

Cardiac patch design: compatibility of nanofiber materials prepared by electrospinning method with stem cells

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Received: 14.08.2015 • Accepted/Published Online: 13.11.2015 • Final Version: 23.02.2016

Abstract: In this article, in order to create a cardiac patch, a biocompatible polyurethane (PU) nanofiber polymer was developed with the electrospinning method. Bone marrow mesenchymal stem cells (BM MSCs) were isolated from whole human bone marrow. Isolated BM MSC homogeneity was determined by flow cytometry using specific markers. BM MSC-PU interactions were studied with a WST-1 kit on the 3rd and 7th days. Cardiomyocyte differentiation was performed with 5-azacytidine. Cell survival and proliferation of cardiomyocyte-like cells that were cultured on nerve growth factor (NGF)-embedded PU were evaluated with the WST-1 kit on the 3rd and 7th days. NGF's effect on the cardiomyocyte-like cell proliferation was investigated using anti-p70 S6 kinase monoclonal antibody. Our data indicate that PU nanofibers provided a suitable environment for human BM MSC, and no evident cytotoxicity was observed. Seven days after seeding, NGF-embedded nanofibers proved to be more competent in cell proliferation compared with non-NGF-embedded nanofibers. Cardiomyocyte-like cells were found to adhere on the scaffolds, showing a spreading geometry and retaining viability. Increases in p70 S6 kinase activity through NGF were monitored by flow cytometry.

Key words: Electrospinning, cardiomyocyte-like cell, mesenchymal stem cells, polyurethane, nerve growth factor

1. Introduction

Cardiomyocytes are one of the most important cellular components of the heart. They are known by their single nucleus, have a cylindrical and elliptical form, and are 80–120 µm in length and 20–30 µm in diameter. After cardiac tissues damage, cardiomyocytes increase the cell size with hypertrophy. Cardiomyocytes that lose self-renewal ability after birth enter into necrosis in damaged tissue that fibroblasts cover and cause scar tissue (Niu et al., 2013). In the treatment of myocardial infarction, it is a promising approach to the implementation of cell therapy with myocardial angioplasty. A study carried out with the cells of fetal cardiomyocytes transplanted into the damaged area showed that these cells could survive in the selected region, prevented the expansion of scar tissue, and stopped congestive heart failure (Benetti et al., 2010). Mesenchymal stem cells (MSCs) are the most commonly used cells for this purpose (Houtgraaf et al., 2013; Williams et al., 2013). Several molecules (cytokines, growth factors, and chemical agents) have been shown to regulate MSC differentiation into mesodermal lineages (osteoblasts, chondroblasts, adipocytes), including cardiomyocyte-

like cells (Celebi et al., 2010). However, in the ischemic heart, a low stem cell viability rate (due to hypoxic conditions and presence of the inflammatory cytokines) limits the restorative impact of stem/progenitor cells in this area. Therefore, the use of stem cells with various biomaterials or coculture of stem cells in the treatment of heart damage is on rise. Some synthetic biomaterials produced for the treatment of cardiovascular diseases are polyurethane (PU), polycaprolactone, polyethylene terephthalate, polypropylene carbonate, and poly-3-hydroxybutyrate-co-4-hydroxybutyrate (Kim et al., 2003; Xue and Greisler, 2003; Stephan et al., 2006; Niu et al., 2013). Biocompatibility of synthesized biomaterials not only depends on its noncytotoxicity; it also depends on its topography (micro, nano), the surface groups that they carry, the conformational change, and resilience against mechanical stimulation. It was shown by Fujimoto et al. that polyester urethane urea patch implantation onto subacute infarcted myocardium induced α -smooth muscle actin and cardiac-specific troponin T in early developmental cardiomyocytes (Fujimoto et al., 2012). McDevitt et al. explained that their biodegradable polyurethane films

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can serve as an appropriate scaffold material to stably support the engineering of spatially organized layers of cardiomyocytes *in vitro*. The resulting cardiomyocyte patterns on polyurethane displayed a similar morphology to those previously achieved on other substrates, such as polystyrene dishes (McDevitt et al., 2003).

It is well known that cardiomyocytes are actively involved in damaged tissue through various signals and cells interactions. Ogawa et al. highlighted that physical contact between a sympathetic neuron and ventricular myocytes increased expression of functional L-type calcium channels and regulated the function of cardiac Ca channels (Ogawa et al., 1992). Cardiac L-type calcium channels play an important role in stimulation and contraction. It is thought that beta-adrenergic receptors (β ARs) play an essential role in this increase of calcium channels (Reuter, 1983). Lockhart et al. reported that nerve growth factor (NGF) plays both acute and long-term roles in the regulation of developing sympathetic synapses in the cardiac system (Lockhart et al., 1997). Recently, it was shown that NGF stimulated the angiogenic activity and supported the cardiomyocyte viability in acute myocardial ischemic injury area (Meloni et al., 2010). Another study performed by Lam et al. indicated that the stimulative regenerative response in failing zebrafish heart of NGF was mediated by stimulation of cardiomyocyte proliferation (Lam et al., 2012). It is thought that NGF increased cardiomyocyte proliferation via inactivating the glycogen synthase kinase-3 beta (GSK-3 β) pathway (Lam et al., 2012). Tseng et al. reported that the β ARs were involved in regulation of neonatal cardiomyocyte proliferation and that this mitogenic control might be mediated via the p70 ribosomal protein S6 kinase (p70 S6K) pathway (Tseng et al., 2001). NGF-mediated β AR signaling regulates cell division in neonatal cardiomyocytes via effects on signaling kinases known to be important in cell cycle regulation.

