

Development of bioartificial myocardium by electrostimulation of 3D collagen scaffolds seeded with stem cells

Kanwal Haneef,¹ Nermine Lila,¹ Samira Benadda,² Fabien Legrand,² Alain Carpentier,¹ Juan C. Chachques¹

¹University Paris Descartes, Pitié-Salpêtrière Hospital, Laboratory of Biosurgical Research (LRB), Paris; ²Institute Claude Bernard, ICB/IFR2 Inserm, Platform of Cellular Imagery, Bichat Hospital, Paris, France

Abstract

Electrostimulation (ES) can be defined as a safe physical method to induce stem cell differentiation. The aim of this study is to evaluate the effectiveness of ES on bone marrow mesenchymal stem cells (BMSCs) seeded in collagen scaffolds in terms of proliferation and differentiation into cardiomyocytes. BMSCs were isolated from Wistar rats and seeded into 3D collagen type 1 templates measuring 25 x 25 x 6 mm. Bipolar *in vitro* ES was performed during 21 days. Electrical impedance and cell

proliferation were measured. Expression of cardiac markers was assessed by immunocytochemistry. Viscoelasticity of collagen matrix was evaluated. Electrical impedance assessments showed a low resistance of 234±41 Ohms which indicates good electrical conductivity of collagen matrix. Cell proliferation at 570 nm as significantly increased in ES groups after seven day (ES 0.129±0.03 *vs* non-stimulated control matrix 0.06±0.01, P=0.002) and after 21 days, (ES 0.22±0.04 *vs* control 0.13±0.01, P=0.01). Immunocytochemistry of BMSCs after 21 days ES showed positive staining of cardiac markers, troponin I, connexin 43, sarcomeric alpha-actinin, slow myosin, fast myosin and desmin. Staining for BMSCs marker CD29 after 21 days was negative. Electrostimulation of cell-seeded collagen matrix changed stem cell morphology and biochemical characteristics, increasing the expression of cardiac markers. Thus, MSC-derived differentiated cells by electrostimulation grafted in biological scaffolds might result in a convenient tissue engineering source for myocardial diseases.

Stem cell based therapies give new hope in the field of regenerative medicine, as stem cells have the ability to differentiate into both same as well as different tissue types, and to regenerate themselves without losing their differentiation potential. This property of differentiation has been explored for the regeneration of several damaged tissues.^{4,5}

Experimental studies of an MI model demonstrated that ischemic cardiac tissue responds to the various populations of stem cell therapy and in particular to mesenchymal stem cells.⁶⁻⁹ The exact beneficial mechanism of cell therapy in ischemic heart diseases is not yet fully understood but possible mechanisms involve paracrine secretion of growth factors and cytokines, improving neovascularization and angiogenesis.^{5,8,10,11} The differentiation potential of cardiomyocytes can be induced by electrostimulation which generates phenotypical and biochemical changes in stem cells and shifts them toward a cardiomyocyte-like phenotype.¹²⁻¹⁶

The poor survival of grafted cells in ischemic heart disease has been a subject of great concern for researchers as transplanted cells cannot survive for a long period of time due to pathologically modified extra cellular matrix (ECM), proapoptotic factors, and inflammatory response. It seems appropriate to provide a safe environment for cell proliferation and differentiation. The use of antioxidant and anti-inflammatory molecules, and overexpression of heat shock proteins and antiapoptotic proteins, may result in better survival of transplanted cells.^{17,18} Ascorbic acid is a power antioxidant stimuli and recent studies suggested that ascorbic acid efficiently reduces cell death in transplanted bioartificial graft by stimulating the production of collagen type IV by endothelial cells and subsequently increase the angiogenesis.¹⁹⁻²²

ECM is mainly composed of collagen that gives structural strength to the left ventricle. Three dimensional collagen matrix scaffolds act as a natural ECM and enhance survival of transplanted cells both *in vitro* as well as *in*

Correspondence: Juan Carlos Chachques, Georges Pompidou Hospital, Laboratory of Biosurgical Research, 56 rue Leblanc, 75015 Paris, France.
Tel: +33.613144398, Fax: +33.156095903
E-mail: j.chachques@egp.aphp.fr

