

Beneficial Roles of Cellulose Patch-Mediated Cell Therapy In Myocardial Infarction: A Preclinical Study

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ABSTRACT

Biologic scaffolds have become an attractive approach for repairing the infarcted myocardium and have been shown to facilitate constructive remodeling in injured tissues. This study aimed to investigate the possible utilization of bacterial cellulose membrane patch containing cocultured cells to limit the myocardium's post-infarction pathology. Myocardial infarction was induced by ligating the left anterior descending coronary artery in 45 Wistar rats, and patches with or without cells were attached to the hearts. After one week, the animals underwent echocardiography for assessing ejection fraction and left ventricular end-diastolic and end-systolic volumes. Following the patch formation, cocultured cells retained viability of >90% over 14 days in culture. The patch was applied to the myocardial surface of the infarcted area after staying 14 days in culture. Interestingly, the bacterial cellulose membrane without cellular treatment showed higher preservation of cardiac dimensions; however, we did not observe improvement in the left ventricular ejection fraction of this group compared to coculture treated membranes. Our results demonstrated an important role for bacterial cellulose in supporting cells known to produce cardioprotective soluble factors and may thus provide effective future therapeutic outcomes for patients suffering from ischemic heart disease.

Keywords: implant, bacterial cellulose patch, myocardial infarction, cell, delivery

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INTRODUCTION

Myocardial infarction (MI) and heart failure are major causes of death in developed countries [1].

Several deleterious effects are known to occur in both the infarcted and non-infarcted myocardial tissues, including cardiomyocyte loss, cardiomyocyte lengthening, left ventricular wall thinning, infarct expansion, cardiomyocyte hypertrophy, and collagen accumulation [2-4].

Molecular studies have revealed that regeneration of tissues in various systems can be augmented by growth factors such as platelet-derived growth factor (PDGF), bone morphogenetic proteins (BMPs), and transforming growth factor- β (TGF- β) [5]. Nevertheless, the expensive cost and rapid growth factor degradation remained the main obstacle. Many types of materials are now under extensive investigations to find promising scaffolds for tissue engineering. The ideal scaffold should meet specific criteria, being biocompatible, matching with surrounding tissues, providing chemical stability or degradability, affording mechanical strength, and allowing cell adhesion and proliferation. In natural tissues, the extracellular matrix composition, cell density, and physiological properties are often non-homogeneous. The great challenge of tissue engineering is the distribution of cells throughout tissue engineering scaffolds. These biomimetic materials should resemble the natural tissue and extracellular matrix (ECM) to provide the targeted area [6]. Mimicking the natural conditions in both the tissue and ECM requires proper adhesion and growth properties that maintain normal tissue structure [7]. Biopolymers involving cellulose were tried previously for developing scaffolds and revealed successful outcomes [8-12].

At the molecular level, bacterial cellulose-based aqueous gel-like biomaterial has been used to quantify transcriptomics and proteomics in cell culture. Interestingly, statistical analysis of 12,475 transcripts and 7831 proteins documented significant differences indicating its role in major gene-dependent functional responsiveness [13]. Cellulose is a linear homopolysaccharide which consists of glucose (d-glucopyranose) linked by glycosidic β (1-4) linkages. Its polymerization degree varies widely from 2,400 in some plants, like the horsetail (*Equisetum arvense*), to 15,300 in the untreated cotton (*Gossypium*) fiber [14, 15]. The hydroxyls linked to the hemiacetalic ring are arranged in an equatorial position that allows extremely stable conformation [16]. The substituents in adjacent rings are in a quasi-planar disposition that allows the formation of a linear chain on which adjacent chains

are aligned in a hydrogen-bond crystalline structure, with a hydrophilic character on the surface but hydrophobic in the interior, making cellulose insoluble in water [14, 17, 18].