In this study, in order to create a cardiac patch, biocompatible polyurethane nanofiber polymer was developed by means of the electrospinning method. Bone marrow mesenchymal stem cells (BM MSCs) were isolated from whole human bone marrow. Isolated BM MSC homogeneity was determined by flow cytometry using specific markers. BM MSC-PU interactions were studied with a WST-1 kit. Cardiomyocyte differentiation was performed with 5-azacytidine. Cell survival and proliferation of cardiomyocyte-like cells that were cultured on NGF-embedded PU were evaluated with the WST-1 kit on the 3rd and 7th days. NGF's effect on the cardiomyocyte-like cell proliferation was investigated using anti-p70 S6 kinase monoclonal antibody. Therefore, the role of β AR in human BM MSC-derived cardiomyocyte-like cells was investigated.

2. Materials and methods

2.1. Production and preparation of polyurethane nanofibers with electrospinning technique

The polymer solution used in the production of nanofibers consisted of mixtures of specific molecular weights of PU granules and dimethylformamide (DMF). The mixture prepared for the experiments contained 10.0%–12.0% PU by weight. A heater-magnetic stirrer was used for the preparation of solution. First, PU granules in DMF solution were heated gradually at 70 °C and at same time stirred for 2 h with the heater-magnetic stirrer. Afterwards, the solution was gradually warmed to room temperature over 1 h. Before use, the prepared polymeric solution was held in a 50-mL syringe at room temperature for 1 day. From the syringe polymer solution was placed on the dosing pump located in electrospinning machine; the cable connected to the tip of the syringe was selected as the (+) pole and coverslips connected to aluminum foil located 15 cm away from the tip were selected as the (-) pole. The production of nanofibers was realized by applying approximately 20 kV between these two poles and at a pumping rate between 1.00 and 1.50 mL/h. At the end of this process, randomly oriented PU nanofibers were accumulated on coverslip (2 cm \times 2 cm). Electrospun scaffolds were disinfected with 70% ethanol and UV treatment for stem cell experiments.

The degradation of PU nanofiber was investigated by thermogravimetric analysis (PerkinElmer Diamond TGA). The analysis was performed from 20 to 1650 °C at a heating rate of 10 °C/min under nitrogen atmosphere using ceramic crucibles. The chemical bonding of nanofiber was investigated in the range of 4000–500 cm^{-1} in the transmission mode using a Fourier transform infrared (FTIR) spectroscopy attenuated total reflectance (ATR) spectrophotometer (PerkinElmer Spectrum One). The surface of the scaffold was analyzed by scanning electron microscopy (SEM) (FEI Quanta 200F). The images were taken at magnifications of 8000 \times and 15,000 \times .

2.2. Human bone marrow mesenchymal stem cell culture

Human MSCs were obtained from whole BM cells (Lonza, Walkersville, MD, USA). The mononuclear cells were obtained using density gradient solution (Ficoll-Paque, GE Healthcare, Life Sciences, Piscataway, NJ, USA). They were cultured in alpha-minimum essential medium (Biochrom, Berlin, Germany) supplemented with 20.0% fetal bovine serum (FBS, GIBCO, Invitrogen Burlington, ON, Canada), 1.0% penicillin and streptomycin, and 1.0% L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and were incubated at 37 °C in a humidified atmosphere containing 5.0% CO₂ for 3 days. Then the culture medium was changed once every 2 to 3 days. At 80.0%–85.0% confluence, adherent MSC cells were trypsinized with TrypLE solution (GIBCO Invitrogen), and cell viability was checked by trypan blue dye exclusion. Passage 2 (P2) BM MSCs were used.

2.3. Characterization of human BM MSCs

Flow cytometric analyses of P2 BM MSCs were performed on a FACS Aria flow cytometer (BD Biosciences, Pharmingen, San Diego, CA, USA) to evaluate BM MSCs in terms of expression of main MSC surface markers CD73 (BD Biosciences), CD90 (BD Biosciences), and CD105 (BD Biosciences) and lack of expression of hematopoietic stem cell markers CD34 and CD45 (BD Biosciences). All markers were conjugated with fluorescent isothiocyanate, allophycocyanin, or phycoerythrin. BM MSCs were trypsinized and washed with PBS. To evaluate BM MSC marker profiles, 1.5×10^5 cells were suspended in 100 mL of PBS-BSA-Na azide with 2 mL of each flow cytometry antibody in a separate tube and incubated for 30 min in the dark. At the end of incubation, cells were washed twice with PBS and finally diluted in 200 mL of PBS-BSA-Na azide. The analysis of cells was performed according to 10,000 event counts with the FACS Aria. The acquired data were analyzed by using BD FACS Diva Software v 6. 1. 2 (BD Biosciences).