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Introduction

Myocardial ischemia (MI) is a leading cause of heart failure all over the world. Following myocardial infarction, the irreparable loss or dysfunction of cardiomyocytes occurs due to sudden deprivation of oxygen to the heart. The myocardium has very limited regeneration capacity as most of the myocytes seems to be terminally differentiated and only a small fraction of myocytes retain the capacity to replicate.^{1,2} Until now, pharmacological therapy, surgical procedures (*e.g.* revascularization, ventricular remodeling and restoration, dynamic cardiomyoplasty), organ transplantation and mechanical circulatory assistance devices have been used to treat hearts that are irreparably damaged. Therefore, there is a need to develop more effective, less invasive, therapeutic strategies for heart failure.³

vivo.^{10,23,24} The aim of this study is to explore the role of *in vitro* electrostimulation of mesenchymal stem cells seeded in collagen scaffolds. Survival, and the organization and differentiation of stem cells implanted in the templates were all evaluated.

Materials and Methods

Isolation and culture of bone marrow mesenchymal stem cells

Bone marrow mesenchymal stem cells (BMSCs) were isolated from femur and tibia of 2-month old Wistar rats. BMSCs were extracted by inserting a 21-gauge needle into the bone diaphysis and flushing the medullar canal with 1 mL of culture medium (DMEM) (Gibco, Boston, USA) combined with 10% fetal bovine serum (FBS) (Biowest, Nuaille, France) and the addition of 100 U/mL penicillin/streptomycin (Sigma). Marrow plug suspension was plated in a T-75 culture flask and incubated at 37°C, in a 5% CO₂ humidified atmosphere. The following day, cultured cells were washed twice with PBS in order to remove non-adherent cells, then 10% FBS DMEM was added. The mesenchymal population was isolated on the basis of its ability to adhere to the culture plate. Culture medium was changed every 3-4 days. At 90% confluence, the cells were trypsinized and first-passage BMSCs were used in all experiments.

Culturing mesenchymal stem cells on collagen scaffolds

Scaffolds were composed of lyophilized, non-denatured, native type I collagen (bovine origin) obtained from a commercially available collagen kit (Pangen 2, Urgo Laboratory, Chenove, France). Cells at the first passage were trypsinized at 90% of confluence, resuspended in 1 mL DMEM medium and counted manually in duplicate using a hemocytometer chamber. Templates of 25×25×6 mm of collagen scaffold were placed in 100 mm Petri dishes. The suspension containing BMCs (1 million cell per cm²) were later seeded on the upper collagen surface by gently dropping. The scaffolds were left at 37°C in a 5% CO₂ humidified atmosphere for 1 h to allow the cells to attach. Afterwards, 10 mL medium supplemented with 50 mol/L of L-ascorbic acid (Sigma, USA) was added and plates were incubated at 37°C in a 5% CO₂.

Optimized cell count for proliferation within scaffold

Before starting experiments, we optimized seeding efficacy on collagen scaffold by selecting 1×10⁶ (optimized number of cells) to be

seeded on 25×25×6 mm patches of porous collagen type I.

Electrostimulation of cell-seeded collagen scaffolds

Two electrodes with curved needles for easy insertion were fixed into opposite borders of the collagen scaffold (Figure 1). Both electrodes were connected to bipolar pacemakers (Transform model, Medtronic Inc., MN, USA). Cells implanted into collagen matrix were stimulated using single pulses at a frequency of 120 × min (rate 2 Hz), pulse amplitude of 7 Volts and pulse width of 5 ms. Cell-seeded matrix were analyzed after one and three weeks of electrostimulation.

Control group

Equal numbers of cells were seeded in separate plates, not treated with electrostimulation, recovered at the same time points, and processed for evaluation of cell viability and phenotypic characterization using appropriate techniques. Cell culture media was changed twice a week. At least five scaffolds were analyzed for each experimental condition and at each time-point (at one and three weeks).

Mesenchymal stem cell characterization

To determine the cells and matrix interaction, collagen scaffold were embedded in OCT and stored at -80°C. Histological studies were performed on a 5 micron section and stained with hematoxylin and eosin. For immunostaining, BMSCs were seeded onto glass coverslips and fixed in 4% paraformaldehyde at room temperature for 15 min and then washed twice with Ca²⁺ and Mg²⁺ free PBS. Non-specific antibody-binding sites were blocked with 2% BSA/2% goat serum/0.1% Tween20/PBS (block-

ing buffer) during 1 h at room temperature. Later, the primary antibodies anti CD29 (R&D) 1:50 in blocking buffer, and CD44 (Invitrogen, USA) 1:50 in blocking buffer were added and incubated overnight at 4°C. Cells were washed for 5 min three times in PBS and incubated for 1 h in Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Invitrogen, USA) at room temperature. Cells were washed for 5 min three times in PBS and cell nuclei labeled with 0.5 µg/mL DAPI for 15 min at room temperature. After staining, cells were mounted on standard glass slides. Images were taken by fluorescence microscope.

