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The transplant of cardiac cells and myoblast skeletal cells in myocardial infarction

O transplante de células mioblásticas esqueléticas e de células cardíacas no infarto do miocárdio

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Abstract

Objective: **To analyze the functional results of adult skeletal muscle cell and cardiac cell transplantations in the hearts of rats that have suffered an infarct.**

Method: **An infarct of the anterolateral wall of the left ventricle was provoked, by left lateral thoracotomy and posterior legation of the left coronary artery. After five days the animals were submitted to a transthoracic echocardiography to evaluate the systolic (LVFSV) and diastolic volumes (LVFDV) of the left ventricle and the left ventricle ejection fraction (LVEF). After that, the animals were divided in three groups: 1) control (n=10), 2) adult cardiac cells (n=8) and 3) adult skeletal muscle cells (n=8). Seven days after the myocardium infarct, all the animals were operated on again by median sternotomy, identifying the region with fibrosis and conducting the injection of 0.15 ml of a culture in group I, $8.5x10^6$/0.15 ml, of heterologous skeletal muscle cells in group III, and $8.5x10^6$/0.15 ml of heterologous adult cardiac cells in group II. All animals received cyclosporin (15mg/kg/day). After two months, an echocardiography was conducted on all mice, assessing the same parameters.**

Results: **Two months after transplantation, group I presented a decrease in the LVEF (48.18% ± 9.95% vs. 33.25% ± 12.41% p=0.0003), with an increase of the LVFSV and the LVFDV (0.308 ml ± 0.072 ml vs. 0.536 ml ± 0.228 ml p=0.0026 and 0.597 ml ± 0.098 ml vs. 0.776 ml ± 0.187 ml p=0.0540, respectively). In group II, there was an stabilization of the LVEF (42.48% ± 7.83% vs. 41.31% ± 8,46% p=0.4968, respectively) an increase in the LVFDV and LVFSV (0.602 ml ± 0.203 ml vs. 0.771 ml ± 0.110 ml p=0.0711 and 0.358 ml vs. 0.450 ml p=0.0400, respectively). Group III presented an increase on the LVEF, LVFSV and LVFDV (40% ± 5.69% vs. 47.35% ± 6.89% p=0.0142, 0.643 ml ± 0.103 vs. 0.931 ml ± 0.226 p=0.0026 and 0.388 ml ± 0.082 vs. 0.491 ml ± 0.149 ml p=0.0557, respectively).**

Conclusion: Two months after cell transplantation, there was a significant improvement in the LVEF in group III, when compared to group I, and there was a preservation of the ventricular contractility and a stabilization of LVEF in group II.

Descriptors: **Cell transplantation. Myocardial infarction. Heart. Myocardium.**

Resumo

Objetivo: **Comparar o resultado funcional e anátomo-patológico entre o transplante de células mioblásticas e cardíacas no infarto do miocárdio.**

Método: **Realizado infarto da parede ântero-lateral do ventrículo esquerdo em 26 ratos Wistar, com ligadura da artéria coronária esquerda. Após cinco dias, os animais foram submetidos a ecocardiografia transtorácica para cálculo dos volumes sistólico (VSFVE) e diastólico (VDFVE) finais e da fração de ejeção do ventrículo esquerdo (FEVE). Os animais foram divididos em três grupos: 1) controle (n=10), 2) células cardíacas adultas (n=8) e 3) células musculares esqueléticas adultas (n=8). Sete dias após o infarto do miocárdio, os animais foram reoperados por esternotomia mediana, sendo identificada a região de fibrose no ventrículo esquerdo e nela, injetado 0.15ml de meio de cultura no grupo I, 8.5x106/0.15ml de células cardíacas heterólogas no grupo II e 8.5x106/0.15ml de células musculares esqueléticas heterólogas no grupo III. Todos os animais receberam ciclosporina (15mg/kg/dia). Após dois meses do transplante, realizou-se nova ecocardiografia avaliando os mesmos parâmetros.**

Resultados: **Após dois meses do transplante celular, o grupo**

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I apresentou um decréscimo da FEVE (48.18% vs. 33.25% p=0.0003), sendo que houve um acréscimo dos VSFVE e VDFVE (0.308ml vs. 0.536ml p=0.026 e 0.597ml vs. 0.776ml p=0.054, respectivamente). No grupo II houve uma estabilização da FEVE (42.48% vs. 41.31% p=0.4968, respectivamente) e um discreto aumento do VDFVE (0.602ml vs. 0.771ml p=0.0711). O VSFVE variou de 0.358ml a 0.450ml p=0.0400. O grupo III apresentou um acréscimo da FEVE, VDFVE e VSFVE (40% vs. 47.35% p=0.0142, 0.643ml vs. 0.931ml p=0.0026 e 0.388ml vs. 0.491ml p=0.0557 (sem significância), respectivamente. O GIII apresentou um maior valor, considerado estatisticamente significativo, da fração de ejeção do ventrículo esquerdo, em comparação ao GI e ao GII (47.35% ± 6.89% vs. 41.31% ± 8.46% vs. 33.25% ± 12.41% p=0.0200, respectivamente). Identificou-se uma diferença estatisticamente significativa da fração de ejeção do ventrículo esquerdo entre o GIII e o GI, após dois meses do transplante (47.35% ± 6.891% vs. 33.25% ± 12.41% p=0.0213, respectivamente). Identificou-se uma diferença da fração de ejeção, após dois meses, entre o GIII e o GII, todavia não foi considerada estatisticamente significativa (47.35% ± 6.891% vs. 41.31% ± 8.46% p= 0.481, respectivamente). Também se identificou uma diferença deste mesmo parâmetro entre o GII e o GI, mas sem ser significativa (41.31% ± 8.461% vs. 33.25% ± 12.41% p=0.245, respectivamente).