Cellulose could be obtained from many sources, such as plants or microorganisms. Bacterial cellulose (BC) has attracted the attention of researchers in the last years. It consists of a gelatinous translucent pellicle grown on the surface of Acetobacter bacterial colonies. These microbial cellulose nanofibrils are about 280 nm in width and several micrometers in length [19]. The amorphous regions in this cellulose occupy almost 90% of its volume; however, as these regions contain up to 99% water, their contribution to final cellulose mass is minimal. BC's overall crystallinity is high and is estimated to be about 70% [18, 20]. BC's water retention capacity is much better than cellulose from other sources, above 1000% [21]. To allow the oxygenation of tissues and fluid draining, the use of controlled production of variable size pores is necessary [22]. The above-mentioned BC properties and their biocompatibility increased the interest in using it for therapeutic purposes.

New studies on cellulose nanomaterials for tissue engineering have employed bacterial cellulose because it is a material with unique properties compared to biomaterials commonly used in tissue engineering scaffolds [12, 21, 23]. For instance, BC has been used in scaffolds applied in microsurgeries to promote burned tissue regeneration. The advancement of micro- and nanotechnologies enable developing tissue scaffolds with a gradient in material composition and properties that enable spatially controlled differentiation of cells and subsequent tissue development [23].

Nanofiber fabrication systems have been developed to mimic such fibrous structures of *in vitro* cell culture for the generation of polymer or composite fibers from natural or synthetic materials. These nanofibers possess a large surface area, which allows cell attachment [24-27]. Nanofibers' physical and chemical properties can easily be tunable under appropriate conditions to facilitate cell growth and subsequent tissue development, thereby imparting gradient features into a nanofiber system, providing an exciting area of research [25, 28]. Micro and nanoscale techniques are versatile tools for developing such gradient biomaterials and could be utilized to design a new generation of engineered grafts for use in interface tissue engineering [25, 29]. The membrane of bacterial cellulose has recently been shown to promote adhesion, the proliferation of skeletal muscle and mesenchymal stem cells, and angiogenesis improvement during tissue regeneration [30-32]. These approaches

could allow the modification of targeted structures via pharmaceutical molecules. For instance, using BC and fluconazole in scaffolds has been used to promote stem cell growth during the regeneration of burned tissues [33].

Emerging cell therapeutic strategies are promising procedures for promoting myocardial regeneration and repair. Different isolated or combined stem cells have been studied both in preclinical models and in clinical trials, including skeletal muscle cells (SMC), mesenchymal stem cells from adipose tissue, hematopoietic cells from the bone marrow or umbilical cord blood, or Wharton Jelly [34]. Various routes have been used to administer cells such as catheterization, epicardial and intramyocardial injection. Cell therapy has been suggested to be greatly improved if accompanied by a 3D scaffold, matrix modifiers, and adhesion molecules [35].

Carvalho et al. [34] have reported beneficial effects of cocultured bone marrow and myoblasts in Chagas myocardial disease and myocardial infarction. These studies provided us with important information required for choosing and applying the cells used in this study. This study aimed to assess potential improvement of infarcted heart function upon using autologous cocultured skeletal muscle cells, and bone marrow mesenchymal stem cells (BMSC) seeded on bacterial cellulose patch. To our knowledge, this is the first study that uses bacterial cellulose membrane as a patch for cellular delivery in myocardial infarction model.

MATERIALS AND METHODS

Animals

An experimental animal study has approved at the Pontifical Catholic University of Paraná Animal Use Committee, numbered 555 (CEUA-PUCPR). The rats were housing under standard conditions with food and water ad libitum on a 12-h day/night cycle (light on at 7 am). All animal experiments were done in the Experimental Laboratory of Institute of Biological and Health Sciences of the Pontifical Catholic University of Paraná, which was structured for Animal Housing as well as with Animal Experimental Surgical Room for the proceedings. All experiments approved were performed following the ARRIVE Guidelines [36].