Confocal microscopy

For immunohistochemical analysis, collagen scaffolds were fixed in 10% formaldehyde for 24 h, washed three times with PBS and then with 0.1% triton for 10 min. Scaffolds were blocked with 1% bovine serum albumin for 2 h at 4°C. Immunohistochemical staining was performed using the following primary antibodies (Sigma): anti-CD44 (1:100), anti-CD29 (1:100), anti-sarcomeric actin (1:100), anti-connexin 43 (1:100), anti-desmin (1:50), anti-slow myosin (1:50), anti-fast myosin (1:50) and anti-cardiac troponin I (1:50). After washing, Alexa 488 conjugated goat secondary antibodies (Invitrogen) were used for nucleus staining (at a dilution of 1:500) and counterstained with 1 µg/mL propidium iodide. Samples were examined by confocal microscopy (Zeiss LSMS10-Axiocvert 200M).

Mesenchymal stem cell proliferation into collagen scaffold

BMSC proliferation was evaluated using an MTT-based cell growth determination kit (Sigma, USA) at Days 7 and 21. After removing the supernatant, the scaffold was washed two times with PBS and MTT solution was added in

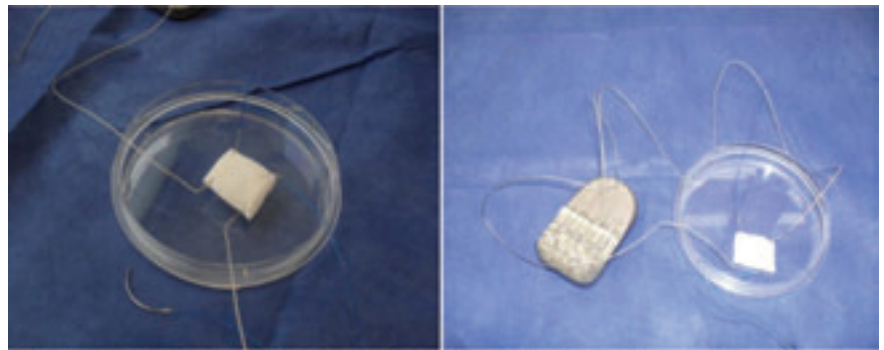


Figure 1. Implantation of electrodes into the 3D collagen matrix and connection with the pacemaker for bipolar *in vitro* electrostimulation.

an amount equal to 10% of the culture volume. Cultures were incubated for 3 h at 37°C in a 5% CO₂ humidified atmosphere. Insoluble MTT formazan crystals are formed by mitochondrial dehydrogenases of viable cells. Each scaffold was then transferred into an Eppendorf tube containing 2 mL of solubilization solution 0.1 N HCl in anhydrous isopropanol and vortexed for 5 min. This solubilization solution dissolves formazan crystals and provoked the release of purple solution from the scaffold. Each sample was centrifuged at 15,000 g for 5 min and the supernatant was read at 570 nm with a spectrophotometer.

Electrical impedance

Electrical impedance of collagen matrix was measured before and after 21 days of electrostimulation. Impedance measurements were assessed through the same electrodes used for matrix electrostimulation, fixed into opposite borders of the collagen matrix. The electrodes were connected to a pacing system analyzer Model 5311 (Medtronic Inc., Minneapolis, USA). Bipolar charge balanced pulses were delivered only for measurements using the following parameters: pulse amplitude 1 Volt, pulse width 0.5 ms, frequency of electrostimulation 70 pulses per minute. Later, electrical impedance within the scaffold was assessed.

Viscoelasticity

A constant stress was imposed to assess viscoelasticity of the collagen matrices. Their stress-strain responses and their temporal dependencies mimic the behavior of the classical Kelvin Standard Linear Solid Model which combines a Voigt system (Hookean spring E₂ in parallel with a viscous dashpot η_2), and a hookean spring (E₁) in series of the Voigt system. Thus, under a constant stress, the material instantaneously deforms according to the strain (the elastic portion of the strain) and then continues to deform until it asymptotically approaches a steady-state strain. This last portion is the viscous part of the strain. In this way, pieces of porous type 1 collagen (n=5) were evaluated; these were cut into pieces of 12 mm x 1 mm, 1 mm thick. The elastic modulus E₁ and E₂ (in mN/mm) and the viscosity coefficient (η_2 in mN/mm.s) were calculated using load clamp force technique before and following collagen hydration.

