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The Role of Biodegradable Engineered Nanofiber Scaffolds Seeded with Hair Follicle Stem Cells for Tissue Engineering

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ABSTRACT

Background: The aim of this study was to fabricate the poly caprolactone (PCL) aligned nanofiber scaffold and to evaluate the survival, adhesion, proliferation, and differentiation of rat hair follicle stem cells (HFSC) in the graft material using electrospun PCL nanofiber scaffold for tissue engineering applications. **Methods:** The bulge region of rat whisker was isolated and cultured in DMEM: nutrient mixture F-12 supplemented with epidermal growth factor. The morphological and biological features of cultured bulge cells were observed by light microscopy using immunocytochemistry methods. Electrospinning was used for production of PCL nanofiber scaffolds. Scanning electron microscopy (SEM), 3-(4, 5-di-methylthiazol- 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, and histology analysis were used to investigate the cell morphology, viability, attachment and infiltration of the HFSC on the PCL nanofiber scaffolds. **Results:** The results of the MTT assay showed cell viability and cell proliferation of the HFSC on PCL nanofiber scaffolds. SEM microscopy images indicated that HFSC are attached, proliferated and spread on PCL nanofiber scaffolds. Also, immunocytochemical analysis showed cell infiltration and cell differentiation on the scaffolds. **Conclusion:** The results of this study reveal that PCL nanofiber scaffolds are suitable for cell culture, proliferation, differentiation and attachment. Furthermore, HFSC are attached and proliferated on PCL nanofiber scaffolds. *Iran. Biomed. J. 16 (4): 193-201, 2012*

Keywords: Nanofiber, Electrospinning, Stem cells, Tissue engineering

INTRODUCTION

Tissue engineering is a new approach to reconstruction and/or regeneration of lost or damaged tissue [1]. There are three key parameters in tissue engineering: the cells, the scaffold type and suitable condition for growing cells in three-dimensional scaffolds. The scaffolds play an important role in tissue engineering by serving as a matrix for cellular ingrowths, proliferation and new tissue formation in three dimensions [2].

The nanofiber scaffolds may serve as suitable environment for cell attachment, proliferation and function [2]. Such scaffolds hold the promise to provide the topographic cues to the seeded cells and may potentially enhance tissue regeneration [3]. To design an ideal scaffold, various factors should be considered, such as pore size and morphology, mechanical properties versus porosity and surface properties and appropriate biodegradability [4].

Currently, there are three basic techniques capable of generating nano-fibrous scaffolding: electrospinning, molecular self-assembly and thermally induced phase separation [5] with an attractive feature of having simple and inexpensive setup [6]. Besides, electrospinning has several advantages, including producing continuous fibers, applicability to various polymers and easy adjustability. The thickness of mat

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can also to be transformed by changing the collection time during the electrospinning. The dimensions and

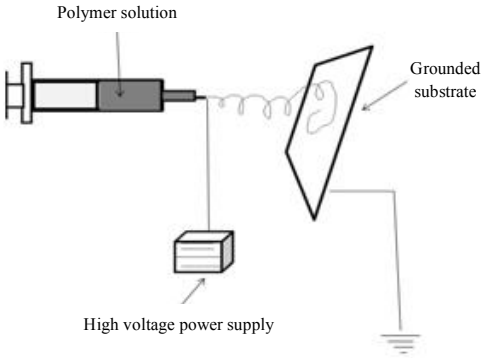

Fig. 1. Schematic illustration of the electrospinning process.

surface morphologies of the electrospun fibers can be adjusted by altering the solution properties as well as by processing parameters. In electrospinning process, a strong electrostatic field is applied to a polymer solution. When the voltage surpasses a threshold value, the electric forces overcome the surface tension of solution and a charged jet of solution is ejected toward a collecting material screen [7]. Caprolactone (PCL) is a biodegradable-biocompatible polymer that has been successfully electrospun. Furthermore, it has been shown that it is capable of supporting a wide variety of cell types [8]. Tissue-specific adult stem cells have long attracted attention, especially since they can be isolated from one individual, expanded and eventually differentiated *in vitro* and transplanted back into the same individual [9]. Accessibility of donor tissue is of considerable importance in such case. Compared to embryonic stem cells, there is no need to use immunosuppressive medication, since they are the patient's own cells and consequently, there will be no concerns for graft rejection and ethical nature. Moreover, compared to induced pluripotent stem cells or nuclear transfer, there is no need for genetic or mechanical manipulation [10]. Hair follicle stem cells (HFSC), located in the hair follicle bulge, possess stem cell characteristics, including multipotency, high proliferative potential, and ability to enter quiescence [11]. The bulge region of the hair follicle has different stem cells for hair and skin such as nestin-expressing cells [12, 13]. In recent years, many researchers have devoted to establishing cultures of hair follicle cells of the mouse, rat and human [14]. The goal of this study was to investigate the suitability of electrospun PCL nanofiber scaffold for the HFSC culture, viability, proliferation, and differentiation and using these nanofibers and cells for potential use in peripheral nerve regeneration applications.