Experimental Design

All animals were from Pontifical Catholic University of Paraná. Forty-five rats, *Rattus norvegicus* – male Wistar (weight, 250–300 g) were submitted to Myocardial infarction (MI) surgery, as described earlier [34]. Seven days after, they were analyzed by echocardiography apparatus to assess baseline heart function. The animals which displayed $\leq 55\%$ LVEF have been randomized into three groups. Group I (n = 15): Myocardial infarction without treatment (control group); Group II (n = 15): Implantation of the bacterial cellulose matrix on the left ventricular surface and Group III (n = 15): Implantation of the bacterial cellulose matrix seeded with cocultured cells on the left ventricular surface. Fourteen days after surgery, the second surgery and the implantation of the patch were performed. One month after MI, the hearts were analyzed by echocardiography apparatus for a second time. The animals were then euthanized, and histopathological analysis was performed (Figure 1).

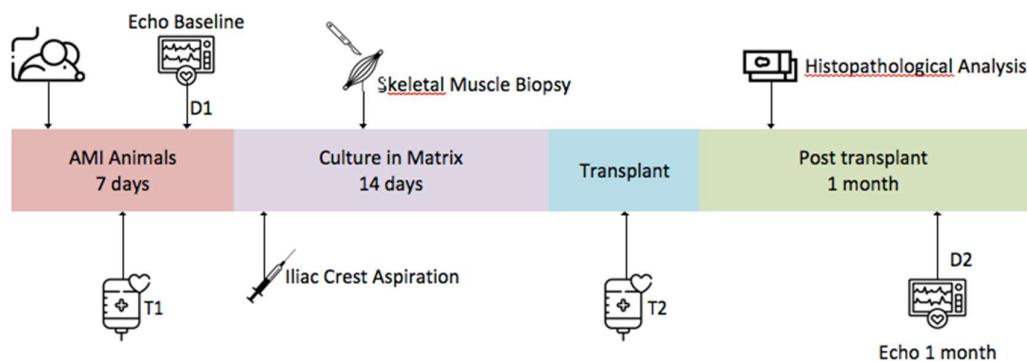


Figure 1. Experimental Design: Acute myocardial infarction (AMI), distinct time point (D), echocardiography (Echo) and thoracotomy (T).

Acute myocardial infarction model

The rats were anesthetized by intramuscular administration of ketamine (Ketalar®) (50 mg/kg) and xylazine (Xylazine®) (10 mg/kg) and were then subjected to mechanical ventilation. These procedures were followed by thoracotomy (T1) at the left 5th intercostal space. To induce myocardial infarction (MI), the left anterior descending coronary artery was directly ligated just beyond the first

diagonal branch. The rats were then maintained in cages and kept under controlled temperature and high oxygen pressure to facilitate postoperative recovery. Both normal respiratory activity and heart rate (350–450 beats/min) were carefully monitored before housing the animals under standard conditions until the next experimental step.

Fourteen days after surgery, the rats were subjected to another left thoracotomy (T2) for membrane implantation in the ventricular surface, with or without co-cultured cells. Immediately after LAD ligation, a bacterial membrane fragment was gently placed onto the left ventricle of Group III animals, combined with cocultured cells in contact with the epicardial surface. The membrane fragment edges were ligated to the ventricle using a suture and placed without any artificial reinforcing effect. Finally, the sternum and skin incisions were sutured (Figure 2).



Figure 2. A cellulose patch is placed on the left ventricle.

Euthanasia

All animals were euthanized with a lethal dose of pentobarbital sodium (thiopental) 200 to 250 mg/kg injected intraperitoneally.

Echocardiographic Analysis

Before echocardiography, the rats were anesthetized (with 10 mg/kg xylazine and 50 mg/kg ketamine, intramuscular injection) and subjected to transthoracic Doppler echocardiographic studies using Sonos 5500 Sonos Agilent echocardiographic model, equipped with a phased array 12-5 MHz probe, with a software specifically designed for studies in small animals. Local and overall left ventricular contractility

was evaluated by assessing left ventricular ejection fraction (LVEF), left ventricular end-systolic volume (LVES), and left ventricular end-diastolic volume (LVED). The echocardiographic analysis was performed 7 days after MI (baseline, D0), 1 month after the MI, and after transplantation of the patch with or without cells (D1) [37, 38].