2.4. Cell viability and proliferation

Cell viability tests were carried out with human BM MSCs that were seeded at a density of 25,000 cells per well and incubated in 6-well tissue culture plates previously coated with PU polymers. Cells were seeded on uncoated surfaces as a negative control. Cultures were continued for 7 days. At least three replicates were studied for each condition. After 72 and 168 h of incubation, the cell metabolic activity was assessed using a water-soluble tetrazolium-based assay [10% WST-1,4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzenedisulfonate] for 2 h, and 100 mL of medium for each condition was transferred into enzyme-linked immunosorbent assay (ELISA) microplates (96-well plates, Corning Life Sciences, Lowell, MA, USA) for spectrophotometric measurement. The absorbances of the solutions were measured spectrophotometrically at 450 nm. Data were expressed as $(OD \text{ value of the sample} - OD \text{ value of the blank}) \times 100 / OD \text{ value of the sample}$ where OD = optical density.

2.5. Cardiomyogenic differentiation of human BM MSCs and NGF-embedded PU interactions

Human BM MSCs were exposed to regular plating medium (DMEM-LG medium supplemented with 10% FBS and 1% penicillin and streptomycin) supplemented with 10 μ M 5-azacytidine (Sigma) for 24 h and then the medium was switched to regular plating medium in the following days up to 28 days. The 5-azacytidine treated MSCs were examined daily using light microscopy to monitor changes in cell morphology. Protein expression of cardiac troponin T at day 28 was assessed via immunofluorescent staining. Cells were fixed with 3.7% neutral buffered formaldehyde (Sigma) for 20 min at 37 °C and then permeabilized with Triton (0.2%, Sigma) and washed with Tween

(0.05%, Sigma). 4',6-Diamidino-2-phenylindole (DAPI, AppliChem GmbH, Darmstadt, Germany) stain was used for nucleus staining. Monoclonal antihuman troponin T antibody (R&D Systems Inc., Minneapolis, MN, USA) and Alexa 488 conjugated secondary antibody were used for receptor staining.

To investigate the role of NGF on cardiomyocyte-like cell expansion, we embedded 50 ng/mL NGF on PU. Cardiomyocyte-like cells were seeded at the density of 25×10^3 cells per well and were incubated in 6-well tissue culture plates up to day 7. Cell proliferation was checked by WST-1 at days 3 and 7. Cells were seeded on nonembedded surfaces as a control. At least three replicates were studied for each condition. Samples were analyzed by SEM to assess PU and cell distribution. The samples were examined using a SEMLEO 1430 VP and images were captured using the energy-dispersive spectroscopy system.

2.6. Detection of beta-adrenergic pathway

It was reported that the β ARs were involved in regulation of neonatal cardiomyocyte-like cell proliferation and this mitogenic control might be mediated via the p70 S6K pathway (Tseng et al., 2001). To understand the role of the p70 S6K pathway on cell proliferation, flow cytometric analysis of 70-kDa S6 kinase was performed on cardiomyocyte-like cells with or without NGF (NGF (+) and NGF (-)).

2.7. Statistical analysis

The data were analyzed with SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was evaluated based on one-way ANOVA and Student's paired t-test, and $P \leq 0.05$ was considered significant. All data were described as mean \pm standard deviation.

3. Results

3.1. Characterization of polyurethane nanofiber

The thermal degradation of PU nanofiber began at 300 °C and continued up to 425 °C with weight loss of 90.0%. Weight loss of 10% was performed between 25 and 350 °C. Different magnifications of PU nanofiber scaffold surfaces fabricated by electrospinning method are shown in Figure 1. Maximum weight loss was observed at 375 °C that was associated with loss of carbon dioxide and followed by benzoic acid up to the end (Figure 1a). FTIR-ATR spectral analysis of the PU nanofiber was performed and the peaks of C - O - C and C = O stretching were found at 1200 and 1650 cm^{-1} while the amide (N - H) stretching was located at 3400 cm^{-1} (Figure 1b). The intensities of CH_2 (asymmetric vibration) and CH_3 (symmetric vibration) were normally located at 2800–3000 cm^{-1} (Park et al., 2012). In this case, only the peak of CH_2 at 2950 cm^{-1} was observed. The nanofibers were randomly oriented and they overlapped each other. The diameter of nanofibers were between 400 and 800 nm (Figure 1c).

