# Tissue cardiomyoplasty using multi-layer cell-seeded nano-structural scaffolds to repair damaged myocardium: an experimental pilot study

Massoud Soleimani[1,2], Yousef Mohammadi[2], Nasser Ahmadbeigi[1,2], Hossein Ahmadi Tafti[3], Seyed Mahdi Nassiri[4], Mohammad Ali Boroumand[3], Shahram Rabbani[3], Mohammad Sahebjam[3], Naghmeh Moshtaghi[3], Ehsan Fayazzadeh[3]

[1]Hematology Department, Faculty of Medicine, Tarbiat Modares University, Tehran, Iran
[2]Stem Cell Technology Co., Tehran, Iran
[3]Experimental Research Department, Tehran Heart Center, Medical Sciences, University of Tehran, Tehran, Iran
[4]Department of Clinical Pathology, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

**Corresponding author:**
Ehsan Fayazzadeh, MD
No. 310, Block C2
Shahrak Ekbatan, Tehran
13956-33559, Iran
Phone/fax: 98-21-4464 1894
E-mail:
efayazzadeh@razi.tums.ac.ir

**Submitted:** 24 November 2007
**Accepted:** 19 February 2008

Arch Med Sci 2008; 4, 4: 364–370
Copyright © 2008 Termedia & Banach

## Abstract

**Introduction:** The emerging fields of tissue engineering and biomaterials have begun to provide potential treatment options for heart failure. Tissue engineering approaches are designed to repair damaged cardiac tissue through the use of cellular transplantation, and biodegradable scaffolds. In an experience for the first time in Iran applying nanobiotechnology in heart tissue engineering, we investigated the outcome of a multi-layer nanostructural scaffold containing cardiac and endothelial cells grafted on the infarcted heart.
**Material and methods:** Myocardial infarction was induced in sheep by ligation of the distal portion of the left anterior descending coronary artery. Biopsy of the left ventricular cardiac muscle and jugular vein was obtained. Tissue samples were isolated and cultured *in vitro*. Cultured cardiac and endothelial cells were seeded onto the layers of poly(ε-caprolactone)/collagen biodegradable scaffolds. After two months, the scaffold was sutured on the surface of the infarcted myocardium.
**Results:** Eight weeks after implantation, there was remarkable thickening as well as decreased paradoxical motion of the ventricular wall in echocardiographic evaluation. There was no significant improvement in global ejection fraction. In microscopic examinations by Masson's trichrome staining and immunohistochemical analysis, viable cells were observed within layers of the scaffold with incorporation of the graft into the adjacent myocardium. There were also spectacular amounts of vascularization in the grafted material.
**Conclusions:** Our data demonstrate that engrafting of multilayer cell-seeded nanostructural scaffolds can induce angiogenesis in the implanted region. Such tissue engineered cells containing scaffolds are a promising means of tissue cardiomyoplasty in the field of regenerative medicine. Further studies are however encouraged to investigate the physiopathological aspects of heart tissue engineering.

**Key words:** tissue scaffolding, heart failure, cell transplantation, myocardial infarction, sheep, polycaprolactone, collagen.

## Introduction

Myocardial infarction (MI) is the leading cause of death in developed countries and the cause of two-thirds of cases of congestive heart failure (CHF) as well. MI and the resulting death of cardiomyocytes lead to increased wall stress in the remaining myocardium. This process results in a sequence of molecular, cellular and tissue pathological responses that cause left ventricular (LV) dilatation. This LV remodelling progresses eventually to heart failure [1]. Cellular therapy has been lately proposed as a possible alternative for conventional cardiac repair and prevention of LV remodelling and consequent CHF. More recently, the emerging field of tissue engineering of the heart has proposed more potential advantages than direct delivery of cells to the heart [2, 3].