***Conclusão: As* células mioblásticas mantiveram suas características morfológicas após o transplante no infarto do miocárdio. Os fibroblastos foram as células encontradas em maior quantidade, durante o processo de cultura celular no grupo das células cardíacas. Após dois meses do transplante das células, houve uma melhora significativa da FEVE do grupo III em comparação ao grupo I, uma preservação da contratilidade ventricular no grupo III e uma estabilização da fração de ejeção do grupo II.**

***Descritores*: Transplante celular. Infarto do miocárdio. Coração. Miocárdio.**

INTRODUCTION

The death of adult cardiomyocytes originating from ischemic accident leads to cardiac muscular fibrosis and its progression can lead to heart failure. This is because from the moment that the cardiomyocyte becomes mature, it looses its capacity to multiply, which occurs after the first years of life. Basically, the growth of the heart from birth until adult suffers a hypertrophic and not a cellular hyperplastic process.

Some authors suggest a capacity of the cardiomyocytes to multiply. However, this capability is very small, around 0.5% of the total of the cardiomyocytes can undergo mitosis, (1) which is too few to obtain an improvement in cardiac function.

Heart failure is a public health problem with high annual morbid-mortality rates, both in developed and underdeveloped countries. It is exactly due to this, associated with the difficulties of clinical treatment (ACE inhibitors, beta-blockers, digitalis and diuretics) and surgery (cardiomyoplasty, heart transplantation, aneurysmectomy, ventriculotomy, pacemaker and artificial heart implantation) of heart failure, that transplantation of cells has been proposed.

The disadvantage of treatments that aim to recover the ventricular remodeling is that they do not treat the fundamental cause which is the loss of mature cardiomyocytes. Currently, among other techniques, two lines of study are being proposed for the treatment of heart failure, thus attempting to rectify the main cause: 1) the administration of angiogenic factors that increase the vascular flow and 2) transplantation of cells which increases the contractile mass.

Interest for these procedures emerged from studies such as that by VAN BEKKUN, (2) in bone marrow transplantation; by OSTMAN et al., (3) in Langerhans' islet transplantation; by PATRIDGE et al., (4) with myoblast transplantations in patients with Duchenne's disease; and VELDE et al. (5) with the of nerve cell transplantation in the therapy of Parkinson's Disease.

Experimental transplantation of skeletal myoblast cells by MURRY et al., (6) smooth muscle cells by LI et al., (7) fetal cardiomyocytes by SCORSIN et al. (8) and mature cardiomyocytes by SAKAI et al., (9) in infarcted myocardial patients, have been performed with the objective of recovering the infarcted region, avoiding the development of dilated myocardiopathy, which can lead to congestive heart failure.

However, there are still some doubts in relation to which is the best muscle cell to be transplanted in infarcted myocardial patients. SCORSIN et al. (10) compared the transplantation of fetal cardiomyocytes and skeletal myoblasts, and observed a similar improvement in the left ventricle ejection fraction in both groups.

Thus, the objective of this study is to compare the functional and anatomicopathological results between the transplantation of myoblastic and cardiac cells in myocardial infarction patients.

METHOD

This experimental research was performed in the Cardiovascular Surgery Department of the Bichat – Claude Bernard Hospital and the INSERM-523 unit (Myology Institute, Grupo Hospitalar Pitié Salpètriére), Paris, France.

All the animals received professional care in accordance with the "Principles of Laboratory Animal Care", formulated by the National Society of Animal Research and "The Guide

to Laboratory Animal Care", prepared by the National Academy of Science and published by the National Institution of Health (NIH Publication Nº 80-23, reviewed 1985).

Experimental Model

Twenty-six Wistar mice with an average weight of 400 grams were operated. Anaesthetic induction was by the administration of 50 mg/kg/weight of Ketamine and 10 mg/kg/weight of xylazine, both via intraperitoneal. Following this the animals underwent endotracheal intubation and mechanical ventilation using a volume respirator (HARVARD® Inc, model 683 respirator, Massachusetts, USA).

In all the animals a left lateral thoracotomy was performed, by which pericardiotomy was made and consequently a ligature of the left coronary artery using polypropylene 7.0 thread (Ethicon®, Inc., Somerville, NJ) was made, inducing infarction of the anterolateral wall of the left ventricle. The effectiveness of this procedure was proven when there was a change in the color (paling in aspect) of the left ventricle wall. (Figures 1a and b)

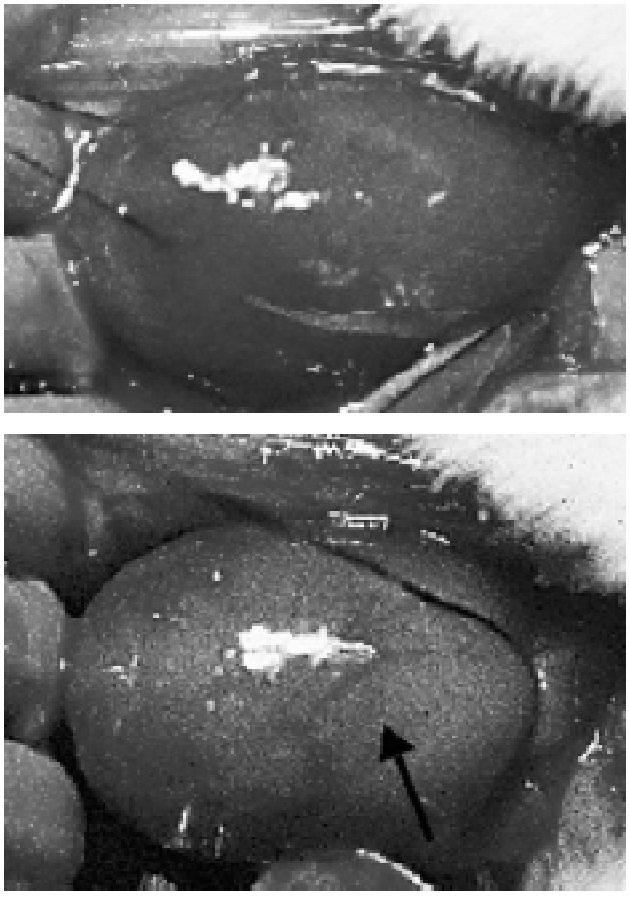


Fig. 1 - (A) Normal heart of rat - (B) Myocardial infarction of rat. Notice the pale aspect of the antero-pateral wall of the left ventricle (arrow)

