

## Cell transplantation: Differential effects of myoblasts and mesenchymal stem cells

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### Abstract

**Background:** Cellular transplantation has emerged as a novel therapeutic option for treatment of ventricular dysfunction. Both skeletal myoblasts (SM) and mesenchymal stem cells (MSC) have been proposed as ideal cell for this aim. The aim of this study is to compare the efficacy of these cells in improving ventricular function and to evaluate the different histological findings in a rat model of severe post-infarct ventricular dysfunction.

**Methods:** Myocardial infarction was induced in Wistar rats by left coronary occlusion. Animals with resulting ejection fraction (EF) lower than 40% were included. Heterologous SM were obtained by lower limb muscle biopsy and MSC by bone marrow aspiration. Nine days after infarction, rats received intramyocardial injection of SM ( $n=8$ ), MSC ( $n=8$ ) or culture medium, as control ( $n=11$ ). Echocardiographic evaluation was performed at baseline and after 1 month. Histological evaluation was performed after HE and Gomori's trichrome staining and immunostaining against desmin, fast myosin and factor VIII.

**Results:** There was no difference in baseline EF and left ventricular end diastolic (LVEDV) and systolic volume (LVESV) between all groups. After 1 month a decrease was observed in the EF in the control group ( $27.0\pm 7.10\%$  to  $21.46\pm 5.96\%$ ,  $p=0.005$ ) while the EF markedly improved in SM group ( $22.66\pm 7.29\%$  to  $29.40\pm 7.01\%$ ,  $p=0.04$ ) and remained unchanged in the MSC group ( $23.88\pm 8.44\%$  to  $23.63\pm 10.28\%$ ,  $p=0.94$ ). Histopathology identified new muscular fibers in the group that received SM and new vessels and endothelial cells in the MSC.

**Conclusion:** Skeletal myoblasts transplantation resulted in myogenesis and improvement of ventricular function. In contrast, treatment with mesenchymal stem cells resulted in neoangiogenesis and no functional effect.

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**Keywords:** Cells; Transplantation; Heart failure; Myocardial infarction

### 1. Introduction

Congestive heart failure is a common final pathway for patients with ischemic cardiomyopathy. Therapeutical options mainly target on the consequences of heart failure, like fluid overload, and neuro-humoral activation,

that is long term deleterious. Although some authors have shown evidence of mitotic division of cardiomyocytes [1], scar formation following myocardial infarction leads to remodeling and permanent loss of ventricular contractile capacity. Recently, the restoration of ventricular contraction by cell transplantation has emerged as a novel and feasible therapeutic option. However, the most appropriate cell for this therapy is still a matter of discussion.

Skeletal myoblasts transplantation has been shown effective in many experimental [2–6] and one clinical [7]

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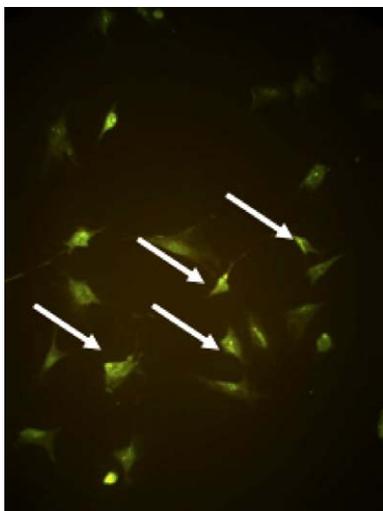


Fig. 1. Mesenchymal stem cells (arrows) in culture identified by anti-vimentin antibody (Immunofluorescence, 200 $\times$ ).

study. These cells differentiate into viable muscle fibers within the scarred tissue and they seem to be less prone to ischemia compared to cardiomyocytes [8]. However, there's still controversy whether this cell is the best option, at least to be used solely, since they lack morphological differentiation into cardiomyocytes and no intercalar discs develop between transplanted cells and the native adult cardiomyocytes. Moreover, in a small clinical study, patients who received this kind of cells had a higher incidence of ventricular arrhythmia [7]. The ongoing MAGIC multicenter clinical trial, where 300 patients were enrolled, will try to clarify some of these unanswered questions.

On the other hand, adult stem cells are pluripotent [9]. They have the ability to differentiate into specific cells depends on surrounding tissue and factors. Some studies suggested a cardiac functional improvement [10] and a differentiation into cardiomyocytes [11], but others only an angiogenic potential [12].