In a novel method, applied for the first time in Iran using nanobiotechnology in tissue engineering of the heart, we used a multilayer nano-structural scaffold made of sheets of poly (ε-caprolactone)/collagen, which were seeded separately by adult cardiomyocytes and endothelial cells. It is expected that these nanofibrous scaffolds would lead to much more efficient cellular attachment and communication. Poly(ε-caprolactone) (PCL) is a biocompatible elastomer belonging to the family of poly (α-hydroxyacids) with hydrophobic properties. This FDA-approved material degrades by passive hydrolysis of ester linkages in the polymer backbone and has shown to be an efficacious biocompatible and biodegradable polymer for tissue engineering applications [4].

## Material and methods

### Animal selection

We chose the ovine model of coronary ligation as being relatively close to human pathology and set up an experimental model in which the size and the functional consequences of coronary ligation are well defined [5, 6]. The experiment was in accordance with the "*Guide for the Care and Use of Laboratory Animals*" published by the US National Institute of Health (NIH Publication NO. 85-23, revised 1996). After institutional review and ethical committee approval, an Iranian adult ewe weighing about 50 kg was used in this study. The animal was adapted for one week in our department's large animal care unit and examined by a veterinary specialist to rule out any cardiac or systemic disorders.

### Echocardiography

Echocardiographic examination (model SSA380A, Toshiba, Tokyo, Japan) was performed before the operation to evaluate ejection fraction (EF), fractional shortening (FS), and segmental wall kinesis and was repeated 2 and 4 months later (i.e. prior to scaffold engraftment and animal sacrificing, respectively).

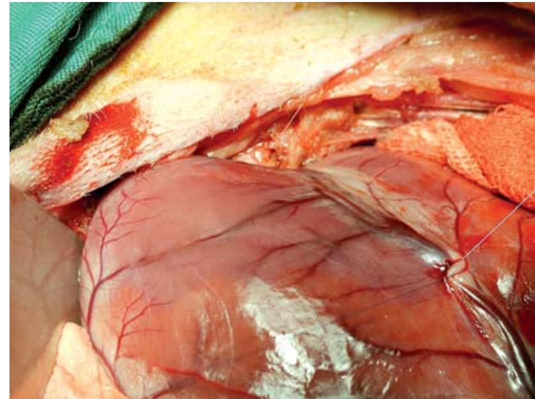

**Figure 1.** The homonymous artery was ligated just beyond bifurcation of the second diagonal branch. Note the cyanotic discoloration distal to the ligation point at an area of 4 × 5 cm

### Surgical procedure

Acute MI was induced by coronary artery ligation. Anaesthetic and pre-operational procedures were done as described before by Rabbani et al. [6]. After prep and drape, under sterile conditions, 10 cm left lateral thoracotomy was made in the 4$^{th}$ intercostal space. After entering the chest and opening the pericardium, the left ventricle was well exposed and the coronary vasculature investigated. The left anterior descending coronary artery (namely the homonymous artery in the sheep) was ligated by 6-0 Prolene™, at a point in the distal two-thirds of its path (just beyond bifurcation of the 2$^{nd}$ diagonal branch). Anti-arrhythmic prophylaxis with lidocaine (Institute Pasteur, Tehran, Iran), as elsewhere described by the authors [6], was administered at the time of vessel ligation (2 mg/kg) and was repeated after 15 min (1 mg/kg). Ischaemic bluish discoloration in the anterior area was immediately evident (Figure 1). EKG changes, notably T wave inversion and ST segment elevation, were also considered after a short while. Another 4 cm skin incision was done on the left side of the animal's neck for jugular vein excision. After harvesting vessel and heart muscle specimens, chest and neck incisions were closed by continuous running 3-0 and 2-0 Vicryl™ sutures (for muscles and skin, respectively). The animal recovered well without any unwanted complications. It was then transferred to the large animals care unit and housed for 2 months.