Five days after the myocardial infarction, the animals were submitted to an evaluation by 2-dimensional transthoracic echocardiogram (Sequóia Acuson Corporation, Mountain View, CA), equipped with a 15 MHz linear transducer, allowing an analysis of up to 160 Hz and specifically developed for the ultrasound study of small animals. The transducer was placed on the left anterolateral portion of the thorax and the heart was visualized in 2-dimensions with axial vision of the left ventricle, including the mitral valve, aorta and the apex in the same image. Digital conversion of the image was achieved by delimitation of the interventricular septum and the posterior wall of the left ventricle. Consequently, the following measurements were made: final systolic and diastolic surface areas, final systolic and diastolic length of the left ventricle and cardiac frequency, for a subsequent calculation of the final systolic and diastolic volumes and the left ventricle ejection fraction. All the measurements were made three times by the same professional, and the average of each parameter was calculated.

FORMULA OF THE VENTRICULAR VOLUMES (11):

$V = 8 \times (S)^2 / (3 \times 3.1415926 \times L)$, where:
V = volume; S = surface area; and L = length.

FORMULA OF THE LEFT VENTRICLE EJECTION FRACTION:

LVEF = LVFDV – LVFSV / LVFDV Where:
LVEF = Left Ventricle Ejection Fraction;
LVFDV = Left Ventricle Final Diastolic Volume; and
LVFSV = Left Ventricle Final Systolic Volume.

At this point the animals were divided in three groups:
-GI = Control group (n=10),
-GII = Mature cardiac cell transplantation group (n=8),
-GIII = Mature skeletal muscle cell transplantation group (n=8).

Seven days after myocardial infarction, the animals were submitted to a median sternotomy. After identification of the myocardial infarction area, a sub-epicardial injection of 0.15 culture medium (57% MEM and 0.5% BSA - Fraction V, Sigma) without cells was applied to the control group (GI).

GII received 7.8×10^6 to 9×10^6 heterologous mature heart cells (mean 8.5×10^6 cells), diluted in 0.15 ml of culture medium. As the connexin-43 protein was not marked in the culture medium, the identification of the isolated number of mature cardiomyocytes was not possible.

GIII received the same amount of culture medium only with 7.5×10^6 to 10×10^6 heterologous mature skeletal muscle cells (mean 8.5×10^6 cells), with the number of myoblasts varying from 8.3×10^5 to 1.88×10^6 (mean 1.39×10^6).

All the animals received cyclosporin (15 ml/kg/weight/day) before sternotomy and the dose was maintained until the animals were sacrificed.

Two days after the cell transplantation, all the animals were resubmitted to an echocardiographic evaluation, when the same parameters as in the pre-transplantation test were assessed. After this, the animals were sacrificed with an overdose of intra-abdominal ketamine and xylazine for anatomicopathological evaluation.

Cell Culture

Preparation of the mature cardiac cells

The mature cardiac cells were prepared in the following stages:

Harvesting of the cells was made by the sacrifice of adult Wister mice and the resection of 2 mg of interventricular septum muscle. Subsequently, the muscle was placed in a sterile medium, and after, in a culture medium (Iscove's modified Dulbecco's medium, Canada Life Technologies Inc., Burlington, Ontario), 10% bovine fetal serum, 0.1 mmol/1-beta-mercaptoenotanol, 100 u/ml penicillin and 100 mg/ml streptomicin). Consequently, the muscle was washed in a PBS solution (NaCl 136 Mm, Kcal 2.7 Mm, Na_2HPO_4 8.1 Mm, KH_2PO_4 1.5 Mm, pH = 7.3). The connective tissue was removed and the muscle was cut in small portions. After the muscle was submitted to digestion with trypsin EDTA (0.25% for 20 minutes; Gibco) to 0.2% and 1A collagenase (2 mg/ml for 1 hour, Sigma, St Louis, MO) for 5 minutes at 37 ºC . The floating segments were collected and put in the culture medium again, and following this submitted to centrifugation at 1000 revolutions/minute for 5 minutes.

The percentage of the mature cardiomyocytes obtained separately in the cellular culture medium was not identified. Despite the use of a specific cardiac muscle cell marker, connexin-43, it was not possible to identify them. The main cells that were identified, were fibroblast (in 50%), endothelium cells, smooth muscle cells and Purkinje cells, with a total of 8×10^6 cells.

Preparation of the mature skeletal muscle cells

The myoblastic cells, which is the most undifferentiated contractile structure in the linage of the formation of the skeletal muscular tissue, were prepared in the INSERM 523 laboratory from skeletal muscular biopsy of adult Wistar Mice. Two grams of tibial skeletal muscle of mice were submitted to enzymatic dissolution at 37 ºC, utilizing initially 1A collagenase (2 mg/ml for 1 hour, Sigma, St Louis, MO) and after trypsin EDTA (0.25% for 20 minutes; Gibco). Following this, filtration and centrifugation at 1000 revolutions/minute for 5 minutes was made. After the cells were placed in a culture medium with 240 ml of F12 solution rich in nutritive factors (glycol, calcium, oligoelements, magnesium and zinc), 60 ml of 20% bovine fetal serum, 1% antibiotic (penicillin / streptomicin) and 5 nanograms/ml of bFGF. The cells were then fractionated and cooled until the following day when the culture medium was changed. This was repeated until completing 7 days.