Statistical analysis

Data are presented as means \pm standard deviation. Differences between groups were tested for significance using Student's t-test. P<0.05 was considered significant.

Results

Characterization and differentiation of mesenchymal stem cells

MSC presented a fibroblast-like shape in culture conditions (Figure 2A). Figure 2B showed MSCs on collagen scaffold stained with propidium iodide at phase and at confocal microscopy. These results indicated the good even distribution of MSCs on collagen scaffold. At passage 1 and before seeding on the matrix, cells were characterized by mesenchymal stem cell antibodies. Our studies showed that this population had a positive staining for CD44 and CD29 cells (Figure 3). Images from confocal microscopy after immunocytochemical staining of MSC marker and different cardiomyocyte markers in the control group, and after seven and 21 days of electrostimulation are shown in Figure 4. We found that in the electrostimulation groups there is a progressive decrease in the expression of CD29 MSCs marker from seven days to 21 days. All cells were negatively stained after 21 days of ES. On the other hand, cardiomyogenic marker expressions, troponin I, connexin 43, sarcomeric actin, desmin, fast myosin and slow myosin, progressively increased from seven to 21 days of ES.

Cell proliferation significantly increased in ST groups after seven days (ST 0.129 ± 0.03 vs control 0.06 ± 0.01 , P=0.002) and after 21 days, (ST 0.22 ± 0.04 vs control 0.13 ± 0.01 , P=0.01) (Figure 5). These results showed that ES treatment gradually increases the proliferation of cells in the ES group as compared to the control group at both seven and 21 days. The increase in the number of cells was directly correlated with the duration of the stimulation.

Electrical impedance

Electrical measurements were taken in cell medium alone and in the collagen matrix. Cell culture medium showed impedance of 292 ± 25 Ohms and for collagen matrix this was 234 ± 41 Ohms at Day 0 (day of cell implantation), and 251 ± 36 Ohms after 21 days of cell electrostimulation (P=NS). These results show that the material evaluated presents electrical conduction properties, *i.e.* resistance, similar to those found in cardiac tissues. Therefore, cell-seeded collagen matrices could potentially be used for myocardial substitution.

Viscoelasticity

All collagen matrices showed the same viscoelastic properties similar to those of the Kelvin Standard Linear Solid Model. Samples showed linear stress-strain relationship, sim-

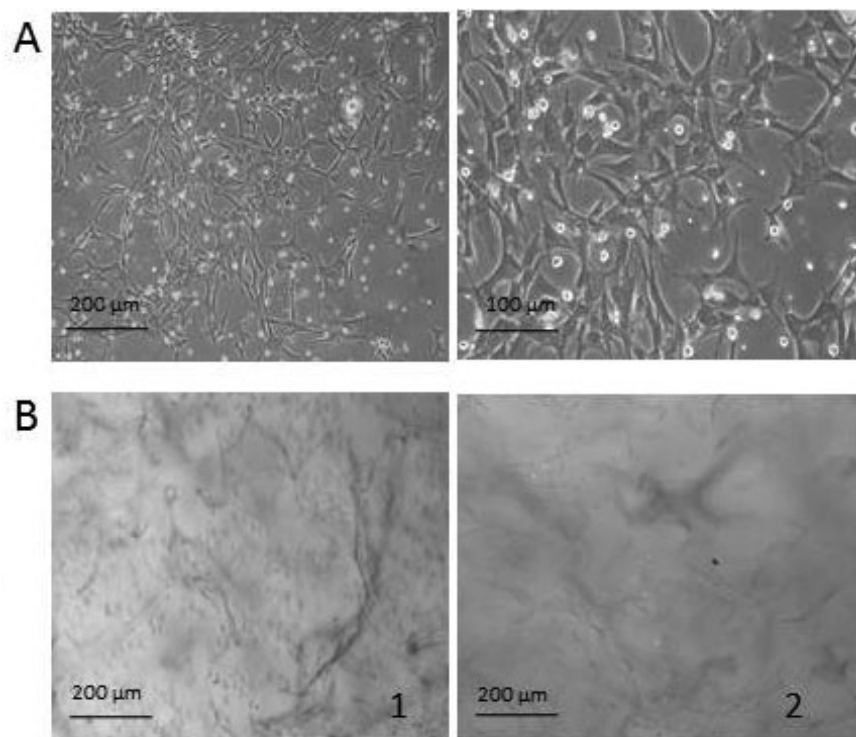


Figure 2. Mesenchymal stem cell culture. (A) Passage 1 mesenchymal stem cells in culture by phase microscopy. (B) passage 1 mesenchymal stem cells into collagen scaffold after 1 day; B1 at phase microscopy, B2 at confocal microscopy stained with propidium iodide.