### Tissue harvesting

During the surgical procedure, a 2 mm piece (3 × 3 mm) of LV myocardial tissue in the intact region was excised with a #15 surgical blade. All care was

taken to avoid damaging visible vessels during the procedure. A 15 mm fragment of the external jugular vein was also excised for endothelial cell culture. Tissue specimens were carried to the laboratory in the animal's own heparinated (100 U/ml; IPDIC, Tehran, Iran) blood in sterile tubes containing penicillin (100 U/ml; Sigma Chemicals, St. Louis, MO, USA) and streptomycin (100 µg/ml; Sigma Chemicals).

### Cellular isolation

In the cell-culture laboratory, one hour after tissue harvesting in the operating room, the specimens were placed into Petri dishes (Nunc, Copenhagen, Denmark) for dissection in a laminar air-flow hood using sterile techniques.

#### Isolation of endothelial cells

The vessel was cleared of surrounding tissue, dissected longitudinally by fine scissors and incubated in medium containing Dulbecco's modified Eagle medium (DMEM; Gibco, Gaithersburg, MD, USA), 20% fetal bovine serum (FBS; Gibco), and 0.2% collagenase II (Sigma Chemicals) at 37°C for 30 min. Detached cells were flushed out by rinsing the vessel thoroughly with phosphate-buffered saline (PBS). The cell suspension was aspirated and washed twice with DMEM 20% FBS. The cells were pelleted by centrifugation and resuspended in M-199 (Biosera, Ringmer, East Sussex, UK) supplemented with 20% FBS, penicillin G (100 U/ml), streptomycin (100 µg/ml), heparin (20 U/ml; Sigma Chemicals), and endothelial cell growth supplement (20 µg/ml; Becton Dickinson, Bedford, MA, USA). The cells were then plated on to 25 cm2 0.2% gelatin-coated culture flasks (Nunc) and incubated with 95% air and 5% $CO_2$ at 37°C. The cell culture underwent a pre-plating step at the first passage to deplete fibroblasts from the culture, based on their quicker attachment property compared to endothelial cells. The medium was changed twice per week. Non-adherent and red blood cells were removed during the first few medium changes, and attached cells were kept in culture until the outgrowth of fibroblastoid cells within the next two weeks. The cells were passaged at subconfluence by detaching them using 0.05% trypsin/EDTA solution (Gibco).

#### Isolation of adult ventricular cardiomyocytes

The cardiac tissue specimen was washed in calcium-and bicarbonate-free Hanks' balanced salt solution with HEPES (CBFHH; Sigma Chemicals) three times and then minced in CBFHH by scalpel to particle sizes of approximately 1 $mm^3$. Tissue fragments were trypsinized at room temperature for 20 min, and then allowed to settle, followed by removing the resultant turbid supernatant. They were subsequently treated with 0.1% trypsin (Sigma Chemicals) and 0.1% collagenase IV (Sigma Chemicals) for 5 min. This cycle was repeated until nearly complete digestion of the heart tissue was achieved. The supernatant of the trypsin/collagenase digested material was then collected in 15 cc centrifuge tubes (Nunc) containing Iscove's modified Dulbecco's medium (IMDM; Sigma Chemicals) and 10% FBS to inactivate the trypsin. Collection tubes were centrifuged at 50 g for 15 min with the supernatant discarded. The cell pellet was then resuspended in complete explant medium (CEM) containing IMDM supplemented with 10% FBS, penicillin G (100 U/ml), streptomycin (100 µg/ml), L-glutamine (2 mmol/l), and 2-mercaptoethanol (2-ME; 0.1 mmol/l), and pooled into one tube. This procedure was repeated once again. The cell suspension was then strained through a 70 µm nylon mesh filter (BD Falcon, Becton Dickinson, USA) into culture dishes and incubated in CEM with 95% air and 5% $CO_2$ at 37°C for one hour. During this time, the fibroblasts and other non-cardiac myocytes would adhere, whereas the cardiomyocytes would remain floating. Non-adherent cells were then transferred to a 50 ml tube, sedimented by centrifugation, resuspended in fresh medium, and finally counted.