Studies where myoblasts and bone marrow cells are compared are lacking. To date, only one study has compared these cells, but in a rabbit model of cryoinjury [13]. The aim of our study is to compare the efficacy and histological findings of cellular transplantation using skeletal myoblasts and mesenchymal stem cells in a rat model of post-infarct ventricular dysfunction.

## 2. Material and methods

### 2.1. Experimental model—myocardial infarction

All experiments were performed in the Experimental Laboratory of Cell Culture in our institution, in accordance with the “Guiding Principles in Care and Use of Animals”, approved by the American Physiological Society.

Wistar rats weighting 250–300 g were anesthetized with xylazine (50 mg/kg) and ketamine (10 mg/kg), ventilated

with a small animal respirator (“683” Harvard model. Apparatus, Inc., USA) and submitted to left lateral thoracotomy. Left coronary artery was occluded with 7.0 prolipropilene string (Ethicon®, Inc., Somerville, NJ, USA), inducing anterior wall infarction of the left ventricle. Immediate procedure success was confirmed by change in myocardium color in the infarcted area (red to whitish).

After 7 days, ventricular function was assessed by bidimensional transthoracic echocardiography (5500 Sonos model Hewlet Packard Company, USA), with S12 (5–12 MHz) setorial transducers and 15L6 (7–15 MHz) limiar, specifically designed for ultrasound studies of small animals. Animals with a left ventricle ejection fraction below 40% were included in the study.

### 2.2. Cell isolation procedures

After bone marrow aspiration (iliac crest) from rats aged 2 to 4 months, mononuclear cells were separated by Ficoll-Hypaque ( $d=1.077$ ) density gradient centrifugation, according to Boyüm [14]. These cells were plated in 25 cm<sup>2</sup> flasks, at a concentration of  $5 \times 10^5$  cells/ml, in DMEM medium supplemented with 15% fetal calf serum, 1% ATB and 10 ng/ml Insulin Growth Factor (all products from Gibco BRL, Life Technologies, Inc., Rockville, USA). Flasks were maintained in incubator with 5% CO<sub>2</sub> at 37 °C. After 48 h, only mesenchymal cells adhered to flask surface, while those of hematopoietic origin did not. Cells were cultured for 10 days, exchanging medium every another day. Identification of mesenchymal cells was performed with primary antibody for immunofluorescence against-vimentin (Peroxidase-Sigma, IMM10–Vimentin S-20 Santa Cruz Biotechnology, FITC, California, USA) (Fig. 1).

Skeletal myoblasts were obtained from four limbs of sacrificed neonatal Wistar rats, followed by enzymatic dissociation technique, as described by Delaporte [15]. Cells were cultured for 10 days, in the same medium as mesenchymal cells. Cells origin was confirmed by anti-desmin for immunofluorescence (Anti-Human Desmin–Clone D33, Dako, Copenhagen, Denmark) (Fig. 2).

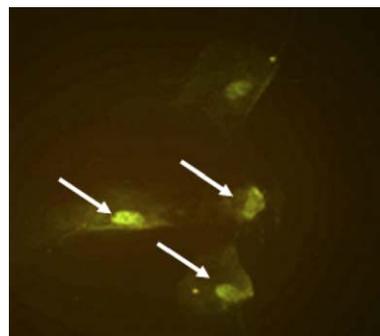


Fig. 2. Skeletal muscle (arrows) in culture identified by antibody anti-desmin (Immunofluorescence, 400 $\times$ ).

Table 1  
Baseline and after 1-month EF

Variable	Control (n=11)	BMSC (n=8)	Myoblast (n=8)	p <sup>a</sup>
	Mean±SD	Mean±SD	Mean±SD	
EF baseline	27.00±7.10	23.88±8.44	22.66±7.29	0.442
EF 1-month	21.46±5.96	23.63±10.28	29.40±7.01	0.008 <sup>b</sup>
p <sup>c</sup>	0.005	0.94	0.04	

p<0.05.

<sup>a</sup> ANOVA.

<sup>b</sup> Adjusted to baseline.

<sup>c</sup> Paired t-test.

For both cell cultures, viability was 85–95% by the Tripan Blue coloration (Sigma, Saint Louis, Missouri, USA).