plifying the evaluation of viscoelasticity. In all hydrated and non-hydrated specimens, E1 ranged from 20 to 40 in mN/mm, E2 ranged from 10 to 100 in mN/mm, and n_2 ranged from 1 to 5 mN/mms. These results show that the viscous and elastic properties of collagen matrix can be compatible with both myocardium contraction and relaxation.

compressive strain,²⁷ as well as by chemical (cytokines) and biological/genetic stimuli (cell co-cultures, genetic manipulations).²⁸ Electrostimulation is a safe method to induce physical and biochemical changes in stem cells moving toward cardiac cells¹²⁻¹⁴ without using any demethylating agents or viral vector, representing a safe alternative to drugs like 5 azacytidine and chemical cytokines.²⁹

Results of clinical trials using stem cell transplantation for myocardial regeneration are showing poor survival of implanted cells without significant improvement of ventricular function^{2,6,30} due to both local ischemia and pathologically modified extracellular matrix (ECM). In addition, the lack of gap junctions due to the absence of electrophysiological connection between donor and host cells, leads to

Discussion

MSC isolation by differential plating is a consolidated technique, as described by different research groups.^{2,4} In our experiments, cells at P0 showed a heterogeneous population, *i.e.* some cells are oval and some are elongated. However, at P1 these cells showed a homogeneous population with fibroblast-like morphology. We used P1 cells for all experiments. Furthermore, microscopic examination of our cells also confirmed the purity of MSCs, verified by differentiation of MSCs into osteogenic and adipogenic lineages (Figure 6). On the basis of these results we confirmed that the cells used in our study made up a pure and homogeneous population of MSCs.

Left ventricular remodeling is a serious consequence of MI which leads to heart failure. Stem cells have the ability to differentiate into various types of lineages which makes them attractive for the regeneration of myocardial tissue. Stem cell differentiation can be achieved by extrinsic physical stimuli (electrostimulation, magnetic fields),^{18,25,26} cyclic

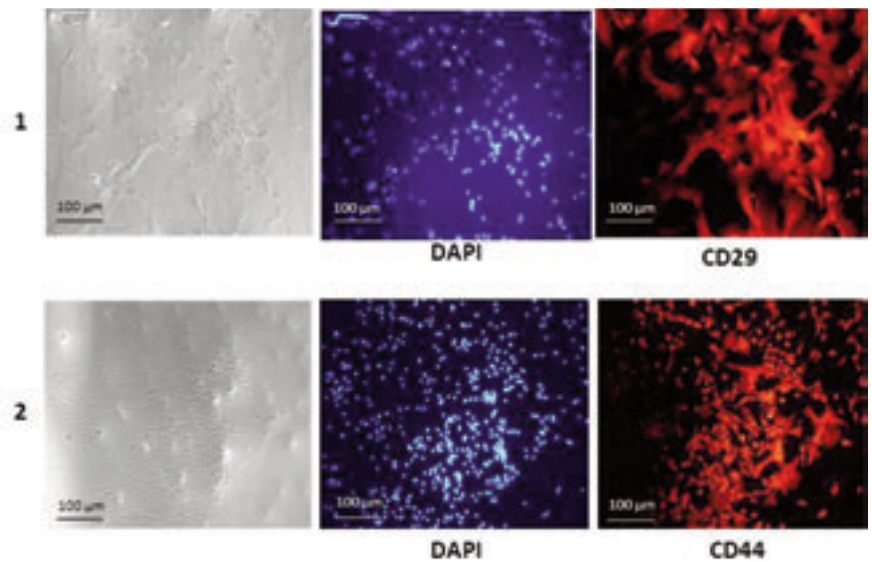


Figure 3. Immunofluorescence staining of mesenchymal stem cells before electrostimulation. Passage 1 mesenchymal stem cells are positively stained with mesenchymal stem cells markers: 1) CD29, 2) CD44. Nucleus were stained with DAPI.