Cellular concentrations were adjusted to $5 \times 10^6$ cells/ml in which 2-3 × $10^6$ were expected to be viable as judged by trypan blue exclusion [7].

### Scaffold fabrication

Poly(ε-caprolactone)/collagen nanofibrous hybrid scaffolds were prepared by the double-jet electrospinning method, as elsewhere described by Khademhosseini et al. [8], and Matthews et al. [9]. Briefly, a high DC voltage was applied to two separate syringe pumps (model SP-500, JMS Co., Tokyo, Japan) in order to charge the polymer solutions contained within. The polymer solutions consisted of PCL (Aldrich Chemical, Steinheim, Germany) and collagen I (Sigma Chemicals), which were then ejected from each syringe in a controlled manner, and a jet stream of the charged polymers was drawn towards a grounded (earthed) rotating collator. The polymers dissolved to form nanofibres with the travelling of the jet stream through the air towards the collator. The charge of the polymers was slowly dissipated after collecting on the collator. The resultant nanostructural hybrid scaffolds were then freeze-dried at 0.02 mbar and –10°C for 48 hours and stored in a desiccator at room temperature. The scaffolds were then cut into 4 × 4 cm sheets and sterilized by decreasing concentrations of ethanol for 1-2 days. The morphology of the electrospun PCL/collagen nonwoven scaffolds (Figure 2) was observed by means of scanning electron microscopy

(model VEGA-LSU, Tescan, Cranberry, PA, USA) at an accelerating voltage of 20 kV. Samples for the morphological study were coated with gold using a sputter coater. The mean diameters of PCL and collagen fibres were 460, and 200 nm respectively. Each layer of the scaffold sheet was 500 µm thick, with a bulk density of 1.09 g/cm$^3$ and void volume of 92%, which allowed maximal nutrient transfer. The percentage of collagen content in the prepared patches was 15.26%.

### Cellular seeding

Before cell seeding, each PCL layer was base-treated to cleave ester bands on the mesh surface, leading to –OH and O=C-O-H- acid groups formation, and thereby increasing the surface hydrophilicity for the host cardiac muscle attachment [4]. Cultured adult cardiac and endothelial cells were then separately seeded onto the collagen-enriched scaffolds. The number of endothelial/cardiac cells was 2 × 10$^5$ per sheet. Ten sheets of scaffold were layered on together alternatively seeded by cardiac and endothelial cells for tissue scaffolds engraftment.

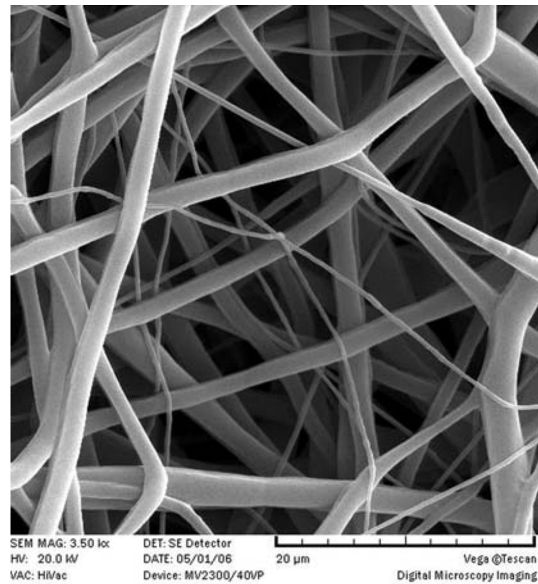


**Figure 2.** Scanning electron photomicrograph of the scaffold shows its hybrid nanostructure consisted of PCL (thicker) and collagen I (thinner) fibers

### Engraftment of tissue scaffolds onto the infarcted myocardium

Two months after myocardial infarction, the animal was re-operated with the standard surgical approach described earlier. A large dyskinetic scar zone (3 × 3 cm) was observed in the anterior region of the left ventricle. After careful trimming of the scaffold sheets, it was implanted on the epicardium with 5-0 Prolene™ sutures, thus covering the whole dyskinetic and border zones (Figure 3).