Cell Isolation Procedures

Skeletal myoblasts were isolated after taking a biopsy from the lower limb's skeletal muscle, as described earlier [39]. Bone marrow mesenchymal stem cells were isolated through bone marrow aspiration of the iliac crest [40]. After cell centrifugation, the isolation was performed using a density gradient, 1.077, Ficoll-Paque PLUS solution (Amersham Biosciences®, USA); then, mononuclear cell fractions were distributed in flasks, kept for 48 hours, and washed with PBS (Gibco Invitrogen®, Life Technologies, USA). Only the mesenchymal stem cells remained adherent, while cells of hematopoietic origin did not. For both cell types, the culture medium used was Dulbecco's Modified Eagle Medium (DMEM) (Gibco Invitrogen® Life Technologies, USA) containing 15% fetal bovine serum (FBS) (Gibco Invitrogen®, Life Technologies, USA), 100U/mL penicillin and 100 μ g/mL streptomycin (Gibco Invitrogen®, Life Technologies, USA). The cultures were maintained in an incubator at 37°C with 5% CO₂.

The assays were performed in 25-cm² polystyrene flasks (TPP®-Germany). The BC (Membracel®, Brazil) and cells were seeded as described by Carvalho et al. [40]. The proportion of skeletal muscle cells to bone marrow mesenchymal stem cells was 2:1, approximately 5 \times 10⁵/mL per 14 days. The coculture medium used was DMEM containing 15% FBS, 1% of antibiotics (100U/mL penicillin and 100 μ g/mL streptomycin), and 10 ng/mL of insulin growth factor (IGF-I) (Gibco BRL®, Life Technologies, Inc, Rockville, MD, USA). The medium was changed every 48 hours, and the cultures were incubated at 37°C with 5% CO₂ [39].

MTT assay

Cells were cocultured in the above-mentioned media (1 \times 10⁴ cm²) on the membrane surface and maintained in 6-well plates. The plates were incubated in standard cell culture conditions at 37°C

temperature with 5% CO₂. Subsequently, 100µL of 5mg/mL 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) (final concentration, 0.5mg/mL) was added to the wells, and the cells were incubated at 37°C with 5% CO₂ for 1,3,7 and 10 days. Supernatants were used for analysis using a spectrophotometry reader (reference wavelength set to 595 and 630 nm) [40].

Cytometric analysis

To verify the bone marrow mesenchymal stem cells' origin and skeletal muscle cells, flow cytometric analysis was performed using the FACSCalibur system (BD Biosciences, San Jose, CA, USA). Immunophenotypic analysis for CD34, CD 45, CD105, CD 90, CD73, and Myo-D was performed with a commercially available kit (Stem Kit, Beckmann Coulter, Krefeld, Germany) as a single-platform method according to the international Society of Hematotherapy and Graft Engineering (SHAGE) guidelines [41]. This Kit consists of anti- CD45-FITC monoclonal antibody (Mab), anti- CD34-PE, CD 105-FITC (clone 266), CD 90-PE (clone OX-7), CD 73-PE, and CD Myo-D-FITC (clone Mab5.8A). The conjugated Mabs were already provided in combinations ready to use.

Euthanasia

All animals were euthanized with a lethal dose of pentobarbital sodium (thiopental) 200 to 250 mg/kg injected intraperitoneally [34].