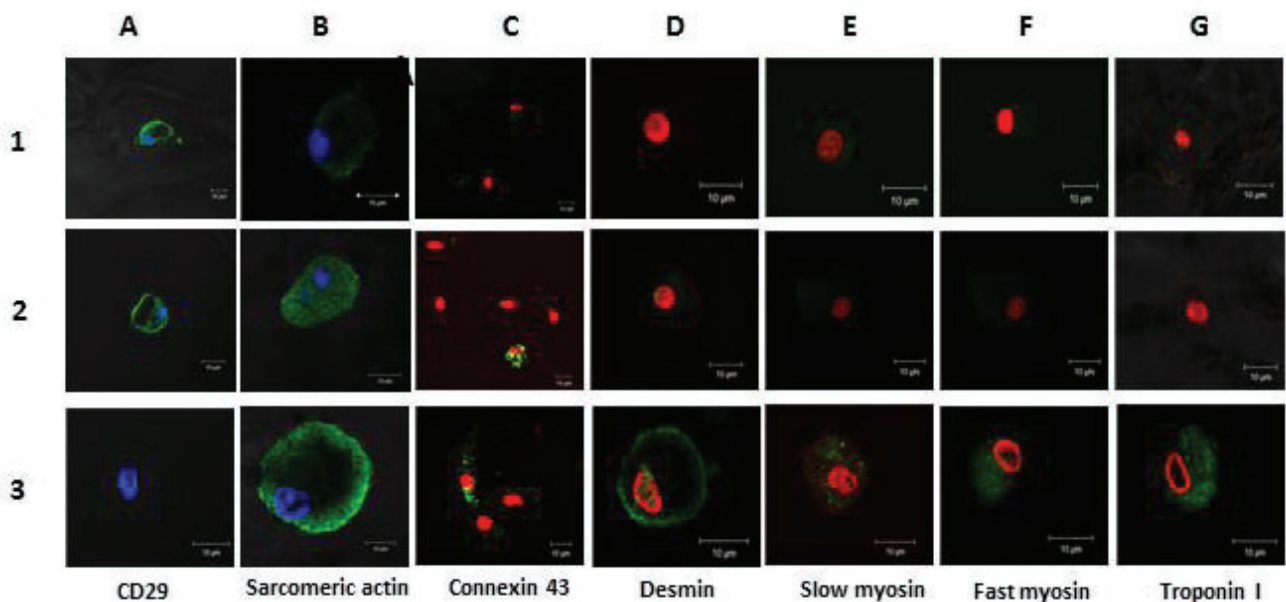


Figure 4. Characterization of mesenchymal stem cells into collagen scaffold by confocal microscopy after electrostimulation: 1) control without electrostimulation; 2) seven days of electrostimulation at 7 volts; 3) 21 days of electrostimulation at 7 volts. Cells were stained with (A) CD29, (B) sarcomeric actin, (C) connexin 43, (D) desmin, (E) slow Myosin, (F) fast myosin and (G) troponin I antibodies.

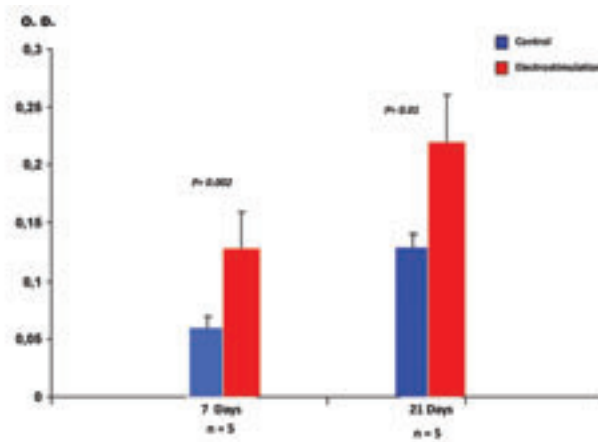


Figure 5. Mesenchymal stem cell proliferation into collagen scaffold. Electrostimulation at 7 volts increased cell proliferation at seven and 21 days compared with control non-stimulated cells. OD, optical density.