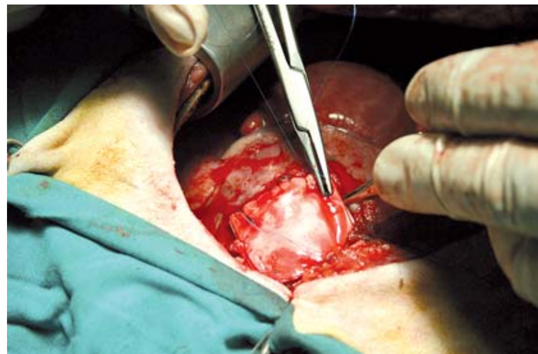

**Figure 3.** The multi-layer scaffold is implanted onto the infarcted heart

### Animal scarification

Two months after engraftment of the tissue scaffolds, the animal was euthanized by a lethal IV dose of sodium thiopental (40 mg/kg; Sandoz GmbH, Kundl, Austria). Blood heparinization (10,000 U; IV) was already performed. The heart was excised *en bloc* and sent in formalin solution to the pathology laboratory for histological examinations.

## Results

### Paraclinical parameters

Pathological Q waves, which were evident on electrocardiography two months after MI, sustained until the time of animal sacrificing. The echocardiographic variables are listed in Table I. The global EF of the animal declined one day after MI from 65 to 43% and remained nearly unchanged (45%) for the next two months. However, there was a large aneurysmal area in the anteroseptal segment at the time of scaffold implantation, which improved substantially yet remained akinetic after two months. Interestingly, the engrafted tissue scaffolds could not be differentiated by sight from the beating heart.

**Table I.** Echocardiographic parameters of the animal. Columns A-C represent variables before MI, two months after MI (at the time of implanting the scaffold), and four months after MI (at the time of animal sacrificing), respectively

| Variable | A | B | C |
|---|---|---|---|
| LVEDV [ml] | 25.9 | 87.6 | 63.8 |
| LVESV [ml] | 8 | 50.8 | 35.1 |
| CO [ml/min] | 2750 | 2580 | 2230 |
| EF [%] | 69 | 42 | 45 |
| FS | 37 | 20 | 22 |

*LVED – left ventricular end diastolic volume, LVESV – left ventricular end systolic volume, CO – cardiac output, EF – ejection fraction, FS – fractional shortening*

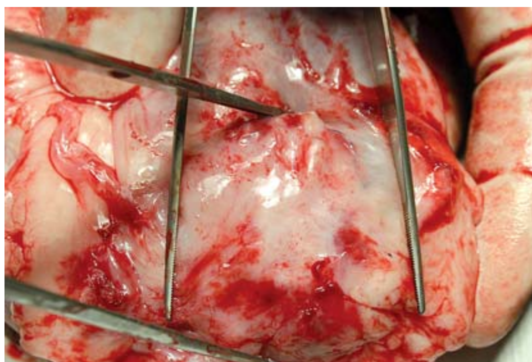

**Figure 4.** Macroscopic examination two months after implantation shows near complete integration of the scaffold in to the host myocardium

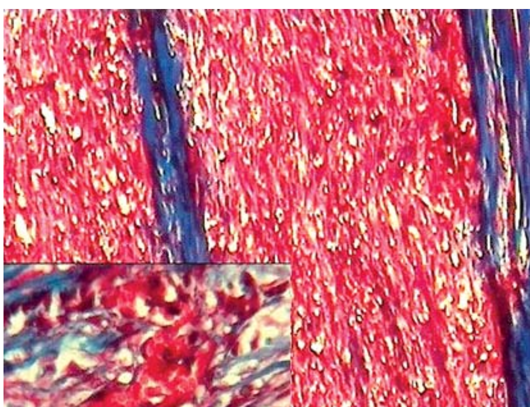

**Figure 5.** Masson's tri-chrome staining of the grafted scaffold. Two months after implantation, there is seen diverse cellular survival and considerable amounts of angiogenesis with RBCs within neovessels (inset)