MATERIALS AND METHODS

Fabrication of caprolactone nanofiber scaffold. Polycaprolactone with average molecular weight of 80,000 Da was purchased from Sigma-Aldrich (St. Louis, USA). The solvents *N*, *N*-dimethylformamide, and chloroform were obtained from Merck (Darmstadt, Germany). The polymer solution with concentration of 8 wt% was prepared by dissolving PCL in a mixture of *N*, *N*-dimethylformamide/chloroform solvents with the ratio of 1/9. The solution was loaded in a 5-ml syringe capped with a 21-G needle. Upon applying a high voltage (25 kv), a fluid jet was ejected from the tip of the needle. As the jet was accelerated toward a target, which was placed 20 cm from the syringe tip, the solvent evaporated and polymer nanofibers were collected on an aluminum foil. The polymer solutions were delivered via a syringe pump to control the mass flow rate. The mass flow rate of the solutions was 0.5 ml/h. All electrospinnings were carried out at room temperature. Figure 1 shows schematic illustration of electrospinning set-up.

Nanofiber morphology studies. The morphology of electrospun PCL aligned nanofibers was studied by scanning electron microscopy (SEM, HITACH S4160, Japan). Before the observation, the scaffolds were coated with gold using a sputter coater (Technics, Hummer II, Japan) and imaged at 15 kv. The diameter of the fibers was measured using Microstructure Measurement Software (Fig. 2).

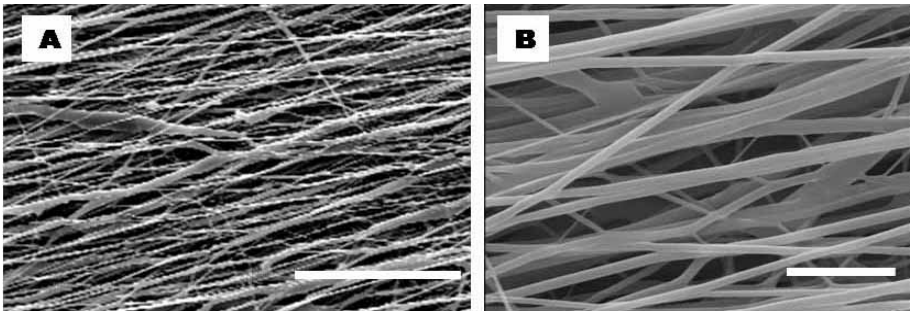

Fig. 2. Scanning electron microscopy micrographs of caprolactone nanofibers. Aligned fabricate the poly caprolactone electrospun

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fibers **(A)** scale bar = 60 and **(B)** scale bar = 6 µm.

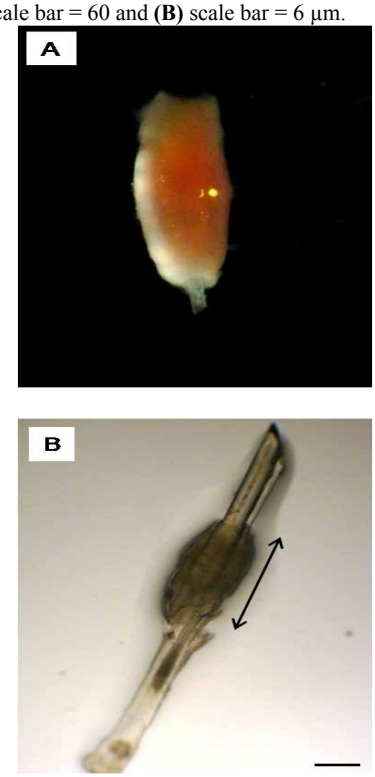

Fig. 3. Dissection of the bulge from adult rat whisker follicle. Photographs of adult rat whisker follicle surrounded by connective tissue **(A)** and capsule **(B)**. Arrow shows bulge region (B). Scale bar = 50 µm.