Histopathological analysis

The hearts were harvested and fixed in 10% neutral buffered formalin for 24 hours. Sections were routinely stained with H&E and Gomory trichrome. To identify the cells, formalin-fixed and paraffin-embedded tissue sections were immunostained using the Vector® M.O.M. Immuno-detection kit (Vector, Burlingame, CA), and monoclonal antibodies specific for muscle alpha-actin (SR 1) and BrdU (BrdU in situ detection kit) (BD Biosciences, San Jose, CA), according to the manufacture instructions. The primary antibody was applied for 1 h at room temperature. Angiogenesis was detected by immunoperoxidase staining for a vascular endothelial growth factor (VEGF) (Abcam, Cambridge, United Kingdom). The slides were then incubated with secondary biotin-labeled, affinity-isolated anti-rabbit and anti-mouse immunoglobulins (LSAB®+ Kit, Peroxidase; DAKO Corp, Carpinteria, CA, USA).

Scanning electron microscopy (SEM) analysis

Cocultured cells (1×10^4 cm 2) were grown on a BC patch for 14 days. The patch was rinsed three times with PBS, fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in a 0.1M phosphate buffer for 15 min, and rinsed in distilled water. Dehydration was performed in a series of ethanol concentrations (50%, 70%, 90%, and twice in 100%). The dehydrated specimens were kept overnight in a vacuum oven at 25°C, after which they were support-coated with gold and examined with A JEOL 6360LV SEM, Japan, Tokyo, Japan operation at a 30 kV accelerating voltage. The experiment was repeated four times, and respective photographs were taken (n=5).

Statistical Analysis

All numerical data are shown as mean values p/ 95% confidence limits. Statistical significance was assessed using a one-way analysis of variance (ANOVA), and the minimum significant difference between means of each group was calculated using the T-test method. For a comparison of 2 groups, a 2-tailed unpaired Student t-test was used; the condition of normality was assessed using the Kolmogorov-Smirnov test. P-values ≤ 0.05 were considered statistically significant.

RESULTS

Cell adhesion and proliferation

Regular monitoring of the cocultured cells confirmed that they adhered very well to the BC membranes. We analyzed the proliferation of cocultured cells using MTT assay (figure 5) and found that the BC membranes could support coculture growth and adhesion. The cocultured cells on the BC membranes showed exponential growth over 14 days. The changes in the membrane, with or without cells, were not due to toxicity as confirmed by SEM analyses, which showed superior cell growth and spread throughout the cocultured BC membranes (Figure 3).

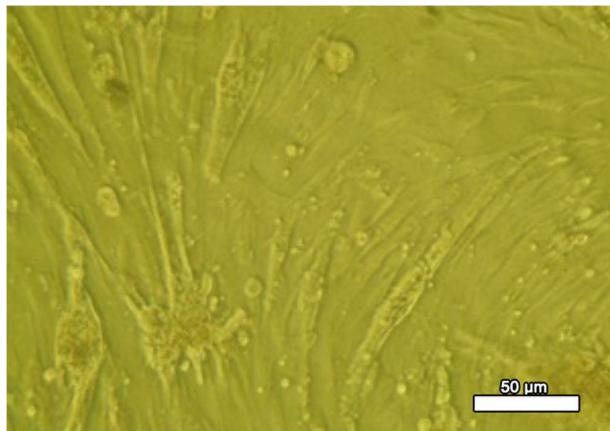


Figure 3. Skeletal muscle cells and bone marrow mesenchymal stem cells cocultured seeded on cellulose membrane, 14 days in cultivation—200X, Optical inverted microscopy.

Echocardiographic findings

Three groups were used in this study (a total of 28 animals), Group I ($n = 9$): myocardial infarction without treatment (control group), Group II ($n = 9$): implantation of the bacterial cellulose matrix on the left ventricular surface, and Group III ($n = 10$): implantation of the bacterial cellulose matrix seeded with cocultured cells on the left ventricular surface. We report that some rats died during the experimental process: 13 after myocardial infarction; 2 in group I, 2 in Group II.