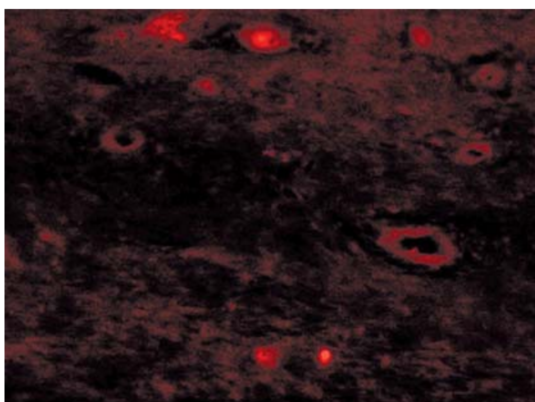

**Figure 6.** Immunohistochemical analysis with PE-conjugated anti-SMA antantibody shows numerous microvessels interspersed within the scaffold
*PE – phycoerythrin, SMA – smooth muscle actin*

## Pathological examination

On macroscopic evaluations, there was fibrous thickening of the ventricular wall at the site of implantation with apparent complete integration of the scaffold patch into the host myocardium (Figure 4).

Microscopic examination of the heart tissue with Masson's trichrome staining (Figure 5) revealed strong vascularization and cellular survival within the tissue scaffolds. The entire tissue scaffolds were penetrated by functional blood vessels, as confirmed by the ultrastructural demonstration of erythrocytes in the blood vessels. The scaffold layers were extensively occupied by bands of cardiac cells aligned in a regular pattern. There were no signs of tissue scaffolds' infiltration by inflammatory cells.

Immunohistochemical analysis of the tissue scaffolds with phycoerythrin-conjugated anti-PECAM-1 (anti-CD31) and anti-smooth muscle actin (SMA) antibodies and diamino-2-phenyl indole (DAPI) (all materials from Dako, Glostrup, Denmark) staining of the nuclei showed angiogenesis in different stages with some vessels fully formed and having lumens (Figures 6, 7).

## Discussion

Myocardial infarction and the resulting heart failure is a major cause of morbidity and mortality worldwide. Over 7 million people die of the disease each year [10]. Despite many conventional medical and surgical options to treat the disease, they fail to respond in complicated cases. In end-stage heart failure, the only currently remaining intervention capable of significant improvement is cardiac transplantation [11]. Hence, there is an obvious need for tissue-engineered myocardial grafts containing cardiac cells to restore left ventricular function and treat heart failure. There are two approaches based on cellular therapy for this purpose: 1) cellular cardiomyoplasty; defined as direct intramyocardial injection or percutaneous coronary artery administration of isolated cells; and 2) tissue cardiomyoplasty; or development of scaffold-based cardiac/endothelial cell constructs *in vitro* that can be engrafted to the damaged myocardium during open-heart surgery. Tissue engineered scaffolds can be provided in the form of either biological or synthetic materials [12].