### 2.3. Cell transplantation

Nine days after myocardial infarction, rats were submitted to cell transplantation. Under same anaesthesia regimen, a median sternotomy was performed and infarcted area was visually identified. Rats were randomly assigned to receive subepicardic injection of one of the following: (1) Skeletal myoblast cells (SM group)— $5.0 \times 10^6$  cells in 0.15 ml of culture medium; (2) Mesenchymal stem cells (MSC group)— $2.5 \times 10^6$  cells in 0.15 ml of culture medium; (3) 0.15 ml of culture medium (Control group). Since cells were heterologous, all animals received cyclosporin (15 mg/kg/weight/day) from cell injection to sacrifice.

### 2.4. Functional assessment

One month after cell transplantation, left ventricular function was assessed by echocardiography, with same device previously described. Under anaesthesia, transducer was positioned in the left anterolateral 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 (LVESV) and end-diastolic volumes (LVEDV) and ejection fraction (EF) were calcu-

Table 2  
Baseline and after 1-month LVEDV

Variable	Control (n=11)	BMSC (n=8)	Myoblast (n=8)	p <sup>a</sup>
	Mean±SD	Mean±SD	Mean±SD	
LVEDV baseline	0.64±0.17	0.54±0.10	0.53±0.09	0.13
LVEDV 1-month	0.75±0.19	0.71±0.16	0.64±0.13	0.74 <sup>b</sup>
p <sup>c</sup>	0.11	0.001	0.04	

p<0.05.

<sup>a</sup> ANOVA.

<sup>b</sup> Adjusted to baseline.

<sup>c</sup> Paired t-test.

Table 3  
Baseline and after 1-month LVESV

Variable	Control (n=11)	BMSC (n=8)	Myoblast (n=8)	p <sup>a</sup>
	Mean±SD	Mean±SD	Mean±SD	
LVESV baseline	0.47±0.15	0.41±0.10	0.41±0.07	0.387
LVESV-1month	0.60±0.18	0.53±0.16	0.45±0.11	0.300 <sup>b</sup>
p <sup>c</sup>	0.025	0.009	0.162	

p<0.05.

<sup>a</sup> ANOVA.

<sup>b</sup> Adjusted to baseline.

<sup>c</sup> Paired t-test.

lated using standard formulas. All measurements were performed three times by the same technician who was not aware of treatment group. Values were averaged. After procedure, animals were euthanized.

### 2.5. Histological analysis

The hearts were removed and washed quickly in PBS (Gibcco-California, USA) and cryopreserved in liquid nitrogen. Serial transversal sections (8 μm) were obtained in Leica cryostat (model cm 1850, USA). Slides were stained with hematoxylin–eosin (H&E) and modified Gomori's trichrome, for morphological assessment.

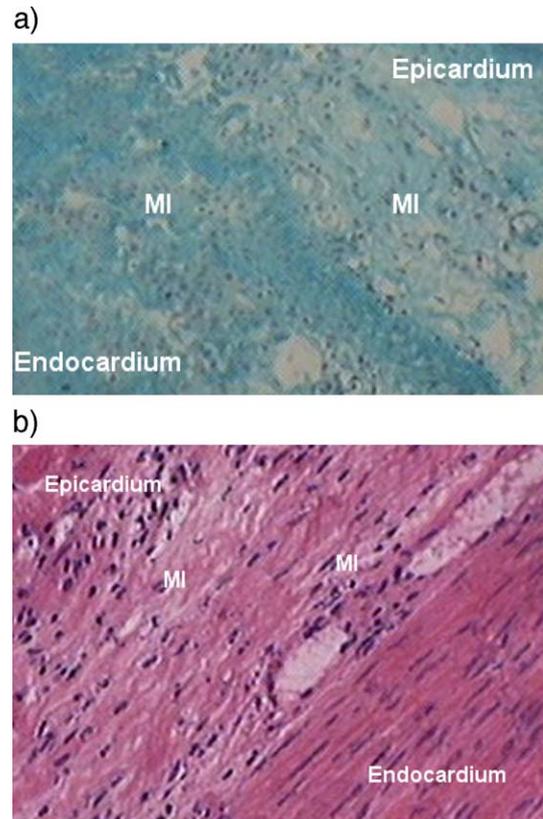


Fig. 3. (a) Myocardial infarction (MI) in control group (Gomori's Trichrome, 200×). (b) Myocardial infarction (MI) in control group (H&E, 400×).

