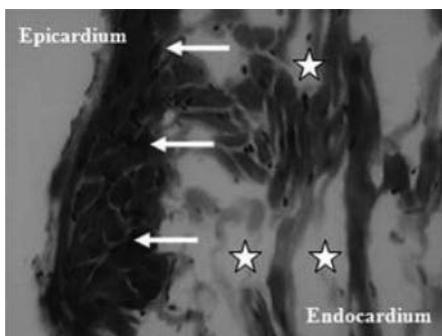


**Figure 6.** Skeletal muscles cells (arrows), endothelial cells, and new vessels in formation (stars) in the epicardium, where cells were transplanted (H&E, 10 000 $\times$ ).

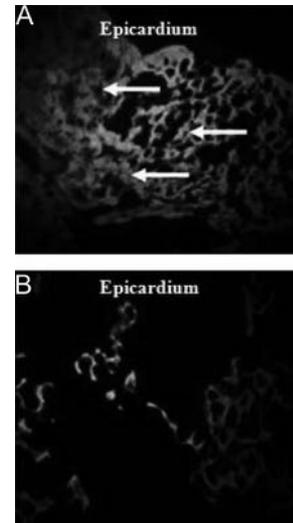
Heart transplantation, one of the few therapeutic options for patients with severe cardiomyopathy, has some peculiar implications in Chagas disease. At first, most patients affected by the disease inhabit poor areas in developing countries, where high costs of heart transplantation may be unaffordable. Second, immunosuppression treatment may reactivate Chagas infection,<sup>22–23</sup> compromising short-term and long-term prognosis. Therefore there is a continuous search for a better therapeutic option to recover ventricular function in these patients. In this study we showed that cell transplantation may be an interesting option.

Data regarding cell transplantation in experimental models of Chagas disease are lacking. Soares et al<sup>24</sup> demonstrated in a mouse model of Chagasic myopathy that bone marrow cells injected intravenously migrated to the heart and caused a significant reduction in the inflammatory infiltrates and in the interstitial fibrosis. Cell therapy induced massive apoptosis of myocardial inflammatory cells. The effect was the same when injected bone marrow cells were obtained from normal or infected mice. Because ventricular function was not assessed, it remains to be proved whether these beneficial histological effects with mononuclear cells transplantation is translated into ventricular function improvement. In the sole case reported in which a patient with Chagas cardiomyopathy received mononuclear cells by intracoronary injection, a consistent improvement in LVEF was observed, but no histological finding was provided.<sup>15</sup>

The idea of using a combination of skeletal myoblasts, which recolonize the infarcted myocardium with new muscle fibers of



**Figure 7.** Skeletal muscles cells. Miotubular feature (arrows), endothelial cells, and new vessels in formation (stars) in the epicardium, where cells were transplanted. (H&E, 400 $\times$ ).



**Figure 8.** A, Skeletal muscle cells (arrows) in the epicardium-treated group (anti-fast myosin, immunofluorescence, 200 $\times$ ). B, Control group, with anti-fast myosin negative (immunofluorescence, 200 $\times$ ).

skeletal origin, and cells derived from the bone marrow, which stimulate the formation of new vessels in the region of fibrosis, was based on the concept of providing an angiomyocardial regeneration and not just an isolated muscular or angiogenic regeneration. Moreover, the option for combined transplantation of skeletal myoblasts and mesenchymal bone marrow cells was based on pathophysiology of Chagasic cardiomyopathy, characterized by chronic inflammation, sites of fibrosis, and subendocardial ischemia. Manasché et al<sup>7</sup> demonstrated in a phase I clinical trial that skeletal myoblasts alone are able to improve ventricular function, but with an incidence of ventricular arrhythmias. Some argue that this harmful effect could be related to the lack of gap junctions between transplanted cells and native undamaged myocardium, as previously demonstrated by other authors.<sup>25</sup> The amount of transplanted cells and inflammation caused by transplanted cell death<sup>2</sup> may be another source for arrhythmias in myoblasts transplantation. Another possible explanation is that when only new muscular fibers are provided (myoblast transplantation), these structures may become ischemic by the lack of vascularization, and thus the tissue become more prone to arrhythmias. Because some authors suggest that bone marrow stem cells have only an angiogenic potential,<sup>11</sup> we have hypothesized that some problems could be eliminated providing contractile and angiogenic cells.

We have recently demonstrated in a model of ischemic cardiomyopathy that transplantation of skeletal myoblasts and mesenchymal stem cells was truly able to provide new contractile cells and vessels, respectively.<sup>17</sup> The histological findings in that model after treatment resembled what we found in our model of Chagasic cardiomyopathy: identification of new skeletal muscular fibers and new endothelial cells and blood vessels in the region of myocardial fibrosis. In the same model of ischemic cardiomyopathy, but comparing the effects between both cells separately,<sup>16</sup> we found that skeletal myoblasts transplantation resulted in myogenesis and improvement of ventricular function. In contrast, treatment with mesenchymal stem cells resulted in neovascularization and no functional effect. In the

current study, co-cultivated cells transplantation provided significant functional improvement. Because we did not evaluate both cells isolated, we are not able to identify which transplanted cell, if not both, is responsible for the increase in LVEF.

Cell transplantation also reduced end-systolic and end-diastolic left ventricle volumes. Vilas-Boas et al<sup>15</sup> demonstrated the same positive effect on ventricular remodeling when bone marrow mononuclear cells were deployed by intracoronary injection in a chagasic patient. Nevertheless, transplantation of the same co-cultured myoblasts and mesenchymal cells to a rat model of postinfarction ventricular dysfunction did not prevent ventricular remodeling despite improvement in ventricular function. Cells were injected only in the anterior wall, differently from the current model in which cells were injected in a more diffuse area (anterior and lateral wall). Thus, the effect on ventricular remodeling seems to be more related to the pathology and the way cells are transplanted into the heart than to the type of transplanted cells. Further studies are necessary to evaluate this point.

### Study Limitation

Rat model of Chagas disease is a difficult model to develop. Only 26% of inoculated rats have developed the ventricular dysfunction, and this is seen only after several months. Because of these limiting factors, we decided to test only the combination of both cells, because effects of isolated cells have been evaluated in another model.

Another study limitation is that despite randomization, groups were different at baseline, with rats in the group that received cell transplantation having lower LVEF and larger LV volumes. However, 1 month after cell transplantation, despite worst baseline function, LVEF and LV volumes were significantly better in the treated group.

### Conclusion

The combined cellular transplantation with myoblasts and mesenchymal stem cells is functionally effective in the Chagas disease ventricular dysfunction.

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### Disclosures

None.

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