Collagen [13], and synthetic polymers [14] have been used to generate scaffolds for cardiac tissue engineering applications. The resultant structures are extremely porous and have very high surface area to volume ratios. Specific nanoscale features play a prominent role both in promoting cell proliferation and in guiding cell growth and general tissue architecture [14-17]. Recent investigations have shown that nanofibres aligned in desired directions and patterns [18] can induce growth and proliferation of muscle cells into biologically relevant contractile spindle structures. In addition, as nanofibres can be generated from well-characterized polymers such as PCL, it is possible to take advantage of properties such as biodegradability

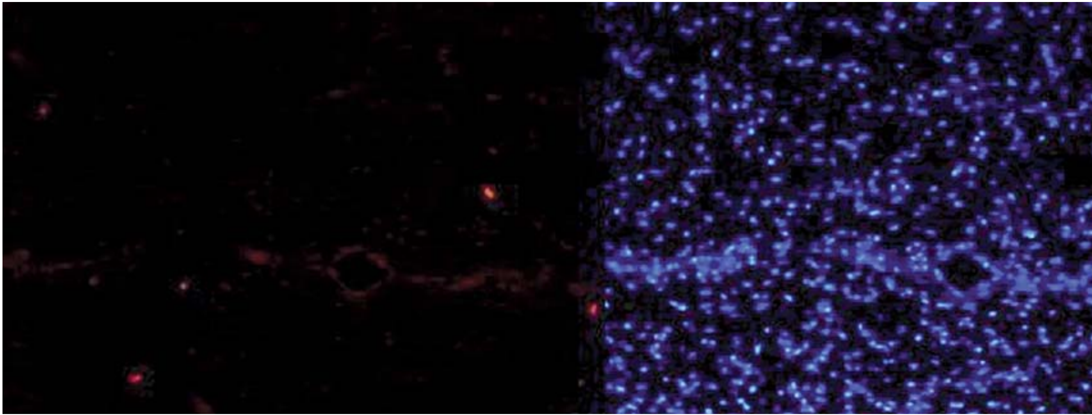

Figure 7. Immunohistochemical analysis with PE-conjugated antibody against CD-31 (PECAM-1) illuminates a capillary within the scaffold (left). Nuclei are counterstained with DAPI (right)
*PE – phycoerythrin, PECAM-1 – platelet endothelial cell adhesion molecule, DAPI – diamino 2-phenylindole*

and surface functionalization. In fact, the size of biodegradable fibres can be used to modulate the degradation rate of the material [14].

We used a combination of cardiac and endothelial cells seeded on layers of nano-structural PCL/collagen I hybrid scaffolds in an ovine model of heart failure. Sheep, like dogs and swine, have a coronary anatomy rather similar to the human heart, but they lack the canine model's disadvantage of rich coronary collateral circulation, and hence could be used as suitable (reproducible yet safe) models of inducing acute heart failure by coronary artery ligation [1, 5, 6].

The presence of viable cardiac cells within the deep layers of scaffold two months after implantation, despite the lack of efficient circulatory mechanisms and poor diffusion, was remarkable. Whether the heavy vascularization of the tissue scaffolds was mainly due to ingrowth of the host blood vessels into the scaffold sheets or because of the formation of new vascular structures from the growth of existing endothelial cells and preformed capillaries remains to be investigated [19, 20].

We did not label the transplanted cardiac and endothelial cells with cellular markers; hence, it is not clear whether the viable cells observed throughout the tissue scaffolds are those that were previously seeded onto the scaffold and survived. There are also many other issues that should be addressed in further studies. The lack of any significant improvement in global EF in our experiment is disappointing. This may be due to the type of the particular scaffolds or cells used. Electromapping studies as well as more sophisticated evaluation methods are required to indicate functional and mechanical coupling of the tissue scaffolds to the host myocardium. Moreover, future *in-vivo* haemodynamic studies should determine whether tissue scaffolds are capable of improving contractile function following myocardial infarction.

## Acknowledgments

The authors would like to thank Roya D. Collins for her contribution in reviewing and editing the article. This work was performed in and technically supported by the research department of Tehran Heart Center, Tehran University of Medical Sciences, Tehran, Iran. The tissue scaffolds were prepared by Stem Cell Tech, Tehran, Iran.

