

Cellular transplant: functional, immunocytochemical and histopathologic analysis in an experimental model of ischemic heart disease using different cells

Transplante celular: análise funcional, imunocitoquímica e histopatológica em modelo experimental de miocardiopatia isquêmica utilizando diferentes células

Paulo R. BROFMAN, Katherine A. CARVALHO, Luiz C. GUARITA-SOUZA, Carmen REBELATTO, Paula HANSEN, Alexandra C. SENEGAGLIA, Nelson MYAGUE, Marcos FURUTA, Júlio C. FRANCISCO, Márcia OLANDOSKI

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Abstract

Objective: To present the functional, immunocytochemical and histopathologic results (*in vitro* or in heart specimens) after isolation, culture and co-culture of mesenchymal stem cells and skeletal myoblast cells transplanted and co-transplanted in experimental animals with ischemic heart disease and left ventricular ejection fractions lower than 40%. **Methods:** We utilized 72 Wistar rats, divided into four groups according to the culture media or injected cells: control group into which only culture media was injected (22 rats); mesenchymal stem cell group (17 rats); myoblast skeletal cell group (16 rats) and co-culture group (17 rats). In the immunohistochemical studies, the cells were stained with anti-vimentin, anti-desmin and anti-myosin. In the histopathologic analysis, slides were stained with Gomori Trichrome, and the neo-vessels and muscle tissues were identified. In the functional analysis the left ventricle ejection fraction was analyzed one week after myocardial infarction and one month after the injection.

Results: The initial left ventricle ejection fraction (control echo) was not statistically significant between the four groups ($P=0.276$), but was significantly different in the follow-up examination ($P=0.001$). This difference was seen between the control and myoblast skeletal cell groups ($P=0.037$), between the control and co-culture groups ($P<0.001$) and between the mesenchymal stem cell and co-culture groups ($P=0.025$). When the initial and final echocardiograms in each group were compared, the control group deteriorated ($P=0.005$) and the co-culture group improved ($P=0.006$). With the immunocytochemical *in vitro* analysis, mesenchymal stem cells were identified when stained with anti-vimentin and muscle cells when stained with anti-desmin. In the heart specimens, muscle tissue, stained with anti-desmin and skeletal myoblast cells, stained with fast anti-myosin were identified. In the histopathologic analysis, new vessels were observed in the mesenchymal stem cell and skeletal myoblast groups, and muscle tissue, angiogenesis and myogenesis in the co-culture group.

Cellular engineering and transplantation laboratory, Pontificia Catholic University, Paraná
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Correspondence address:
Paulo R Brofman
Rua Gumercindo Mares 150 Vista Alegre
Curitiba - Brazil 80810 220
Phone (041) 2711657
E-mail: paulo.brofman@pucpr.br

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Conclusion: The left ventricle ejection fraction improved in the group in which muscle cells were injected and more strikingly, in the co-culture group. The immunohistochemical findings in the culture and co-culture groups evidenced the corresponding cells. In the heart specimens, muscle and skeletal myoblast cells were found. In the histopathologic examination, new vessels and muscle tissue were found in the mesenchymal stem cell, skeletal myoblast cell and co-culture groups.

Descriptors: Cell transplantation. Myocardial infarction, therapy. Myocardium. Myocardial ischemia. Cell culture, utilization.

Resumo

Objetivo: Apresentar os resultados funcionais, imunocitoquímicos e histopatológicos, *in vitro* ou em espécimes cardíacas após isolamento, cultura e co-cultura de células tronco mesenquimais, células mioblásticas esqueléticas e transplantadas e co-transplantadas em animais de laboratório com miocardiopatia isquêmica e fração de ejeção do ventrículo esquerdo menor de 40%.

Método: Foram empregados 72 ratos Wistar, divididos em quatro grupos de acordo com o meio de cultura ou das células injetáveis: Grupo controle em que foi injetado apenas o meio de cultura (22 ratos); Grupo de células tronco mesenquimais (17 ratos); Grupo de células mioblásticas esqueléticas (16 ratos) e grupo co-cultura (17 ratos). Nos estudos imunocitoquímicos, as células foram marcadas com anti-vimentina, anti-desmina e anti-miosina. Nos estudos histopatológicos, as lâminas foram coradas com Tricômio de Gomori e identificados neovasos e tecido muscular. Na análise funcional, foi medida a fração de ejeção do ventrículo esquerdo em dois momentos do seguimento, uma semana após o infarto

do miocárdio e um mês após a injeção.