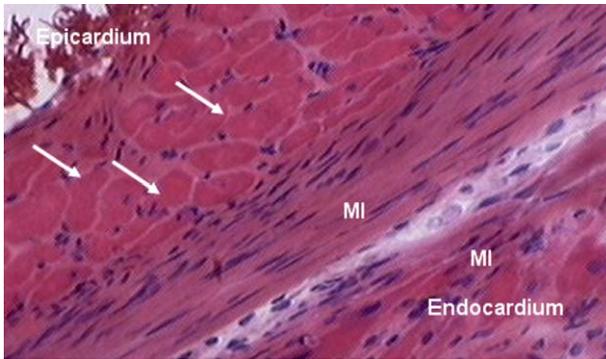


Fig. 4. Skeletal muscle fibers (arrows) with multiple peripheral nuclei in the myocardium infarction (MI) for the SM group (H&E, 400 $\times$ ).

Muscle fibers were identified in the scar area by immunofluorescence with anti-fast myosin antibody (monoclonal anti-skeletal myosin—clone MY 32 (Sigma, Saint Louis, Missouri, USA).

Vessels within in the scarred area were identified by immunostaining with anti-factor VIII antibody (Dako, Copenhagen, Denmark).

### 3. Statistics

All data are presented as average and standard deviation. Intra group comparison of baseline versus 1-month follow-up was performed with a paired *t*-test. After having evaluated the homogeneity of variance and normal distribution of data, analysis of variance was performed to determine differences among groups in each stage of analysis (baseline and 1 month), and adjusted to baseline results for a 1-month analysis. LSD tests were used for multiple comparisons between groups. The Fisher's test was used to compare proportion of cases where EF

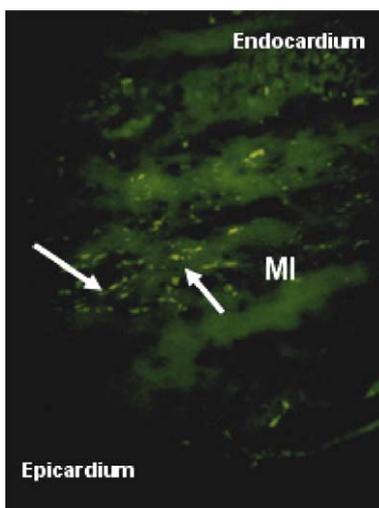


Fig. 5. Skeletal muscle fibers (arrows) in the myocardial infarction (MI) for the SM group with fast myosin positive (Immunofluorescence, 200 $\times$ ).

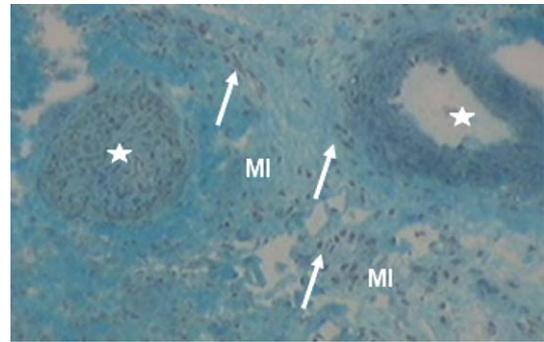


Fig. 6. New vessels (stars) and endothelial cells (arrows) in the myocardium infarction (MI) in MSC group (Gomery Trichrome, 200 $\times$ ).

decreased 1 month after treatment. Significance is defined as  $p < 0.05$ .

## 4. Results

### 4.1. Echocardiographic study

The three groups were homogeneous at baseline, with similar EF, LVESV and LVEDV in all groups.

One month after transplantation, EF decreased in the control group ( $27.0 \pm 7.10\%$  to  $21.46 \pm 5.96\%$ ,  $p = 0.005$ ), enhanced in myoblast group ( $22.66 \pm 7.29\%$  to  $29.40 \pm 7.01\%$ ,  $p = 0.04$ ) and remained unchanged in mesenchymal group ( $23.88 \pm 8.44$  to  $23.63 \pm 10.28\%$ ,  $p = 0.94$ ). The resulting EF in myoblast group was significantly higher compared to other groups ( $p = 0.008$ , respectively).

Ventricular diastolic volumes increased in all three groups, 1 month after transplantation.

In the same period ventricular systolic volumes increased in control and mesenchymal groups ( $p = 0.025$  and  $p = 0.009$ , respectively), but this dilation was not significant in myoblast group ( $p = 0.162$ ) (echocardiographic measurements are listed in Tables 1–3).