## References

1. Falk V, Garbade J, Walther T. Experimental models of heart failure. In: Dhein S, Mohr FW, Delmar M, eds. Practical methods in cardiovascular research. Berlin/Heidelberg: Springer-Verlag 2005; 88-90.
2. Zimmermann WH, Melnichenko I, Eschenhagen T. Engineered heart tissue for regeneration of diseased hearts. Biomaterials 2004; 25: 1639-47.
3. Eschenhagen T, Didié M, Münzel F, Schubert P, Schneiderbanger K, Zimmermann WH. 3D engineered heart tissue for replacement therapy. Basic Res Cardiol 2002; 97 Suppl 1: I146-52.
4. Li S. Degradation of biodegradable aliphatic polyesters. In: Ma PX, Elisseeff J. Scaffolding in tissue engineering. Boca Raton: CRC Press 2006; 335-52.
5. Hasnat AK, van der Velde ET, Hon JK, Yacoub MH. Reproducible model of post-infarction left ventricular dysfunction: haemodynamic characterization by conductance catheter. Eur J Cardiothorac Surg 2003; 24: 98-104.
6. Rabbani S, Ahmadi H, Fayazzadeh E, et al. Development of an ovine model of myocardial infarction. ANZ J Surg 2008; 78: 78-81.
7. Eschenhagen T, Zimmermann WH. Engineered heart tissue. In: Vunjak-Novakovic G, Freshney RI. Culture of cells for tissue engineering. Hoboken: Wiley 2006; 259-91.
8. Khademhosseini A, Ling Y, Karp JM, Langer R. Micro- and nanoscale control of cellular environment for tissue engineering. In: Mirkin CA, Niemeyer CM (eds.). Nanobiotechnology II. Weinheim: Wiley-VCH 2007; 352-7.
9. Matthews JA, Wnek GE, Simpson DG, Bowlin GL. Electrospinning of collagen nanofibers. Biomacromolecules 2002; 3: 232-8.
10. Yellon DM, Hausenloy DJ. Myocardial reperfusion injury. N Engl J Med 2007; 357: 1121-35.

11. Miniati DN, Robbins RC. Heart transplantation: a thirty-year perspective. Annu Rev Med 2002; 53: 189-205.
12. Daly CD, Campbell GR, Campbell JH. Tissue engineering. In: Pasterkamp G, de Kleijn DP (eds.). Cardiovascular research: new technologies, methods, and applications. Springer, New York 2006; 214-7.
13. Xiang Z, Liao R, Kelly MS, Spector M. Collagen-GAG scaffolds grafted onto myocardial infarcts in a rat model: a delivery vehicle for mesenchymal stem cells. Tissue Eng 2006; 12: 2467-78.
14. Zong X, Bien H, Chung CY, et al. Electrospun fine-textured scaffolds for heart tissue constructs. Biomaterials 2005; 26: 5330-8.
15. Li WJ, Laurencin CT, Caterson EJ, Tuan RS, Ko FK. Electrospun nanofibrous structures: A novel scaffold for tissue engineering. J Biomed Mater Res 2002; 64: 613.
16. Yim EK, Leong KW. Significance of synthetic nanostructures in dictating cellular response. Nanomedicine Med 2005; 1: 10-21.
17. Shih YR, Chen CN, Tsai SW, Wang YJ, Lee OK. Growth of mesenchymal stem cells on electrospun type I collagen nanofibers. Stem Cells 2006; 24: 2391-7.
18. Xu CY, Inai R, Kotaki M, Ramakrishna S. Aligned biodegradable nanofibrous structure: a potential scaffold for blood vessel engineering. Biomaterials 2004; 25: 877-86.
19. Zimmermann WH, Schneiderbanger K, Schubert P, et al. Tissue engineering of a differentiated cardiac muscle construct. Circ Res 2002; 90: 223-30.
20. Zimmermann WH, Didie M, Wasmeier GH, et al. Cardiac grafting of engineered heart tissue. Circulation 2002; 106 (12 Suppl 1): I151-7.