Left ventricular (LV) function was analyzed 24 hours after myocardial infarction (baseline) (D0) and 30 days after (D1). At baseline and after infarction, the mean ejection function was similar in the three groups (Table 1). Over the course of 30 days, the membrane implantation decreased LVED dimension compared with the baseline value (Figure 4). Also, a significant attenuation of LV dilatation was achieved when the membrane was implanted without cells (group II), compared with the two other groups. There was no detectable dilation of the left ventricle in the untreated control and the membrane groups, as revealed by decreased LVES and LVED (Figures 4 and 5). In contrast to the cellulose patch group, the control samples revealed remarkable remodeling of myocardium. The cellulose patch group's functional analysis did not reveal any improvement of left ventricular ejection fraction compared with the patch with cocultured cells and with the control (Table 1, Figures 4 and 5).

Table 1. Echocardiographic Results in the 3 Groups at the 2-time points.

Variable	Group	D0	D1	P Value (D0 vs D1)
LVEF (%)	I	28.21± 5.07	32.72± 8.62	0.316
	II	22.79± 5.80	26.74± 4.92	0.065
	III	27.37± 8.40	27.92± 9.77	0.865
LVES (mL)	I	0.509± 0.106	0.582± 0.149	0.241
	II	0.596± 0.171	0.598± 0.141	0.982
	III	0.780± 0.112	0.780± 0.206	0.001 *
LVED (mL)	I	0.718± 0.170	0.848± 0.149	0.326
	II	0.747± 0.196	0.768± 0.158	0.043
	III	0.698± 0.102	1.056± 0.183	0.004 *

Table 1. Distinct time point (D); left ventricular fraction ejection (LVEF); left ventricular end-systolic (LVES); left ventricular end-diastolic (LVED). Data are shown as mean ± SD. P< 0.05 was considered statistically significant. Group I: Myocardial infarction without treatment as a control group; Group II: Implantation of the bacterial cellulose matrix on left ventricle surface and Group III: Implantation of the bacterial cellulose matrix seeded with cocultured cells on the left ventricle surface.

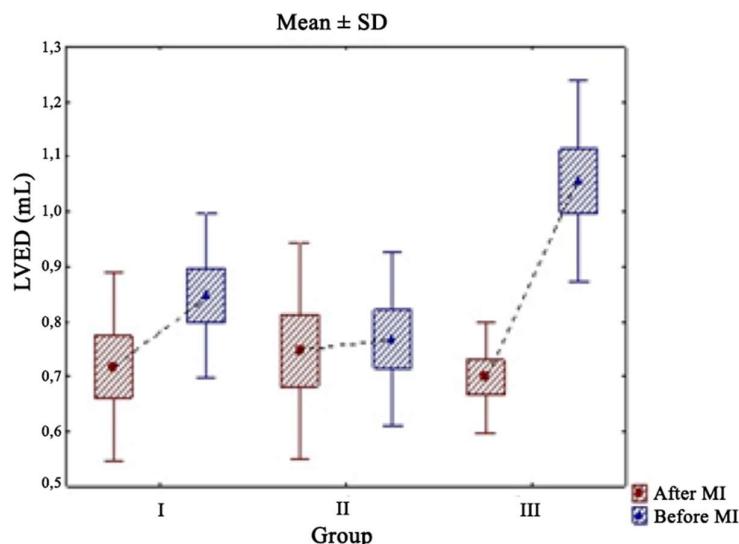


Figure 4. Graphs show echocardiographic measurements of cardiac function: left ventricular end-diastolic (LVED), mean values were calculated for each distinct group. Myocardial Infarction (MI); Group I: Myocardial infarction without treatment as a control group, Group II: Implantation of the bacterial cellulose matrix on left ventricle surface and Group III: Implantation of the bacterial cellulose matrix seeded with cocultured cells on the left ventricle surface; Standard Deviation: SD.