On the day of transplantation, the culture medium was separated from the cells by means of sucction and washed in PBS. Consequently, the cells were replaced in contact with trypsin for 2 minutes at 37 ºC. They were then recovered using a pipette and centrifuged at 1000 revolutions/minute. Twenty-five million cells were produced from each gram of cultivated skeletal muscle. After warming the cells, containers with 5×10^6 cells were prepared and put in a transportation medium with 57% MEM and 0.5% BSA (Fraction V, Sigma). Dead cells did not exceed 5%.

Using an inverted lens on an optical microscope (OLYMPUS IX70, Rugins, France), the prepared solution was randomized, photographed and submitted to fluorescent illumination. The percentage of myoblastic cells obtained was calculated dividing the total number of cells counted in the contrast phase by the total number of cells marked by immunofluorescence with positive dismin. Of the 8.5×10^6 cells injected into the medium, 30% of separated myoblasts were obtained. The other identified cells were fibroblasts, endothelium cells and smooth muscle cells.

Pathologic Anatomy

The animals were sacrificed two months after transplantation, and the hearts were removed and split into two parts: the apex containing the infarcted area, and the base. Consequently, each part was placed in contact with isopentanol and cooled with liquid nitrogen to –20 ºC. After, the two parts of the heart were conserved at –80 ºC. Within one week, the anatomic parts were submitted to fractionated eight-micron sequential cuts of the infarcted region and the transitional zone between the myocardial infarction and the whole myocardium.

Identification of the infarcted area was made by staining of slides with hematoxylin-eosin.

Identification of the quantity of transplanted cells in the mature cardiac cell group (GII) was made by staining of slides with hematoxylin-eosin.

Identification of the viable muscle fibers in the infarcted region and the transition zone between the infarction and whole myocardium, was made by means of immunofluorescence, utilizing the specific anti-desmin antibody to mark the desmin protein.

To identify the skeletal muscle fibers specific antibodies, embryonary anti-myosin were used to identify the embryonary myosin protein and rapid anti-isoform of myosin to identify the rapid isoform of myosin.

The anti-connexin-43 antibody was used to mark the connexin-43 protein, present in 90% of the GAP junctions, which are a part of the intercalated disk, a characteristic structure of the cardiomyocytes, both in the mature cardiac cell group (GII) and the mature skeletal cell group (GIII).

With the objective of identifying transplanted cells and some type of connection between the skeletal cells and the native mature cardiomyocytes, sequential fractional cuts at each 8 microns were made, analyzing the myocardial infarction zone and the transitional zone between the myocardial infarction and the native cardiac muscle.

Statistical Analysis

To compare the results of the ejection fraction, left ventricle final systolic and diastolic volumes soon after myocardial infarction and two months after cell transplantation, Student T-test was used for paired samples.

For the comparison among the groups at the different time intervals, Variance analysis was used assessing the homogeneity of the variance by means of the ANOVA test. When there was a significant difference among the groups, the SCHEFFE test was adopted to investigate the difference. In all tests a 5% difference was considered significant.

RESULTS

Methodology

A mortality rate of 55% was observed after myocardial infarction and the mortality rate after cell transplantation was 40%.

Echocardiographic Analysis

Control Group (GI)

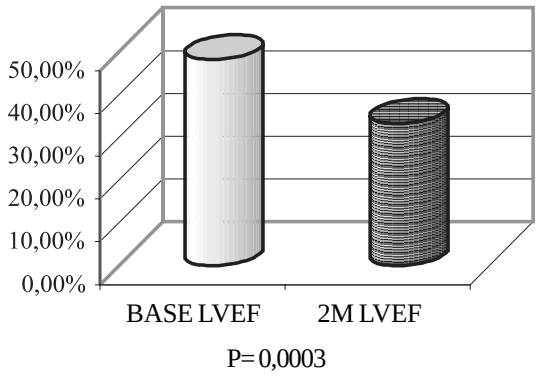
Analyzing the control group individually using the student T-test, a significant reduction in the left ventricle ejection fraction was evidenced two months after the injection of the culture medium in the infarcted region (48.18% ± 9.95% vs. 33.25% ± 12.4% p=0.0003)(figure 2). In the same group, the final left ventricle systolic and diastolic volumes presented an increase (0.308 ml ± 0.072 ml vs. 0.536 ml ± 0.228 ml p=0.0026 and 0.597 ml ± 0.098 ml vs. 0.776 ml ± 0.187 ml p=0.0054, respectively).

Mature Cardiac Cell Group (GII)

In this group no statistically significant difference was evidenced in the left ventricle ejection faction two months after transplantation (42.48% ± 7.83% vs. 41.31% ± 8.46% p=0.4968)(figure 3). The left ventricle final diastolic volume presented with a slight increase, but without statistical significance in this period (0.602 ml ± 0.203 ml vs. 0.771 ml ±0.110 ml p=0.0711), suggesting a limitation in the left ventricular dilation. However, the left ventricle final systolic volume presented with a significant increase (0.358 ml ± 0.154 ml vs. 0.450 ml ± 0.083 ml p=0.0400).

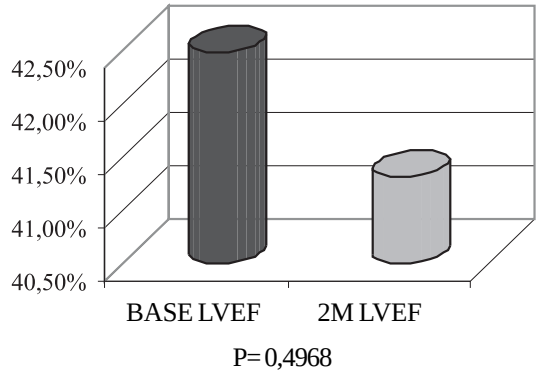
Mature Skeletal Muscle Cell Group (GIII)

This group presented statistically significant improvement in the left ventricle ejection fraction two months after transplantation (40% ± 5.69% vs. 47.35% ± 6.89% p=0.0142)(figure 4). Analyzing the relative values, an increase of 18.37% was observed (range 7.35 to 40%). The left ventricle final systolic volume presented with a slight increase seen by 2-dimensional echocardiograph (Figure 2), however, this value was not considered significant (0.388 ml ± 0.082 ml vs. 0.491 ml ± 0.149 ml p=0.0557)(Figure 5), as it was viewed by the bidimensional echocardiography (Figure 6).