Resultados: A fração de ejeção do ventrículo esquerdo entre os quatro grupos não apresentou diferença estatística significativa ($P=0,276$), o ecocardiograma de seguimento demonstrou diferença estatística significativa ($P=0,001$). Essa diferença ocorreu entre o grupo controle e o grupo de células mioblásticas esqueléticas ($P=0,037$), entre o grupo controle e o grupo co-cultura ($P<0,001$) e o grupo de células tronco mesenquimais e o grupo co-cultura ($P=0,025$). Quando se compararam as medidas obtidas dos dois ecocardiogramas em cada grupo, encontrou-se diferença no grupo controle ($P=0,005$) para pior e no grupo co-cultura ($P=0,006$) para melhor. No estudo imunocitoquímico *in vitro*, identificou-se células tronco mesenquimais quando marcou-se com anti-vimentina e células musculares, com anti-desmina. Nas espécimes cardíacas, identificou-se tecido muscular marcada com anti-desmina e células mioblásticas esqueléticas marcadas com anti-miosina rápida. No estudo histopatológico, observaram-se novos vasos no grupo de células tronco mesenquimais, no grupo de células mioblásticas esqueléticas, tecido muscular e angiogênese e miogênese no grupo co-cultura.

Conclusão: A fração de ejeção do ventrículo esquerdo melhorou no grupo em que foram injetadas células musculares, mais acentuadamente no grupo co-cultura. Nos achados imunocitoquímicos, na cultura e no co-cultivo encontraram-se as células correspondentes. Nas espécimes cardíacas, foram encontradas células musculares e mioblásticas esqueléticas. Na histopatologia, encontraram-se novos vasos, tecido muscular e ambos quando se injetou células tronco mesenquimais, células mioblásticas esqueléticas e a co-cultura das duas células, respectivamente.

Descritores: Transplante de células. Infarto do miocárdio, terapia. Miocárdio. Isquemia miocárdica. Cultura de células, utilização.

INTRODUCTION

Cellular therapy has been utilized in the repair of fibrotic areas caused by myocardial infarction (MI) and different types of cells have been tested [1]. Cellular cardiomyoplasty has been studied in respect to organ function recovery through two lines of research: cells for myogenesis and cells for cardiac angiogenesis [2-4].

For the first, smooth muscle cells, adult bone marrow stem cells, skeletal myoblasts and neonatal and fetal cardiomyocytes have been used. For cardiac angiogenesis, endothelial cells, which are removed from the intimal layer of arteries or veins, bone marrow stem cells and progenitor blood cells have been employed [5].

These cells were injected either individually or in combination in different types of experimental animals [6-9]

and in clinical series [10,11] with ischemic [12] or dilated cardiomyopathy [13].

A new option for the cellular treatment of cardiomyoplasty in experimental animals with left ventricle ejection fractions of less than 40% caused by ischemic cardiomyopathy is proposed in this study.

Mesenchymal bone marrow stem cells (MSC) and skeletal myoblastic cells (SM) that were separately cultivated or in co-cultures and transplanted individually or co-transplanted were used in this work. The functional response was evaluated using bidimensional echocardiography, which is used to measure the left ventricle ejection fractions (LVEF) at two points in the follow-up. Also *in vitro* immunocytochemical studies and after euthanasia one month after cardiomyoplasty histopathologic specimens of the heart were analyzed.

METHOD

All experiments were performed according to the principles of experimental animal care laid down in the Brazilian law n° 6638 that regulates the norms of scientific-educative practices of animal vivisection [14].

The model utilized to cause ischemic cardiomyopathy was ligation of the coronary artery and consequent myocardial infarction.