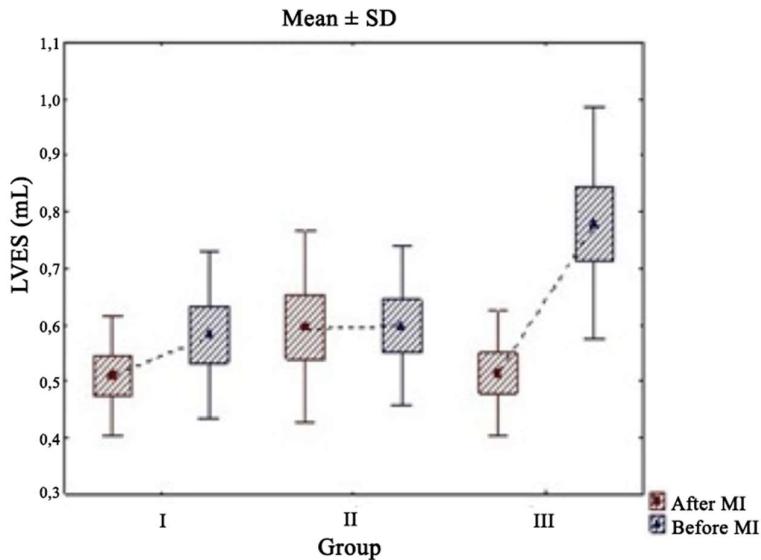


Figure 5. Graphs show echocardiographic measurements of cardiac function: left ventricular end-systolic (LVES), mean values were calculated for each distinct group. Myocardial Infarction (MI); Group I: Myocardial infarction without treatment as a control group; Group II: Implantation of the bacterial cellulose matrix on left ventricle surface and Group III: Implantation of the bacterial cellulose matrix seeded with cocultured cells on left ventricle surface.

Histopathological findings

Assessment of the heart chambers, externally and internally, showed ischemic lesions in the left ventricle's anterior wall and the interventricular septum, with no apparent differences between samples, confirming the *in vivo* morphologic and functional data.

To characterize the cells used in our experimental model, a histopathological analysis was performed and indicated that the isolated cocultured cells adhered to the BC membrane and acquired a skeletal morphology after 14 days in culture (Figure 3). We also detected a notable proliferation of the cocultured cells (BrdU labeling) accompanied by evident angiogenesis (VEGF labeling) (Figure 6). We continued assessing these cells' condition via SEM analysis, which indicated that cocultured cells adhered well and grew intensively in the cellulose membrane (Figure 7).

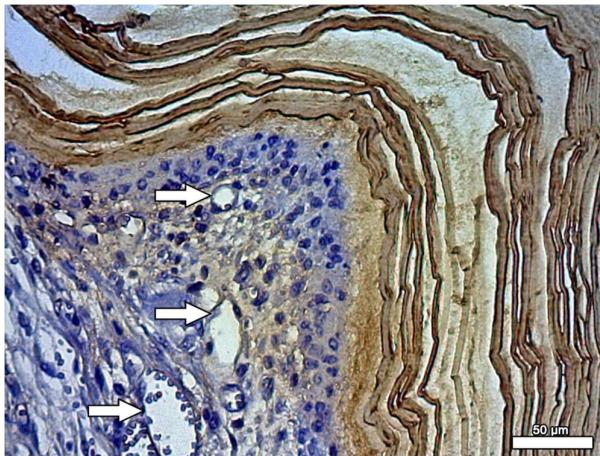


Figure 6. Photomicrography show coculture cells grafted in the post-transplantation cardiac scar tissue. Sections were stained with anti-BrdU antibodies (dark) to identify the engrafted coculture cells and vessels (arrows) 400x (Optical light microscopy).