Significant reduction of the LVEF (%)

Fig. 2 - Variation of the LVEF control group.



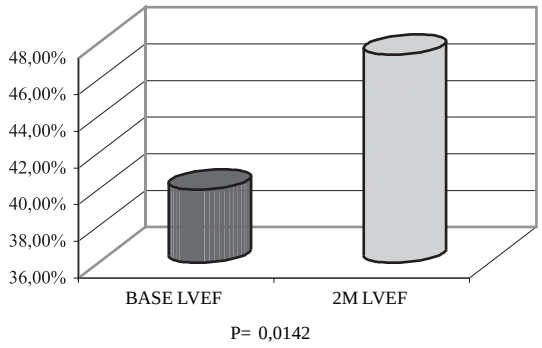
Stabilization of the LVEF (%)

Fig. 3 - Variation of the LVEF - cardiac cell group.

Comparison among the Groups

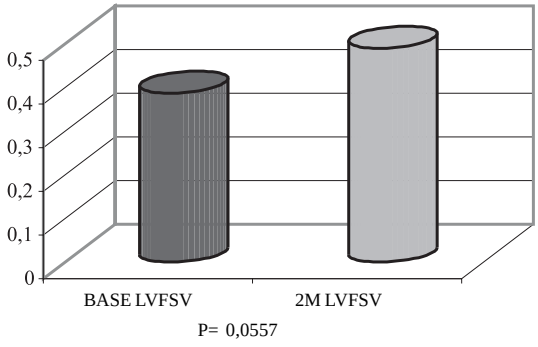
Comparing the three groups using ANOVA, no significant statistically differences were evidenced in the echocardiograph values soon after myocardial infarction. All the animals presented with similar average ejection fraction values (Figure 7) and similar left ventricle final systolic and diastolic volumes. The three groups were considered to be homogeneous (Table 1).

Two months after transplantation, GIII presented with a higher left ventricle ejection fraction, which was considered statistically significant when compared with GI and GII (GII



Significant increase in the LVEF (%)

Fig. 4 - Variation of the LVEF - skeletal cell group.



Non-significant increase in the LVSFV (ml)

Fig. 5 - Variation of the LVFSV - skeletal cell group.

(47.35% ± 6.89% vs. 41.31% ± 8.46% vs. 33.25% ± 12.41% p=0.0200, respectively)(Figure 7).

As there was a significant alteration of this parameter among the groups, the SCHEFFÉ method was adopted to investigate differences. This study showed a statistically significant difference of the left ventricle ejection fraction between GIII and GI two months after transplantation (47.35% ± 6.891% vs. 33.25% ± 12.41% p=0.0213, respectively). Also a difference in the left ventricle ejection fraction two months after transplantation between GIII and GII was observed, although this was not considered statistically significant (47.35% ± 6.891% vs. 41.31%± 8.46% p= 0.481, respectively). A difference of this parameter was evidenced between GII and GI too, but it was not significant (41.31% ± 8.461% vs. 33.25% ± 12.41% p=0.245, respectively).

An analysis of the left ventricle final diastolic volume comparing the period soon after myocardial infarction with two months after transplantation, showed all the groups had an increase of the parameter. However, there was no significant difference among the three groups, both in the period soon after infarction and two months after transplantation. The three groups were considered homogeneous in both analyses (Table 2).

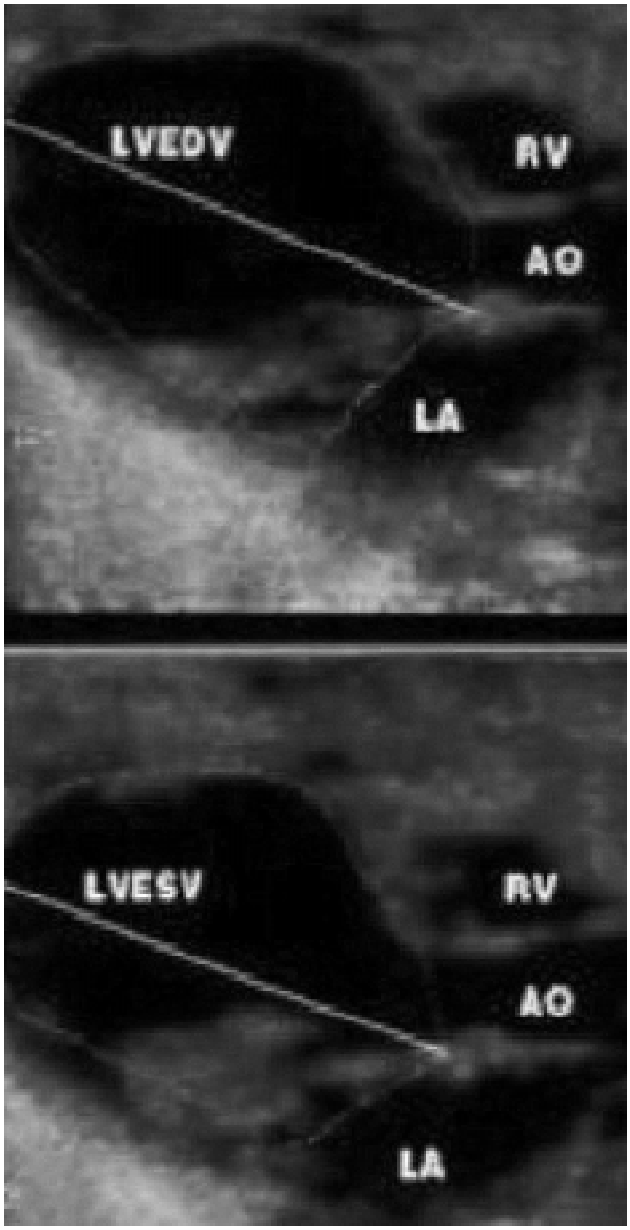
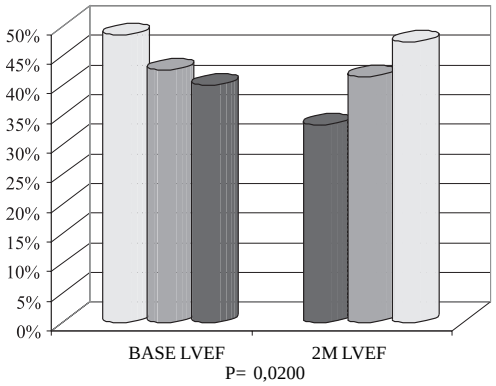