Male Wistar rats weighing between 250 and 300 grams were employed as the experimental animals. Myocardial infarction was caused under general anesthesia with the animals intubated and ventilated by respiratory apparatus (Harvard Apparatus, USA), by left lateral thoracotomy. After heart exposure, the anterior interventricular coronary artery was ligated, between the left atrium and the right ventricle outflow tract using a 7-0 polypropylene thread (Ethicon, USA).

Cell transplantation or an injection of the culture medium was achieved by median sternotomy seven days after myocardial infarction, with a single injection under the same anesthetic and ventilatory conditions.

Echocardiographic valuation

The animals were analyzed using a Sonos 5500 bidimensional echocardiographic apparatus (Hewlett Packard, USA), with S12 (5-12 MHz) sector transducers and 15L6 (7-15MHz) entrance. The left ventricle ejection fraction was measured in the longitudinal para-sternal position according to Simpson's method, with the animals under general anesthesia.

Cell culture method

Harvesting of cells was achieved by two different methods depending on the cell type.

1) Myoblastic skeletal cells.

Muscle cell maceration and cleaning

Anterior tibial muscle is removed from the animals using a laminar flow cell culture hood and is placed on a slide with culture medium and 1-% antibiotics (penicillin 100 U/mL and streptomycin 100 µg/mL). After, the blood vessels and the conjunctive tissues, the aponeurosis and fatty tissues, are removed using a magnifying glass and the fragments are placed on another slide and subsequently macerated.

Enzymatic digestion

The tissue cell specimen is digested in type IA Collagenase (Sigma, USA) and placed for 1 hour in a 5% CO₂ incubator at 37° C and agitated at 10-minute intervals.

The cells are centrifuged and the cell pellet is recovered in trypsin-0.25% EDTA (Gibco, USA) in an incubator. Enzymatic digestion is interrupted using bovine fetal serum (Gibco, USA).

Filtration and seeding

The material is filtrated and centrifuged, the cell pellet is recovered and diluted in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% bovine fetal serum, 1% antibiotics and 10% IGF-I (insulin type I growth factor) and 10⁻⁷ M dexametazon. Cell counting is performed using a Neubauer camera.

The cells are cultivated in a culture medium for, on average, 14 days and kept in a 5% CO₂ incubator at 37 °C. The medium is changed two or three times per week and sub-cultures are obtained according to cellular confluence.

2) Mononuclear bone marrow cells

The cell harvesting is achieved by the puncture and aspiration technique.

Cell Collection

The animal, under general anesthesia, is placed in the lateral decubitus position with the posterior limb in flexion. Puncture of iliac crest is performed with a 5-mL syringe containing heparin (liquemine 5000 U/mL) with a 25x8 - 21G1 needle for aspiration. The collected material is processed using a density gradient, Ficoll-Hipaque (d=1.077) according to the method described by BOYUM in 1968 [15]. After 48 hours the hematopoietic line and debris floating of the culture are suctioned leaving the MSC. Counting of the mononuclear bone marrow cells is made using a Neubauer camera. The culture method is similar for all cells [16].

Co-Culture

After cellular isolation according to the previously described techniques, SM and mononuclear cells are distributed in a proportion of 2:1 and morphologic observations were made, in respect to the survival, adhesion to the substrate and confluence. After 48 hours the hematopoietic line and debris floating on the culture are suctioned from the flasks leaving only the SM, the MSC and fibroblasts. The cells are cultivated according to the previously described method [17].

Experimental Group

A total of 72 rats were included in this study and were divided into four groups according to the culture medium or the injected cells. The 22 rats of the control group were injected with the culture medium alone. The MSC group consisted of 17 rats, in which 2.5 x 10⁶ cells were injected, the SM cell group had 16 rats, in which 5 x 10⁶ cells were injected and the co-culture cell group of 17 rats were injected with 7.5x 10⁶ cells.

Immunocytochemical and histopathologic studies

During the *in vitro* culture the cells are marked with anti-vimentin to confirm the presence of MSC cells and with

anti-desmin for muscle cells and in the specimens the cells are identified with the previously mentioned markers and increased with fast anti-myosin which is a specific marker for SM.

In the histopathologic studies after euthanasia of the animals (one month after the procedure) the specimens are stained using Gomori's trichrome and the results are interpreted by optic microscopy.