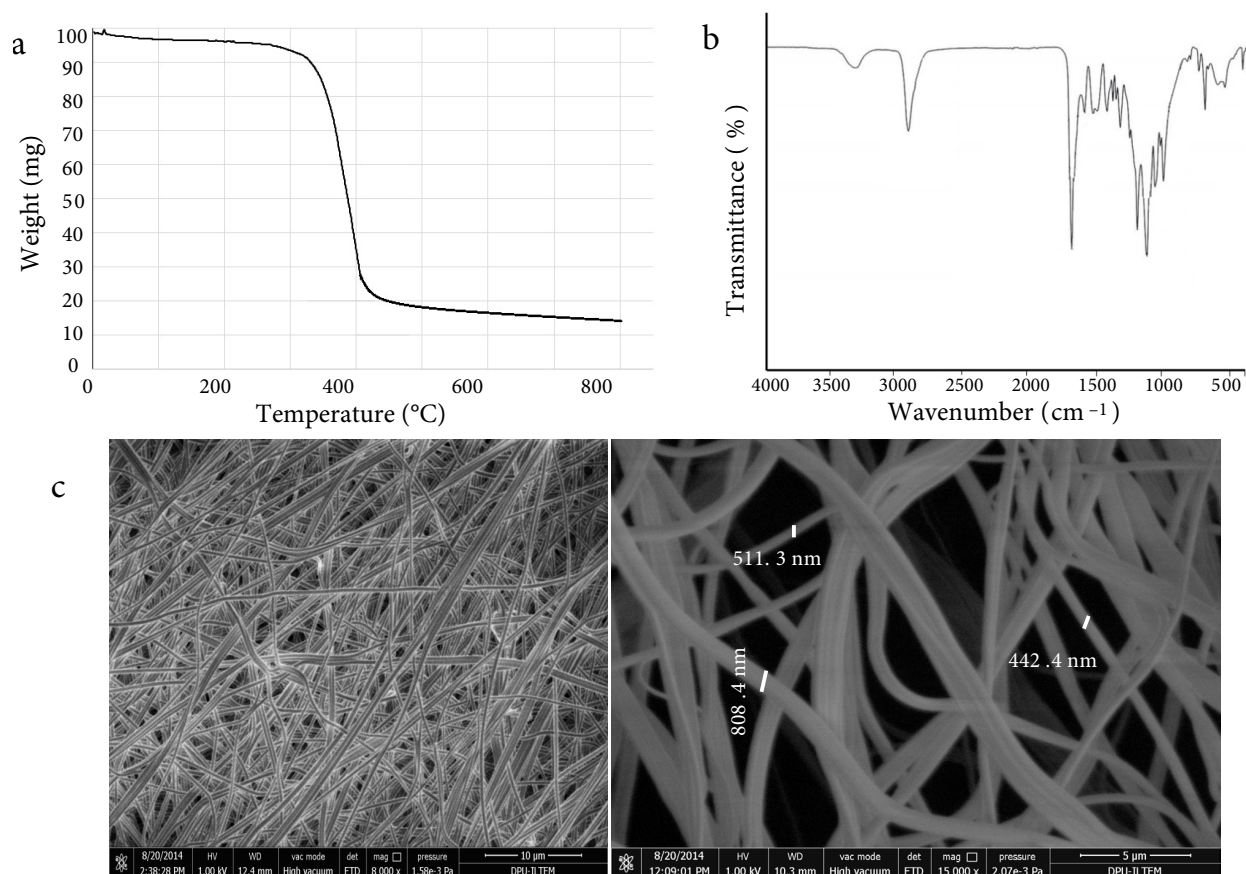


Figure 1. Characterization of polyurethane nanofibers. a) TGA behavior of PU nanofiber scaffold. b) FTIR-ATR spectrum of the electrospun polyurethane nanofiber. c) Scanning electron microscopic images of PU nanofiber scaffold with magnification of 8000 \times and magnification of 15,000 \times .

3.2. Characterization of human BM MSCs

Undifferentiated human BM MSCs maintained spindle-shaped morphology (Figure 2a). Cytometry analysis confirmed that the BM MSCs (passages 2–3) expressed the mesenchymal markers CD90, CD105, and CD73 ($\geq 98.0\%$), but not the hematopoietic markers CD34 and CD45 ($\leq 1.0\%$) (Figure 2b).

3.3. Human BM MSC proliferation on nanofibrous scaffolds

To assess the response of the BM MSCs toward the PU nanofibers, cells were seeded on coated surfaces and their morphologies were checked at different time points (third and seventh days of culture). Uncoated coverslips were used as the control. Representative images of cultures at different times after seeding are shown in Figure 3. The typical spindle-shaped morphology and the gradual formation of a monolayer without substantial difference in comparison to the uncoated control surface was evident (Figure 3a). A significant difference on cell proliferation was observed in BM MSC cultures on PU nanofibers versus cultures on noncoated surfaces ($70.6 \pm 1.0\%$ vs. 60.6

$\pm 2.0\%$, $P = 0.02$). The proliferation of the BM MSCs after 7 days of culture averaged 80.0% for all tested conditions (Figure 3b). Overall, our data indicate that PU nanofibers provided a suitable environment for BM MSCs, and no evident cytotoxicity was observed.

3.4. In vitro interaction of nanofibers with cardiomyocyte-like cells

Cardiomyogenic differentiation was performed using 5-azacytidine treatment for 24 h. Cell morphology did not change during the first week with similarity to control cultures. However, cardiomyocyte-like morphology could be observed between days 14 and 21, and antiscardiac troponin T staining at day 28 confirmed the cardiomyogenic differentiation of BM MSCs (Figure 4a). To establish whether NGF-embedded PU scaffolds could support cardiac cell culture, cell proliferation assay was carried out.