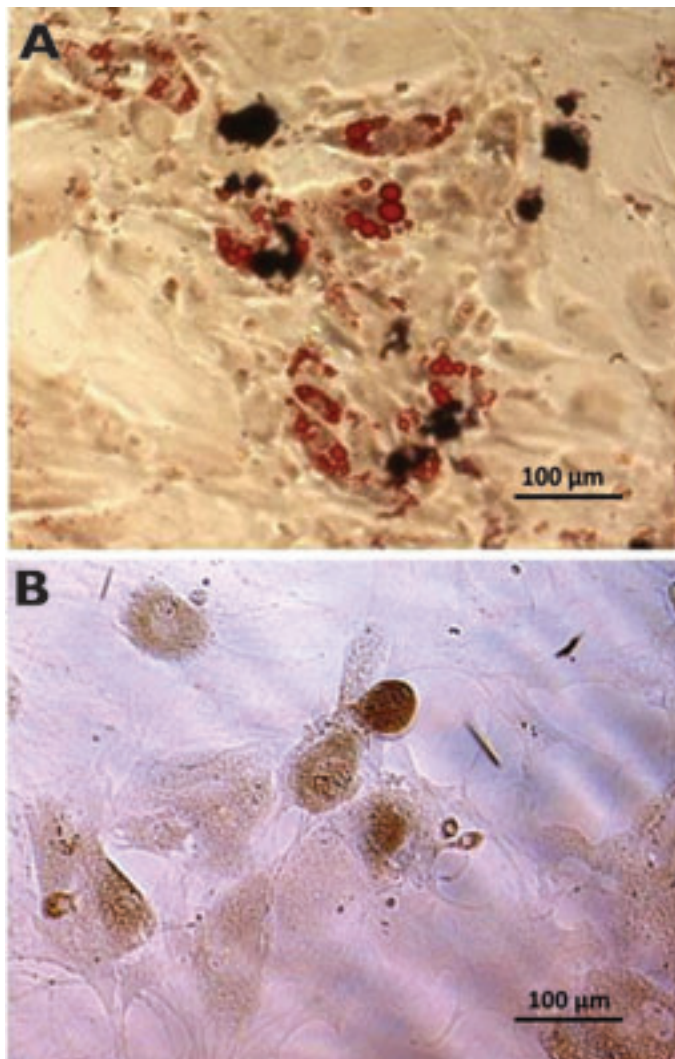


Figure 6. Purity of our stem cells was verified by mesenchymal differentiation into osteogenic and adipogenic cells. (A) Mesenchymal stem cells adipogenic differentiation. Cells were stained with Oil Red O solution (nuclei were stained with hematoxylin) and observed at 10X magnification under phase contrast. (B) Osteogenic differentiation. Cells were stained with alizarin red solution and observed at 10X magnification under phase contrast.

failed cell integration into the myocardium. Improvement of cell survival could be achieved by using 3D collagen matrix as they have the ability to act as a natural ECM, providing structural strength and resistance to deformation. Furthermore, the native ECM and myocardial therapeutic scaffolds are exposed to different inflammatory responses as well as pro-apoptotic stimuli from surrounding ischemic tissues.

In our study, the basic approach was to evaluate the effectiveness of using electrostimulation for conditioning MSCs-seeded in collagen scaffold, and check their proliferation and differentiation into cardiomyocytes. Our selection of an electrostimulation period of up to three weeks was based on data previously reported that MSCs take at least 1-4 weeks to complete their differentiation into cardiomyocytes within heart.³¹ Firstly, we studied the effect of seven and 21 days of electrostimulation on MSC proliferation. We found that cell proliferation after seven days of electrostimulation was 53% higher than control non-stimulated cells ($P=0.002$). Using 21 days of electrostimulation, cell proliferation was 40% higher than control non-stimulated cells ($P=0.01$). Previous studies showed a high incidence of cell death after electrostimulation.¹²⁻¹⁴ This is the first time that higher cell proliferation after three weeks of electrostimulation than control group has been demonstrated. We suggest that this improvement in cell proliferation is related to: i) our electrostimulation programming (7 Volts, 5 ms pulses at a frequency of $120 \times \text{min}$) which was lower than the programming used previously. These values are quite similar to those used in clinical cardiac pacing; higher voltage may cause cell death; ii) the combinations of collagen matrix with electrostimulation which give not only structural strength to the cells but also a 3D microenvironment *niche*, resulting in better proliferation and survival of grafted cells; iii) the use of ascorbic acid. This is more than an antioxidant as it has a dramatically powerful antiapoptotic property; thus reducing cell death and significantly enhancing neovascularization of grafted cells.¹⁹⁻²²