Statistical analysis

The four groups were compared both together and individually, in respect to the LVEF one week after MI and one month after the cell injection. The ANOVA test, student t-test for paired values and Fisher exact test were utilized and statistical differences were considered significant when p<0.05.

RESULTS

Ventricular function analysis

Left ventricle ejection fraction

When comparing the mean LVEF of the groups in the control echocardiograms, no statistical differences were evidenced (p=0.276).

In echocardiographic evaluations one month after the cell injections, significant statistical differences were confirmed between the mean ejection fractions of different groups (p=0.001). These differences were seen between the Control Group and the SM Group (p=0.037), between the Control Group and in the Co-culture Group (p<0.001) and between the MSC Group and the Co-culture Group (p=0.025). The differences between the Control Group and the MSC Group (p=0.372), between the SM Group and the MSC Group (p=0.263) and the SM Group and the Co-culture Group (p=0.267) were not considered statistically significant.

When the measurements between the first and second echocardiograms were compared within each group, significant differences were verified in the Control (p=0.005) and co-culture Groups (p=0.006). Significant differences were not found in the MSC (0.65) and SM (0.09) Groups.

The results obtained in respect to the ejection fractions are showed in the Tables 1, 2 and Figure 1.

Immunocytochemical Study

In the in vitro immunocytochemical study the MSC were identified by staining using anti-vimentin and the muscle cells using anti-desmin stain.

In the evaluation of specimens one month after transplantation, muscle cells were identified with anti-desmin stain and SM with fast anti-myosin stain.

Table 1. Results of left ventricle ejection fraction after 1 week and after 1 month of myocardial infarction

Variable	Control (n=22)	MSC (n=17)	ME (n=16)	Co-culture (n=17)	p*
	Mean ± SD	Mean ± DP	Mean ± DP	Mean ± DP	
EF 1 week	26.68 ± 6.92	26.80 ± 8.17	22.90 ± 6.25	23.52 ± 8.67	0.276
EF 1 month	22.32 ± 6.94	24.80 ± 10.20	28.21 ± 9.15	31.45 ± 8.87	0.001**
p ⇄	0.005	0.649	0.091	0.006	

(*) ANOVA (** adjusted for 1 week (⇄) paired student t-test p<0.05

Table 2. Ejection fraction after 1 month – comparisons between groups

compared groups	p*
Control x SM	0.037
Control x MSC	0.372
Control x Co-culture	<0.001
ME x MSC	0.263
ME x Co-culture	0.267
MSC x Co-culture	0.025

(*) LSD Test (p<0.05)

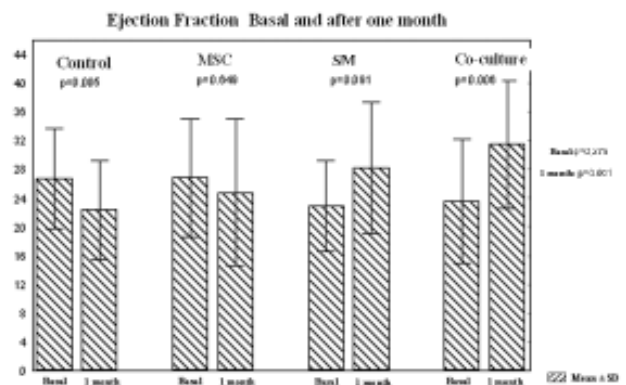


Fig. 1 - Ejection fraction after 1 month – comparison of the ejection fraction of each group between the control echocardiogram and one month after the transplantation (*) LSD test (p<0.05)

Histopathologic Study

In the interpretation of the sections stained with Gomori trichrome, muscle tissue was identified in the group in which these cells were injected. In the group in which MSC were injected, only angiogenesis was identified in the region of MI. In the co-culture group both angiogenesis and myogenesis were verified in the fibrotic area of the left ventricle (Fig. 2).