Representative images of cultures at different times after seeding are shown in Figure 4b. Our WST results for cell proliferation of the prepared nanofibers (NGF (-) and NGF (+)) on cardiomyocyte-like cells showed that the

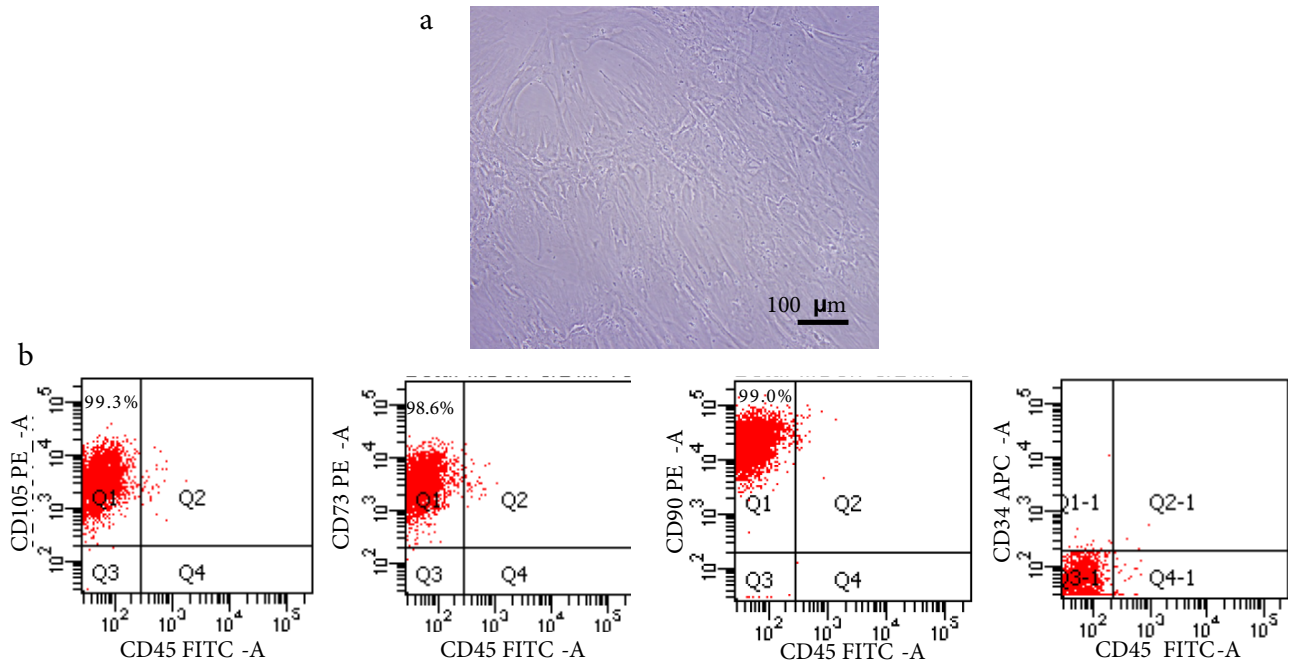


Figure 2. Morphology and characterization of human bone marrow mesenchymal stem cells. a) Phase-contrast microphotograph showing human bone marrow mesenchymal stem cells, scale bar = 100 μm. b) Representative FACS analysis of human bone marrow mesenchymal stem cells. Cells highly expressed CD105, CD90, and CD73 (>98.0%) and lacked CD45 (<=1.0%) markers.

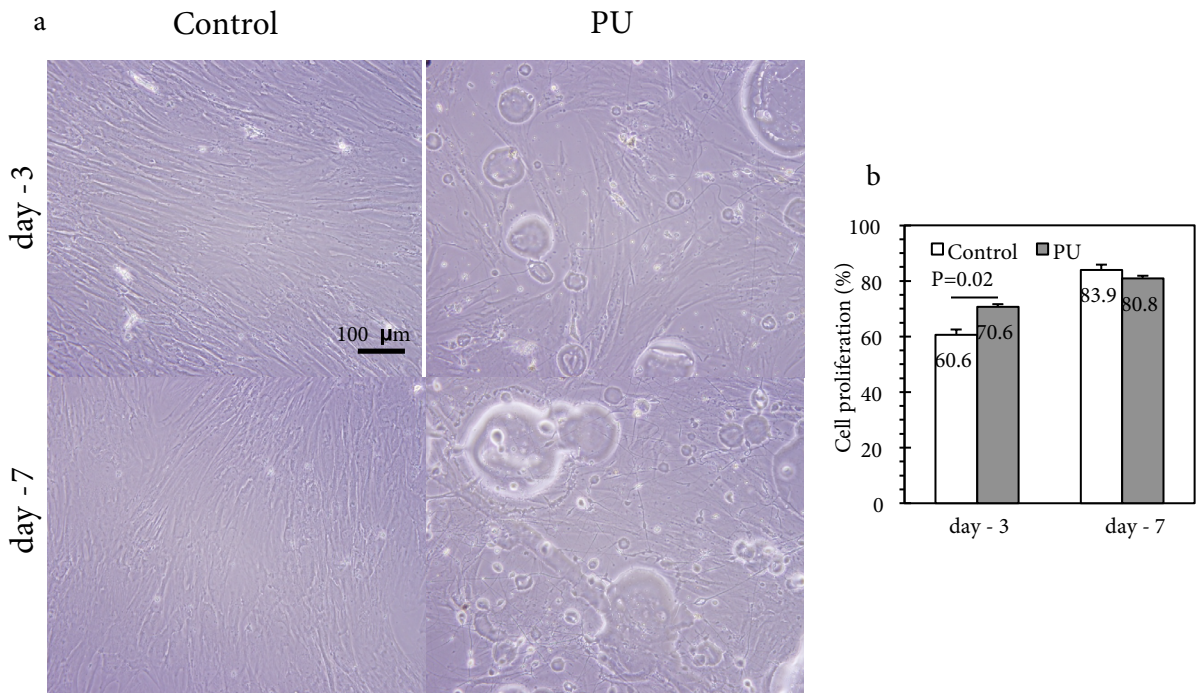


Figure 3. Cell morphology and proliferation. a) Phase-contrast microphotographs showing human bone marrow mesenchymal stem cells on uncoated and PU nanofibers at days 3 and 7, scale bar = 100 μm. b) Cell proliferation at days 3 and 7.