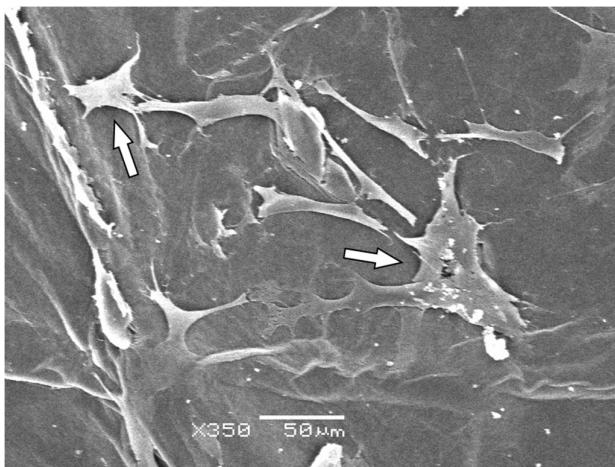


Figure 7. SEM analysis of the BC containing cocultured (skeletal muscle cells and bone marrow mesenchymal stem cells) for 14 days, showing cell adhesion (arrows). Scanning electron microscopy (SEM) and bacterial cellulose (BC).

DISCUSSION

To mimic fibrous structures of in vitro cell culture, nanofiber fabrication systems have been developed for generating a polymer or composite fibers from natural or synthetic materials. These nanofibers possess a large surface area, which is favorable for cell attachment [16]. Nanofibers' physical and chemical properties can easily be tunable under appropriate conditions to facilitate cell growth and subsequent tissue development, thereby imparting gradient features into a nanofiber

system and thus offering an exciting area of research [20, 28, 32]. Micro and nanoscale techniques are versatile tools for developing such gradient biomaterials and could be utilized to design a new generation of engineered grafts for use in interface tissue engineering [12, 25].

The present study's findings could be developed further by investigating the incorporation of biomolecules as growth factor TGF- β 1, which is known to promote cell differentiation and proliferation. Also, the use of bioinformatics databases should be uncovered the expression and effect of genes generally implicated in tissue regeneration such as TGF β 1, MMP2, MMP9, CTNNB1, Wnt4, hsa-miR-29b-3p, and hsa-miR-29c-3p [42]. Gene expression studies have been developed to ensure successful tissue regeneration, such as using bacterial cellulose to release BMP-2 and promote optimal tissue formation. Interestingly, bacterial cellulose+BMP-2 combination enhanced bone regeneration and appeared to be a promising clinical procedure approach [43]. These tools may help preserve the native phenotype, which remained a complex challenge in the field.

On the other hand, studies have suggested the benefits of cocultured cell transplantation in promoting cardiac tissue regeneration and restoring infarcted heart [25, 28]. Previous preclinical studies and clinical trials have used different cell types and biomaterials to test cell therapy's effect on cardiac repair and reported promising results [44-50]. Several types of cells have been proposed for cardiomyoplasty [33, 45, 47, 50]. Using an autologous model of cocultured skeletal muscle cells and bone marrow mesenchymal stem cells is based on each cell type's potentiality: skeletal muscle cells are known to be resistant to ischemia mesenchymal stem cells were shown to be considerably angiogenic. Thus, combining these cells would allow better angiomuscular myocardial regeneration [51-53]. Schussler et al. [25] have shown that transplantation of stem cells combined with three-dimensional (3D) collagen scaffolds into ischemic rat hearts can prevent and reverse heart failure progression. Other investigators have used echocardiography and histology and reported that the collagen matrix in myocardial infarction models did not improve pathological post-ischemic remodeling, EF, and LV wall thickness. However, the latter authors found that using a combination of matrix and cells could prevent ventricular wall thinning [54]. Recently, bacterial cellulose has been demonstrated to enhance the adhesion and proliferation of skeletal muscle and mesenchymal cells [31]. This study found that using a cellulose patch without cells can protect the myocardium against deleterious effects and pathological remodeling of the ischemic heart. Our findings demonstrated

that cellulose patch combined with cells results in beneficial effects not provided by cell therapy. The cellulose patch likely supports cells known to produce paracrine effects *in situ* or allows mobilizing autologous resident stem cells to the site of injury, as shown in other systems [54-57]. In conclusion, the present study suggests that using cellulose as a patch is effective for cell delivery into the myocardium, preventing deleterious remodeling of the ischemic heart. Thus, the cellulose patch is a biomaterial with significant potential in repairing heart damage.

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