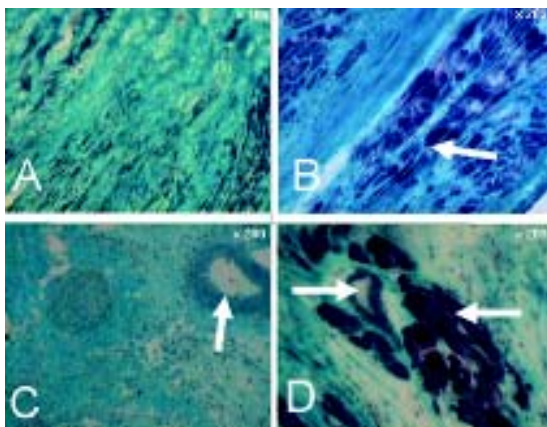


Fig. 2 A – Microphotography of the scar after 1 month of myocardial infarction. B – Microphotography of histopathologic study of the SM group where the transplanted muscle tissue is identified by the arrow. C – Microphotography of histopathologic study of the MSC group, where neo-angiogenesis is identified by the arrow. D – Microphotography of the histopathologic study of the co-culture group with angiogenesis and myogenesis indicated by arrows. The histologic sections were stained with Gomori Trichrome and the magnification is identified in each microphotography.

DISCUSSION

Cardiac insufficiency is determined, among other causes, by ischemic cardiomyopathy [18]. The therapeutic use of percutaneous angioplasty or of coronary artery bypass grafting surgery employing vascular grafts has been adopted to relieve symptoms or to improve the offer of nutrients to the ischemic area but this does not, however, definitively regenerating the injured cardiomyocytes which are responsible for the affected heart contractile function [19].

Heart transplantation has been, until now, the only surgical treatment that treats the cause and not the effect of the injury to the cardiac cells, as the organ is replaced. However, this is restricted by the small number of donors and the difficult postoperative follow-up [20].

With the knowledge of molecular and cellular biology, cell therapy has been used in different diseases that, until now, were incurable and intractable [21]. The development of cell therapy for heart disease started in the 1990s with the experimental utilization of fetal cardiomyocytes evolving to the use of SM and MSC [22,23].

Recovery of the supply of nutrients and metabolic substances through angiogenesis and of muscle mass through myogenesis has been tried. At the beginning of 2000 its utilization in clinical series was started and in 2001 MENASCHE et al. [10] described the utilization of SM as an option for heart contractile function recovery.

A study developed in PUCPR was based on the idea of

trying to promote synergism among cells during culture and to offer two different cells in the regeneration of myocardial infarction scarring with the aim of developing new vessels for perfusion and also muscle mass. With this, the study aimed at reducing the high mortality of cells injected in an area with low nutrients, as has been described in the literature [1].

Thus, MSC and SM are utilized for angiogenesis and myogenesis respectively.

The *in vitro* immunocytochemical findings and results from tests in heart specimens obtained one month after transplantation, confirmed the presence of both isolated and co-culture cells cultivated in a laboratory.

In the histopathologic studies also performed in this period, new vessels were found in the group in which MSC had been injected, muscle cells were found in the SM group and angiogenesis and myogenesis were evidenced in the group in which the two co-cultivated cells were transplanted.

In the evaluation of the LVEF, statistical differences were not observed when comparing control echocardiograms between the four different groups.

Statistical differences were identified among the groups in the evaluation one month after transplantation: between the Control and SM Groups, between the Control and the Co-culture Groups and between the MSC and Co-culture Groups which suggests that in the group transplanted with isolated muscle cells and in the Co-culture Group improvement in the cardiac function occurs.

The comparison between the two evaluations gave significantly worse performance in the Control Group and significantly better performance in the Co-culture Group. In the MSC and SM Groups no significant difference was identified, although the ejection fraction increased in the latter group.

CONCLUSION

The histopathologic, immunocytochemical and echocardiographic findings demonstrated that:

The left ventricle ejection fraction improved only in the group in which isolated muscle cells were implanted or in the Co-culture Group and this latter group showed the most significant recovery compared to the other groups which were utilized in this study.

During co-cultivation, two cells, TMC and SM, were present, that the different types of cells were found separately or together according to the injected cells and that in the MSC Group only new vessels were found, in the SM Group only muscle cells were identified and but in the Co-culture Group both angiogenesis and myogenesis were verified.

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