NGF-embedded nanofibers had no significant effects on cell proliferation of these cells at day 3 (NGF (-) $31.8 \pm 6.7\%$ vs. NGF (+) $37.3 \pm 3.2\%$). However, 7 days after seeding

NGF-embedded nanofibers proved to be more competent in cell proliferation compared with NGF (-) nanofibers (NGF (-) $47.8 \pm 1.2\%$ vs. NGF (+) $69.1 \pm 1.7\%$, $P < 0.001$;

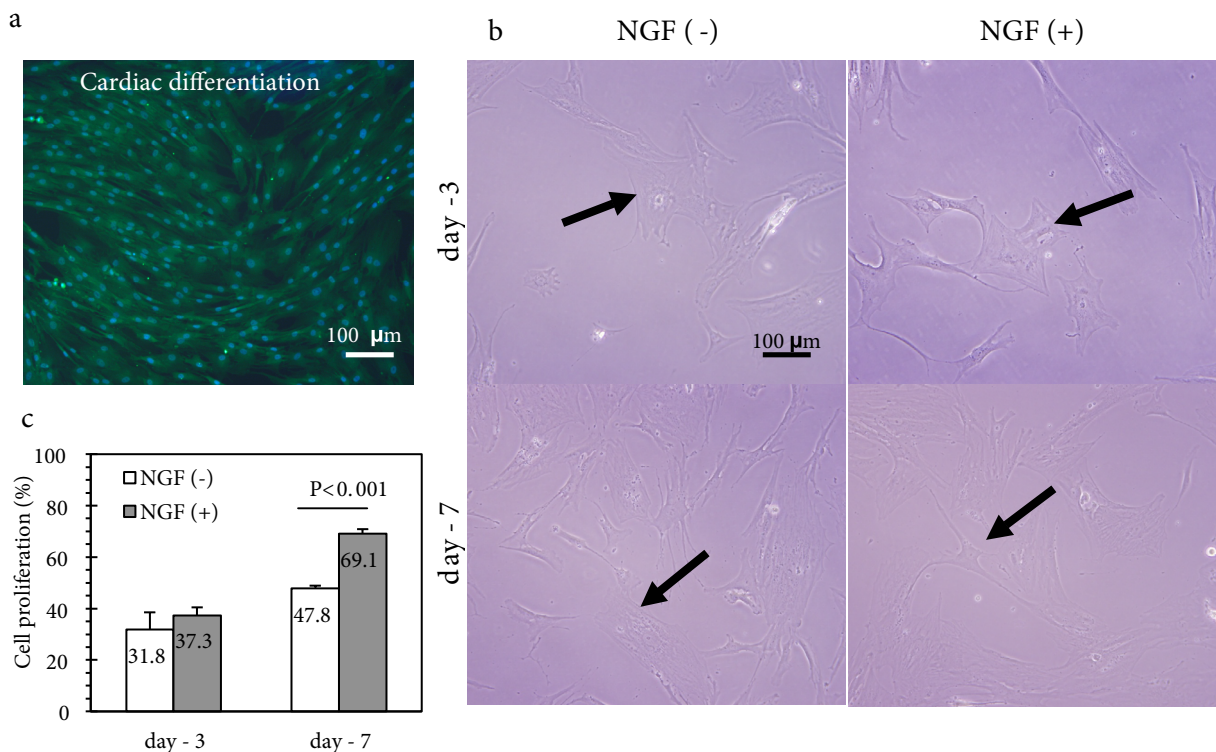


Figure 4. Cardiac differentiation and proliferation on neural growth factor (NGF)-embedded polyurethane. a) Immunofluorescent staining of cardiomyocyte-like cells derived from human bone marrow stem cells with anti-troponin T-Alexa 488 (green) and DAPI (nucleus, blue) at day 28, scale bar = 100 μm . b) Phase-contrast microphotographs showing cardiomyocyte-like cells on NGF embedded or not PU nanofibers at days 3 and 7, scale bar = 100 μm . c) Proliferation of human cardiomyocyte-like cells in contact with PU scaffolds for 3 and 7 days.

Figure 4c). In order to visualize cardiomyocyte-like cell distribution on the surface of the nanofibers, samples were analyzed using SEM and the results are presented in Figures 5a–5c. It was observed that the cardiomyocyte-like cells were apparently attached to samples and associated with fibers.