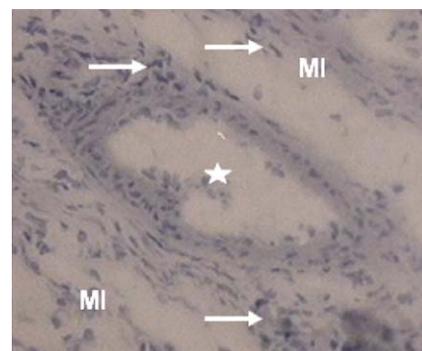


Fig. 7. New vessel (star) and endothelial cells (arrows) in the myocardium infarction (MI) in MSC group (Factor VIII, immunoperoxidase, 400 $\times$ ).

## 5. Histology

Histology showed different components in the infarcted area according to treatment received; in control group, only scar tissue was seen (Fig. 3a,b). In the myoblast group, skeletal muscle fibres were identified, as demonstrated by hematoxylin–eosin staining—fibers with several peripheral nuclei (Fig. 4) and fast myosin immunostaining (monoclonal anti-skeletal myosin—clone MY 32, Sigma, Saint Louis, Missouri, USA) (Fig 5).

In the group that received mesenchymal stem cells, new vessels and endothelial cells were identified, surrounded by collagenous tissue as demonstrated by Trichrome staining (Fig. 6) and factor VIII immunostaining (anti-factor VIII antibody, Dako, Copenhagen, Denmark) (Fig 7).

## 6. Discussion

The aim of cell transplantation in ischemic cardiomyopathy is to colonize the myocardial scar with new cells to recover ventricular contractile function. In our study, we found that skeletal myoblasts and mesenchymal stem cells have different effects when transplanted to the chronic infarcted myocardium. While myoblast cells improved ventricular function, mesenchymal cells only provided stabilization, avoiding further dysfunction. In mice where no cells were injected, ejection fraction worsened as a natural consequence of evolving ischemic cardiomyopathy.

Similar functional results with myoblasts have been already described in several experimental [2–6] and one clinical study, where Menasché et al. [7] showed functional improvement after engraftment of cultured adult skeletal muscle precursor cells (myoblasts) into myocardium scar. In this phase I clinical trial, 10 patients with severe postinfarct ventricular dysfunction received autologous myoblasts cultured for 16 days after taken from thigh. The authors demonstrated a recovery of function in areas previously akinetic and non-viable, but the mechanism of improvement was not completely understood. The authors hypothesized that it could happen either by a change in cell phenotype, since they expressed slow myosin instead of fast myosin, or simply by colonizing the infarcted area and avoiding further dysfunction. Despite the good results regarding contractile function, four patients had ventricular arrhythmia. Although patients with severe ischemic cardiomyopathy are considered high risk for these life-threatening arrhythmias [16], some other reasonable explanations have been considered. Some argue that it could be related to the lack of gap junctions between transplanted cells and native undamaged myocardium [17], suggesting there's no synchronicity in contraction between these tissues. Also ischemia [18], the amount of transplanted cells [19] and inflammation due to transplanted cell death [20], may be another source for arrhythmias in myoblasts transplantation.

Although in a simplistic view improvement in ventricular function can be attributed to new contractile cells, the mechanisms seem to be complex. Murry et al. [21] have performed an *in vitro* myographic analysis of transplanted muscle fibers; they can contract after an electrical stimulation and develop a typical physiologic reaction of skeletal muscles. This can be structurally proven by the fact that myoblasts present a specific isoform of myosin in the skeletal muscle. A conversion of rapid to slow isoform was observed 2 weeks after transplantation, which might suggest an adaptation of the skeletal muscle fibers as a consequence of the frequency of contractions of the cardiac muscle. Whether these fibers can synchronically contract with native fibers is a question that remains to be answered. Reinecke et al. [22] have demonstrated that when neonatal or adult cardiomyocytes are cocultured with skeletal muscle, some electromechanical junctions can be formed and induce synchronous contraction. Despite this *in vitro* study, stronger evidences from animal studies show no coupling between transplanted myoblasts and native myocardium [17]. In the same way, usually no differentiation into cardiac myocyte takes place when myoblasts are injected into myocardium [17]. In our study, histological evaluation of infarcted area where myoblasts were injected showed fibers with several peripheral nuclei, a typical feature of skeletal cells, suggesting no differentiation into cardiac myocyte has occurred.