Fig. 6 - Systolic and diastolic volumes of left ventricle (LVESV LVEDV) after skeletal cell transplantation.

An analysis of the left ventricle final systolic volume evidenced that there were no statistically significant differences among the three groups soon after myocardial infarction. Two months after transplantation, an increase in the final systolic volume was seen in all three groups but without significant differences. However, the group that received skeletal cells (GIII), presented with a smaller variation of the final systolic volume when compared with the control group (p = 0.0557)(Table 3).



Values of the base LVEF without significant difference
Better significance of the 2M LVEF of G III compared to GI

Fig. 7 - comparation between LVEF groups (%).

Table 1. Base mean echocardiographic values

GROUP	LVEF	LVFDV	LVFSV
control	48.18% ± 9.951%	0.597 ml ± 0.098 ml	0.308 ml ± 0.072 ml
cardiac cells	42.48% ± 7.831%	0.602 ml ± 0.203 ml	0.358 ml ± 0.154 ml
Skeletal cells	40.00% ± 5.691%	0.643 ml ± 0.103 ml	0.388 ml ± 0.082 ml
p=	0.1147	0.7611	0.2982

The percentage variation of the LVEF from the period soon after myocardial infarction and two months after transplantation for the control group (GI) was -62.36% ± 56.12%, for the mature cardiac cell group (GII) it was -3.88% ± 13.32% and for the skeletal muscle group (GIII) it was 14.55% + 12.48%; p = 0.0004. There was a significant increase between the skeletal cell group (GIII) with the control group (GI) in respect to this parameter of 76.91%; p = 0.0008. The group that received the cardiac cells (GII) presented a significantly smaller increase in relation to the control group (GI) of 58.47%; p = 0.0098. There was a variation in the ejection fraction between the skeletal cell group (GIII) and the cardiac cell group (GII), but this was not considered significant (18.43%; p = 0.6073).

Table 2. LVFDV mean echocardiográphic values

GROUP	LVFDV BASE	LVFDV 2M
control	0.597 ml ± 0.098 ml	0.776 ml ± 0.187 ml
cardiac cells	0.602 ml ± 0.203 ml	0.771 ml ± 0.110 ml
Skeletal cells	0.643 ml ± 0.103 ml	0.931 ml ± 0.226 ml
p=	0.7611	0.1476

Tabela 3. Valores ecocardiográficos médios do LVFSV

GROUP	LVFSV BASE	LVFSV 2M
control	0.308 ml ± 0.072 ml	0.536 ml ± 0.228 ml
cardiac cells	0.358 ml ± 0.154 ml	0.450 ml ± 0.083 ml
Skeletal cells	0.388 ml ± 0.082 ml	0.491 ml ± 0.149 ml
p=	0.2982	0.5783

Pathologic Anatomy

Control Group (GI)

The infarcted zone of the animals was identified by means of staining their respective slides using hematoxylin-eosin, which proved myocardial infarction in all of the animals.

Mature Cardiac Cell Group (GII)

Use of the anti-desmin antibody to identify the type of muscle fiber in the infarcted region was not efficient, nor was the anti-connexin-43 antibody to identify possible connections between the transplanted cardiac muscle fibers and the native cardiac fibers. Not one type of viable muscle fiber was identified in the transplanted region.

Because of this, the infarcted area was investigated using hematein-eosin to check the presence or absence of transplanted cells. In the mature cardiac cell group (GII), despite not specifically identifying the transplanted muscle cells, an expressive number of cells in the region was observed when compared to the control group.

Mature Skeletal Muscle Cell group

In the investigated region, myocardial infarction was observed and hematoxylin-eosin was utilized to identify the extension of the tissue necrosis and also the presence of any transplanted skeletal muscle fibers (Figure 8).

Immunofluorescence of the embryonary myosin protein and the rapid isoform of myosin was utilized, which identified the presence of skeletal muscle fibers in the infarcted region.

Immunofluorescence of the desmin protein was employed to identify all the types of viable muscle fibers in this region: smooth, skeletal and cardiac fibers (Figure 9). The anti-connexin-43 antibody, which marked the connexin-43 protein, was used, and identified the presence of GAP junctions only between the native mature cardiac cells. Connexin-43 protein was not evidenced between transplanted skeletal muscle cells or between the transplanted cells and native mature cardiomyocytes (Figure 10).

COMMENTS

Transplantation of cells involves some mechanisms, which are still not well understood, as for example, the survival of transplanted cells. We suggest there could be a local angiogenisis mechanism, induced by the transplantation of the cells that is responsible for the nutrition and oxygenation of the transplanted cells.