3.5. p70 S6 kinase activity of cardiomyocyte-like cells

Cardiomyocyte-like cells were found to adhere on the scaffolds, showing a spread geometry and retaining viability. We investigated whether proliferation of cardiomyocyte-like cells with NGF was mediated by p70 S6 kinase activation. Increases in p70 S6 kinase activation were monitored by flow cytometry analysis. This activity is shown in Figure 6 with cardiomyocyte-like cells on NGF-embedded scaffolds compared to cardiomyocyte-like cells on NGF (-) nanofiber (NGF (-) 0.0% vs. NGF (+) 1.8%).

4. Discussion

Tissue engineering strategies focus on the use of prefabricated three-dimensional porous scaffolds or dense patches of synthetic and/or natural polymers to support the diseased region of the heart and help in the transfer of exogenous cells into it (Baheiraei et al., 2014). A tissue-

engineered “cardiac patch” should predominantly be stable but flexible and mechanically strong to support the cardiac tissue. PU-based materials have also been studied for cardiovascular tissue engineering applications due to their elastic mechanical properties, biodegradability, processability, and biocompatibility (Alperin et al., 2005; Silvestri et al., 2011). In this study, we set out first to compare the potential of PU-coated and noncoated surfaces to support the growth of BM MSCs. The adhesion and proliferation assay of BM MSCs showed that the proliferation efficiency of MSCs was maintained at 70.0% on PU electrospun nanofibers measured at day 3. Additionally, WST-1 assay showed similar amounts of viable cells in all the evaluated cultures at day 7 (~80.0%). The current results indicated that MSCs could be seeded without the help of plasma treatment or extracellular matrix coating and could proliferate well on the PU electrospun nanofibers. These results fit well with previous *in vitro* cytocompatibility tests carried out on PU fibers (Kuo et al., 2014).

It was previously reported that MSCs can differentiate into cardiomyocyte-like cells after 5-azacytidine treatment (Antonitsis et al., 2007; Zhang et al., 2009; Li et al., 2013;

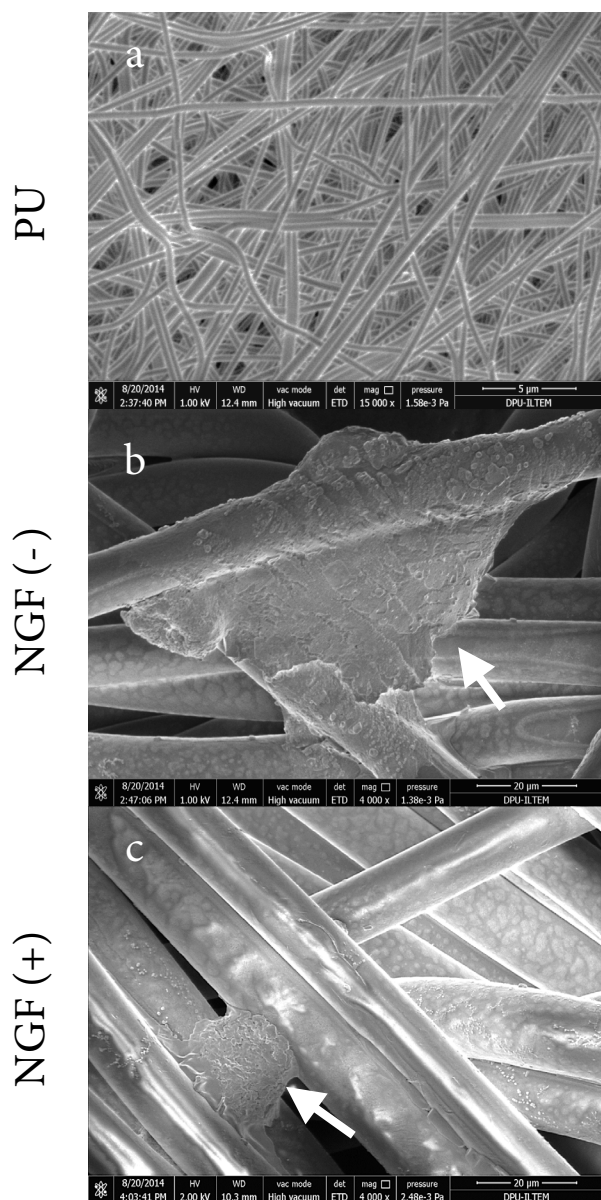


Figure 5. SEM micrographs of polyurethane (PU). a) The morphology of PU electrospun nanofibers and cardiomyocyte-like cells on b) NGF (-) and c) NGF (+) PU.

Supokawej et al., 2013; Wu et al., 2013; Piryaei et al., 2015). Nartprayut et al. showed the ability of perinatally derived MSC differentiation into cardiomyocyte-like cells (after 5-azacytidine treatment) that expressed the cardiac-specific genes *Nkx2.5*, α -cardiac actin, and cardiac troponin T (Nartprayut et al., 2013). BM MSCs are multipotent progenitor cells that can differentiate into cardiac muscle (Orlic et al., 2001). When an acute myocardial infarction occurs, these cells can sense it and move to the infarcted myocardial tissues, differentiating into new cardiomyocytes. However, the newly formed

cardiomyocytes are too few in number to effectively repair the infarcted tissues and this adds importance to the study of cardiomyocyte development through inducement and differentiation in vitro (Cao et al., 2004). In our study, we set out secondly to compare the potential of PU to support the growth of cardiomyocyte-like cells. For this, human BM MSCs were treated with 5-azacytidine to investigate their differentiation into cardiomyocyte-like cells. Characteristics of the myogenic cells were determined by immunofluorescent staining with cardiac troponin T.