If studies with myoblasts show improvement in ventricular contraction, studies with bone marrow cells transplantation have no such consistent results. These cells may undergo the following two processes of differentiation: the hematopoietic cell, which generates blood cells, and mesenchymal line, which may generate muscle cells, hepatocytes, osteocytes and chondrocytes [23]. Studies with bone marrow cells have been performed with different methodologies, with different cells (mononuclear, mesenchymal and hematopoietic), and with different injury models (ischemic myocardium, post-infarct and cryoinjury).

The differentiation capacity of bone marrow cells is not completely understood. Some studies have shown these cells are able to differentiate into cardiomyocyte [11] but in another study only neoangiogenesis was seen [12]. Different results have been also observed according to the studied model. In a model of myocardial infarction, Orlic et al. [24] have demonstrated the beneficial effect of bone marrow cells injection in the border of infarcted myocardium shortly after coronary ligation. Nine days after transplantation, newly formed tissue occupied 68% of the infarcted portion of the ventricle. In this tissue, proliferating myocytes and vascular structures were noted. In this study, bone marrow cells were transplanted in an area with viable myocardium, and not scar tissue, and this may justify why they differentiated into cardiomyocytes. In a clinical trial, Wollert et al. [25] evaluated patients with acute myocardial infarction that, after acute treatment with PTCA, were randomized to standard clinical treatment alone or associ-

ated to bone marrow cell transplantation. Ventricular function significantly improved in patients who received cells, in comparison to those who did not.

In patients with established fibrosis, results are conflicting. Perin et al. [26] showed that transendocardial injection of autologous mononuclear bone marrow cells in patients with end-stage ischemic heart disease improves perfusion and mechanical function of the injected segments. Different results were demonstrated by Marzullo [27], who has demonstrated that bone marrow cells can improve perfusion but not contraction by scintigraphy. The author evaluated patients submitted to CABG with cell injection in fibrosis area. In areas where reperfusion was achieved with graft, improvement was seen in perfusion and contraction. On the other hand, in areas where only cell injection was performed, only perfusion improvement was seen. This is in accordance with our findings of new vessels but no contractile cells within the myocardium scar where mesenchymal stem cells were injected. These histological findings on mesenchymal group may justify stabilization on ejection fraction, since new vessels were identified within infarcted area. These vessels may have improved blood flow to border zone between scarred and normal myocardium, restoring flow to hibernating myocardium and avoiding apoptosis. Some authors state that bone marrow cells may have an anti-inflammatory effect, as shown in a model of Chagas disease [28]. Others suggest that these cells may stimulate production of protective proteins [29].

Despite some beneficial effects of cellular transplantation on ventricular remodelling as proposed by Garot et al. [30], in our study neither mesenchymal nor myoblast cells prevented ventricular dilation. As cell injections were performed only in anterior wall, maybe the effect was restricted to this area. Another explanation for remodelling observed may be the model we chose: as all animals included in the study presented severe ventricular dysfunction (EF 27%, in average) by the time of treatment, with large scar area, maybe it was too late to see some effect on remodeling.

Overall, distinct functional and histological findings in our study in comparison to others may be attributed to the model we studied, of chronic myocardial infarction. In this model, fibrosis is already established, different of previous studied models, as acute myocardial infarction [31] or ischemic myocardium [32].

In the study where myoblast and bone marrow cells were compared, Thompson et al. [13] performed transplantation in rabbits subjected to cryoinjury of the left ventricle. Different from our study, both myoblast and bone marrow groups presented a similar degree of improvement in relation to control group. This distinct result may either be a consequence of the ventricular dysfunction model (cryoinjury vs. post-infarction) or the result of different methods to evaluate ventricular function (micromanometry vs. echocardiography). A point worth mentioning is that also in this study, in the group that received bone marrow

cells, no cardiomyocyte was observed within the transplanted area, but in border zone.

In conclusion, myoblasts cell transplantation improves ventricular function while mesenchymal cells avoid further contractile dysfunction in a model of severe post-infarction ventricular dysfunction. Further studies are necessary to better explore the potential of myoblasts to provide new contractile cells and the angiogenic potential of mesenchymal bone marrow cells.

### 6.1. Study limitation

In our study, the quantity of transplanted mesenchymal cells was lower than myoblast cells, since mesenchymal is slow growing. We decided on transplanting cells at the same time-point, not depending on cell number. To reach the same number, mesenchymal cells should be cultured for more than 30 days.

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