A 3D environment is completely different from the 2D culturing settings and this is the reason for the different cellular morphology in 2D and in 3D environments.^{2,7,15} Data suggest that cells showed a flattened morphology in a 2D setting while round, oval or ellipsoidal shapes are to be observed in a 3D setting. This seems to be the reason for the cells with variable shapes we found in collagen scaffold (Figure 4). MSCs immunocytochemistry results indicated that the stem cell specific CD29 expression was reduced in relation to increased electrostimulation time. After three weeks of electrostimulation, most of the MSCs were not showing CD29 expression but actins were more highly expressed. Immunostaining

results of gap junction protein (connexin 43) indicated that there are more gap junctions and connections between cells after three weeks' electrostimulation as compared to control group. These results demonstrated that electrostimulation induces biochemical and physical changes in MSCs grafted in 3D collagen matrix which shifted them toward a cardiac-like cell phenotype. Furthermore, MSCs stimulated during three weeks become negative for MSC markers. These partially differentiated cells may complete further ongoing differentiation processes within the cardiac microenvironment after transplantation into the myocardium.

The goal of myocardial tissue engineering is to develop scaffolds for ventricular support and myocardial regeneration. Electrophysiological properties of these materials are of paramount importance in therapeutic cardiology.³² Measurement of electrical impedance in collagen matrices showed good electric conductivity representing an ideal model for our study. In addition, biomaterials for cardiac tissue engineering need to feature biocompatibility and mechanical properties in order to be sufficiently elastic to match the myocardium contraction-distractive activity and allow structural and functional biointegration. The viscoelastic behavior of myocardial tissue is a measure that has recently been found to be a determining factor in quality of contraction and relaxation.³³ Stiffening of the left ventricle can compromise the ability of the heart to pump sufficient amounts of blood into the systemic circulation, leading to heart failure. Parameters imposing the viscoelastic behavior of the heart are influenced in part by sarcomere function and myocardial composition. A negative correlation between viscoelastic parameters and ventricular function parameters has been demonstrated. In the present study, the evaluation of viscoelastic properties of the 3D collagen matrix showed mechanical properties to be sufficiently elastic to match the myocardium contraction-distractive activity.^{34,35}

Future directions

The optimal cell-matrix combination for myocardial tissue engineering remains a major medical challenge. Current approaches such as patches of collagen are compromised by the complete biodegradation of the grafted material. Nanomaterials are emerging as the main candidates to ensure the achievement of a proper instructive cellular niche. Good progress in the design and fabrication of synthetic biomaterials with biomimetic characteristics has been accomplished in the field of nanotechnology for direct applications in biomedicine. The main purpose of these materials is to display structural and functional properties similar to extracellular matrices, containing truly 3-dimensional nanonetworks.

Synthetic nanomaterials are attractive platforms because of their pure composition, predictive toxicology, target specificity, low manufacturing costs, and control in degradability. Proteins, peptides, and polysaccharides are at the top of the list for their versatility in nanomaterial design and fabrication.^{2,8}

Our group has been involved in the development of *bioactive implants* for myocardial regeneration and ventricular support (RECATABI European Project <http://www.recatabi.com/>). This approach includes an elastomeric microporous membrane (patch) having one synthetic non-degradable polymer and one partially degradable polymer (biological or synthetic), all associated with a peptide nanofiber hydrogel and stem cells. This *bioactive implant* should provide a suitable environment for cell homing, growth and differentiation (myocardial repair), as well as mechanical support to the heart. The combination of degradable and non-degradable polymers should be advantageous because cells implanted in niches will organize, connect and contract more easily if they are surrounded by material that partially degrades with time. The association of a multielectrode network for local pacing should improve the coupling of stem cells and scaffolds with host cardiomyocytes, becoming a dynamic tissue engineered support.^{18,36}

Conclusions

We have shown that electrostimulation of stem cells seeded in collagen matrix is a safe and effective approach for cell survival and differentiation into cardiac cells. We propose low voltage electrostimulation for longer periods of time to obtain a stable and differentiated phenotype. Associating cardiac electrophysiology seems to be of paramount importance for the development of therapeutic tools for heart failure. Pre-conditioned biological matrix scaffolds could be useful for myocardial support and regeneration in patients suffering from ischemic heart disease. Supported by relevant scientific background, the development of myocardial tissue engineering and nanobiotechnologies may constitute a new road and fresh hope for the treatment of myocardial diseases.³⁷⁻⁴⁰

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