It is well known that the heart is highly innervated by autonomic nervous systems, including sympathetic, parasympathetic, and sensory nerves, derived from neural crest cells. In contrast to cardiac fibroblasts, postnatal cardiomyocytes have little or no regenerative capacity. Loss of terminally differentiated cardiomyocytes as a result of heart disease is irreversible; consequently, new therapeutic approaches are demanded (Ieda, 2013). Lockhart et al. reported that NGF plays both acute and long-term roles in the regulation of developing sympathetic synapses in the cardiac system (Lockhart et al., 1997). It was previously shown that NGF augments cardiomyocyte proliferation and may play an important role in cardiac regeneration (Lam et al., 2012). Quirici et al. explained that low-affinity nerve growth factor receptor (L-NGFR or CD271) defines a subset of cells with high proliferative, clonogenic, and multipotential differentiation ability in adult bone marrow and adipose tissue (Quirici et al., 2002, 2010). We then investigated whether NGF-embedded nanofibers could improve cell adhesion and proliferation. In our study, we note that cardiomyocyte-like cells that were cultured on NGF-embedded PU nanofibers exhibited higher proliferation rates compared to cells that were cultured on non-NGF-embedded PU nanofibers at each time point (days 3 and 7). It was reported by Tseng et al. that β ARs are involved in the regulation of neonatal cardiomyocyte proliferation and that this mitogenic control may be mediated via the p70 S6K pathway (Tseng et al., 2001). In this regard, we investigated the role of β ARs in cardiomyocyte-like cell proliferation. Flow cytometry analysis demonstrated that there was an increase of the p70 S6K pathway via NGF in cardiomyocyte-like cells that might play a critical role in cardiomyocyte-like cell proliferation.

In conclusion, our data indicate that PU nanofibers provided a suitable environment for human BM MSCs, and no evident cytotoxicity was observed. Seven days after seeding, NGF-embedded nanofibers proved to be more competent in cell proliferation compared with nonembedded nanofibers. Cardiomyocyte-like cells were found to adhere on the scaffolds, showing a spreading geometry and retaining viability. Increases in p70 S6 kinase activity through NGF were monitored by flow

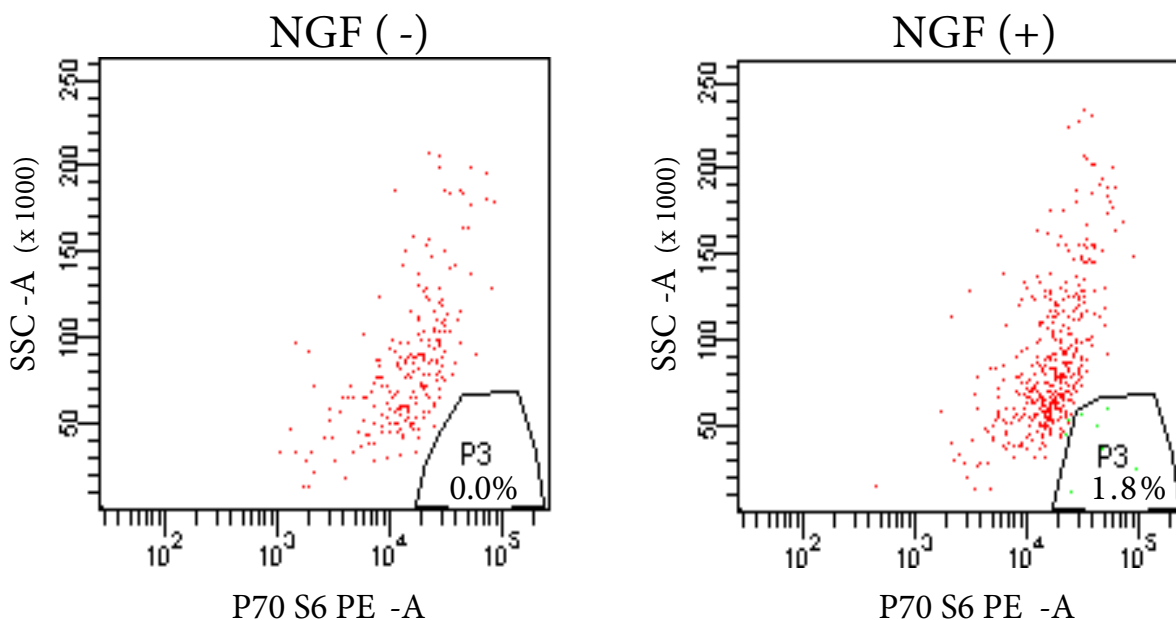


Figure 6. Flow cytometry analysis of p70 S6.

cytometry. Our studies suggest that β ARs are involved in the regulation of MSC-derived cardiomyocyte-like cell proliferation and that this mitogenic control mediated via the p70 S6K pathway.

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Acknowledgment

The authors wish to thank the Research Science Commission of Hacettepe University (Project Number: 013D11102001) for financial support.

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