In this study, it was observed that the group of animals

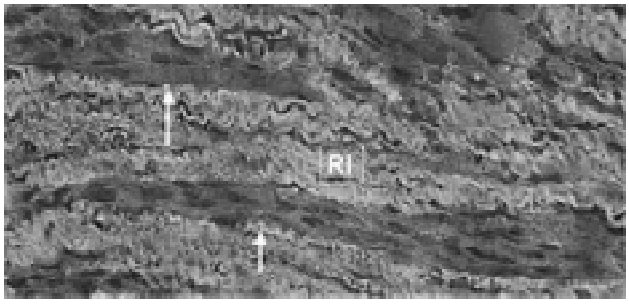


Fig. 8 - Skeletal muscle fibers (arrows), transplanted in the infarcted region (ri) (he magnification 40x)

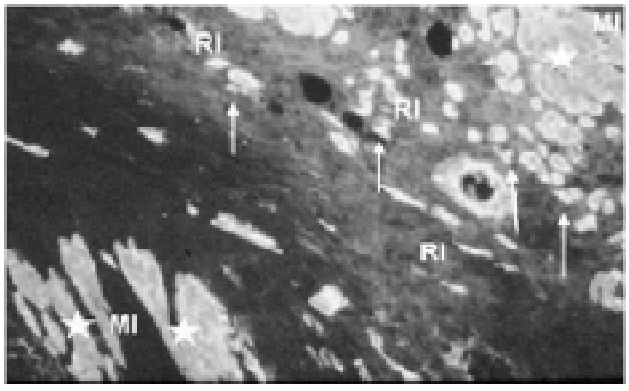


Fig. 9 - Immunofluorescence of desmin. Notice the viable muscle fibers (arrows and stars) in the infarcted region (ri) and in the integral myocardium (mi) (magnification 150x)

that received skeletal cells presented with an improvement in the left ventricle ejection fraction in the two-month period following transplantation. The control group presented a reduction of the left ventricle ejection fraction in the same period, a result which support the findings of LEOR et al., (13) as after the establishing of myocardial fibrosis, suggested that the mature cardiomyocytes did not regenerate, starting left ventricular remodeling. The group that received mature cardiac cells presented with a reduction in the ejection fraction, which was not considered to be statistically significant. We suggest there was a stabilization of the left ventricle ejection fraction, limiting the progression of the ventricular remodeling.

In relation to the left ventricle final diastolic volume, all

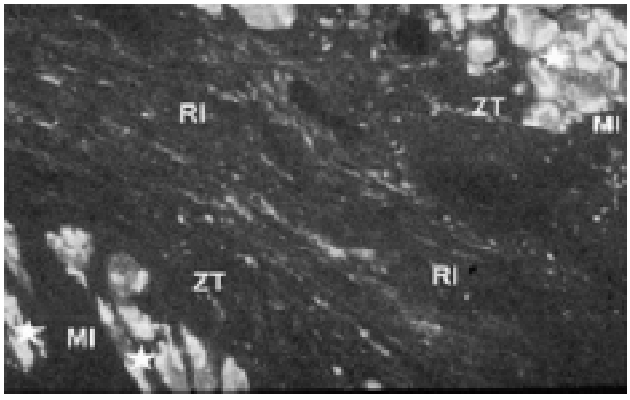


Fig. 10 - Immunofluorescence of the conexin-43 protein. Conexin-43 is not identified in the transition zone (zt) and in the central region (ri) of the slide, where the transplanted skeletal muscle fibers can be identified. Notice the presence of conexin-43 (stars) only in the integral myocardium (mi) (magnification 150x)

the animals presented with an increase two months after transplantation. The final diastolic volume was similar among the three groups, as there was no significant difference between them. This data suggests that the left ventricle dilates in an attempt to normalize the cardiac output, a fact characteristic of ventricular remodeling.

Analyzing a potential contractile capacity of the myocardium, after cellular transplantation, the left ventricle final systolic volume was studied. In the three groups, an increase of this parameter was observed two months after transplantation. However, in the skeletal cell group (GIII), a stabilization of the left ventricle final systolic volume was evidenced, as the difference between the period immediately after myocardial infarction and two months after transplantation was not considered to be significant. This suggests that although the left ventricle was dilated, it maintained its contractile capacity.

Comparing the groups two by two, the difference of the left ventricle ejection fraction was considered significant only between the mature skeletal muscle cell group (GIII) and the control group (GI). Statistically there was no difference between the mature cardiac cell group (GII) and the control group (GI) and between the mature cardiac cell group (GII) and the mature skeletal muscle group (GIII).

Also when studying the percentage of variation of the left ventricle ejection fraction among the three groups, when they were compared in twos, between the period soon after infarction and two months after transplantation, interesting results were observed. The mature skeletal muscle cell group presented with a significant increase of this parameter when compared with the control group. The mature cardiac cell group presented with a smaller decrease compared with the control group. There was a variation between the mature skeletal muscle cell group and the mature cardiac cell group although it was not statistically significant.

As the animals were functionally homogenous soon after myocardial infarction, it is difficult to understand how a group which presented with a stabilization of the left ventricle ejection fraction, that is the mature cardiac cell group, after transplantation did not have a significant difference compared with a group, the mature skeletal muscle group, which presented with a significant increase of this parameter. This situation can be explained statistically as the number of animals in both groups was small.

The exact mechanism involved in the improvement of the left ventricle function after cell transplantation, whether fetal cardiomyocytes, mature cardiac cells, smooth or skeletal muscle cells, is not yet well understood. However, there is a direct association between the presence of the contractile cells and the improvement in the cardiac function. (14)

In relation to the difficulties of cellular expansion of the adult cardiomyocytes, we believe that they reflect the basic characteristics of the cellular difference of the kariomyocyte, as has been explained previously, the adult cardiomyocytes are totally differentiated cells and they do not undergo any multiplication process. We observed that a great number of the adult cardiac cells transplanted were actually fibroblasts, as these were identified in the cell culture soon after transplantation in a greater percentage. The anatomicopathological study did not identify the cardiac muscle cells transplanted, not by means of anti-desmin nor by anti-connexin-43, proving the absence of viable muscle cells in the infarcted region.

In the group that received adult cardiac cells (GII), the slides marked with hematein-eosin identified an important cellularity in the infarcted region when compared with the control group, suggesting the presence of transplanted cells and the efficiency of this procedure. Starting from the fact that transplanted muscle cells were not identified in the infarcted region, we suggest that a transplant of fibroblasts caused the increase in the cellularity identified by the hematein-eosin. Both adult cardiomyocytes and fibroblasts present with a single central nucleus, and they differ in relation to specific cellular marking (desamin and connexin-43 for the cardiomyocyte and fibronectin for fibroblasts) and also distinct physical and mechanical characteristics, as fibroblasts present with a greater diameter, are more elongated and do not contract.

An immunoflorescence with fibronectin marker, the protein found in fibroblasts, was not performed because it would be impossible to distinguish the transplanted fibroblasts from the fibroblasts of the myocardial fibrosis itself.

In truth, the cells we thought were cardiomyocytes at the start of the project, are really fibroblasts. In this way, the stabilization and not the improvement in the left ventricle ejection fraction of these animals is coherent. Fibroblasts are cells that are found in the connective tissue, giving sustenance to it and do not present a contractile structure. The fact that the cardiac cellular group presented with a stabilization of the ejection fraction and a limitation of the left ventricle dilation, two months after transplantation when compared to the control group, is explained by this characteristic of the fibroblasts.

In an attempt to find new types of cells to be transplanted, studies were directed to analysis of skeletal muscle cells which, although they are different to the striated cardiac cells in several properties including morphology, mechanical characteristics, stimulation-contraction mechanism, embryological origin and response to injury, present characteristics of regeneration by means of myoblasts.

Skeletal cell transplantation received considerable attention mainly due to the fact it is an autologous procedure. The first study with myoblasts was performed by CHIU et al. (15) who reported that the myoblast can be transplanted in myocardial infarction with good results, but the number of isolated satellite cells in this work was not satisfactory, prejudicing the results. Marking with beta-galactosidase identified that the transplanted cells presented with a single central nucleus, similar to the native cardiac cell, suggesting a phenotypic alteration of the transplanted myoblasts.

TAYLOR et al., (16) DORFMAN et al., (17) and ROBINSON et al. (18) also suggested a phenotypic alteration of the myoblasts after transplantation. In this study, the skeletal muscle fibers identified by pathologic anatomy presented cells with several peripheral nuclei, a typical characteristic of skeletal muscle fibers.

In the skeletal muscle group (GIII), the skeletal muscle cells in the infarcted myocardium were identified by specific proteins, embryonary and rapid isoform of myosin. Also marking with desmin was performed, which although it is not specific for skeletal muscle fiber, it marked all the muscle fibers in the infarcted region and the transition zone, indicating the presence of viable muscle fiber, proving the efficiency of cell transplantation in this group.

There are indications that after myoblast transplantation and the development of skeletal muscle fibers in the myocardium, these fibers continue to present with satellite cells in their structure. This supposition is based on the physiopathology of skeletal cell regeneration. We can suggest that, in the case that the region where the myoblast was transplanted suffers another ischemic process and consequently cellular aggression, it may be benefited by muscle regeneration. According to REINLIB & FIELD, (19) the myoblasts can, besides differentiate themselves in muscle fiber, perform neo-angiogenisis at the site of the injection of cells.

We believe that, if we had transplanted autologous cells, we would have achieved a greater number of available myoblasts in the myocardium and, as a consequence, a greater number of muscle fibers, as the

results of the functional analysis would have been better, which was suggested by POUZET et al., (20) where these authors reported that the greater the number of myoblast cells transplanted, the better was the recovery of the left ventricle function. This affirmation is fundamental in the increased death rate of cells after transplant, and if there are more cells available after the death of the majority, there will be a greater chance of colonization of the fibrous region. In our work, the number of myoblasts transplanted separately varied from 8.3×10^5 to 1.88×10^6 with a mean of 1.39×10^6, where in two animals 8.3×10^5 were transplanted. We observed that one of them presented with a stabilization of the left ventricle ejection fraction in the period between the cell transplantation and the sacrifice of the animal (35.81% to 34.73% respectively). As this animal was the only one in the group to present with similar values, we suggest that it was caused by a smaller number of myoblasts injected separately.

In relation to the analysis of the contractility of the skeletal fibers in the myocardium, it was not possible to identify connexin-43, and consequently the GAP junctions, between the transplanted skeletal muscle cells and the mature cardiomyocytes. Thus, we can not affirm that there is really a contraction of the transplanted fibers, as differently to fetal cardiomyocytes, which contract spontaneously, skeletal muscle fibers depend on a stimulus to contract. However, in an in-vitro myographic analysis performed by MURRY et al., (6) it was proved that the transplanted muscle fibers can contract after an electrical stimulation and develop a typical physiologic reaction of skeletal muscles. This can be structurally proved by the fact that myoblasts present a specific isoform of myosin in the skeletal muscle. A conversion of rapid to slow isoform was observed two 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.

Interference

There are some observations which should be made to analyze the results of cell transplantation in experimental animals better and to establish some basic points: 1) define the type of cardiac cellular lesion, whether by ligature of the coronary artery, or by administration of drugs or by cellular cold-injury; 2) identify the percentage of viable myoblasts at the time of transplantation; 3) analyze the quantity of live cells after transplantation; 4) identify the electro-mechanical stimulation between the transplanted cells and the native cells, defining what type of connection exists between them; 5) if the transplant affected the left ventricle function; 6) identify in which cardiac diseases cell transplantation is best adapted: ischemic or dilated cardiomyopathies; 7) determine the advantages and disadvantages between the different types of cells to be transplanted, fetal cardiomyocytes, myoblasts, smooth muscle cells, bone marrow cells or even non-contractile structures such as fibroblasts, analyzing the evolution of the infarcted region and the left ventricle remodeling; 8) analyze the development of the cells, their viability and their capacity for angiogenesis and 9) in which evolutional phase of heart failure, the transplantation is most effective.

CONCLUSIONS

Based on completed studies we can conclude that two months after transplantation, the mature skeletal muscle group presented with a statistically significant improvement of the left ventricle function when compared to the control group and the pre-transplantation period.

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