Preparation of nanofiber scaffolds for cell culture. The nanofiber scaffolds were sterilized by an immersion in a 70% ethanol solution for a period of 60 min and then exposed to UV radiation for 60 min. Thereafter, the scaffolds were soaked in a culture medium overnight prior to cell seeding in order to facilitate protein adsorption and cell attachment on the nanofiber surface.

Hair follicle isolation and cultivation. Adult male Albino Wistar rats (n = 30, weighing 250-300 g, 8-10 weeks old, The Animal Center of Tehran Medical University, Tehran, Iran) were used. The rats were sacrificed with ether and the whisker follicles were dissected as described by Sieber and Grim [15]. The tissues were trimmed into small pieces (4 × 8 mm^2) and the samples were incubated in 2 mg/ml collagenase I/dispase II solution (Sigma-Aldrich, USA). Most of the connective tissues and dermis around the follicles was removed and the whisker follicles lifted out (Fig. 3). The bulge region was then amputated from the upper follicle by making two transversal cuts at the site of enlargement spots of outer root sheath with a fine needle. The culture procedure was performed as previously described by Yang and colleagues [16] with a slight modification. Briefly, 20 isolated bulges were cut into small pieces, plated into flask culture plates, percolated with collagen type I (Sigma-Aldrich, USA) and immersed in a 3:1 DMEM: nutrient mixture F-12 supplemented with epidermal growth factor containing 10% fetal bovine serum. All dissection and cultivation procedures were performed under sterile conditions and incubation was at 37°C (5% CO_2). Within approximately 4 days, initiation of outgrowth of bulge cells from the bulges was observed (Fig. 4). One week after onset of this outgrowth, the bulges were removed from the culture plates and the cells were collected by incubation with a mixture (1:1) of 0.125% trypsin (Sigma-Aldrich, USA) and 0.02% EDTA (Sigma-Aldrich, USA) at 37°C for 2 minutes. The dispersed cells were centrifuged at 259 ×g for 10 minute, placed in other collagen coated plates and incubated for another one week with a medium change every 3-4 days. After reaching sufficient confluency, the cells were detached by trypsin and counted by a neobar lam. Then, the cells were transferred to a 24-well tissue culture plate (TCP) as control with a density of 1 × 10^5 cell per well.

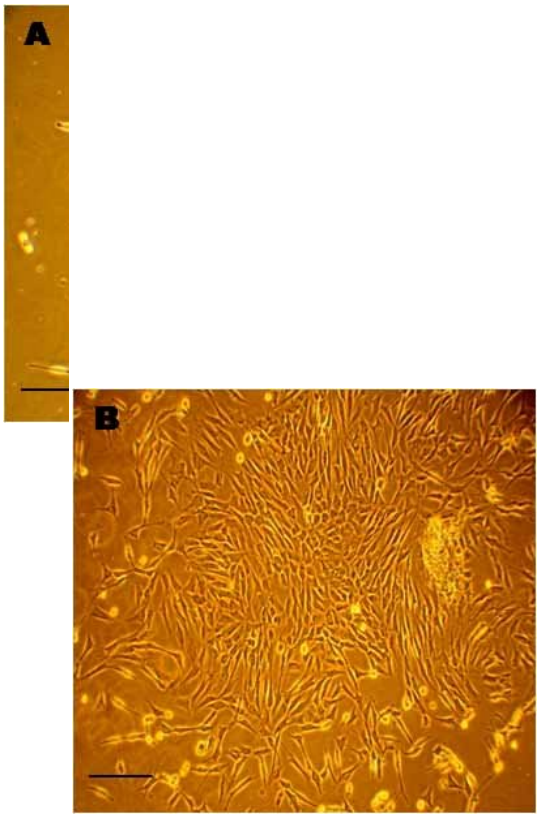

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Fig. 4. The primary culture of bulge cells from rat hair follicles. The growth of bulge cells after 4 **(A)** and 8 **(B)** days. Scale bars = 50 µm (A and B).

Scanning electron microscopy. The morphology and adhesion of HFSC on the PCL nanofiber scaffolds was observed by SEM (HITACH S4160, Japan). Cells (4×10^4) per 100 µl of culture medium were cultured on a sample (5×5 mm^2) and incubated at 37ºC (5% CO_2). After 1 hour, the culture medium was added to cover the sample surface. After 1 day of cell seeding, samples were fixed in 4% paraformaldehyde at room temperature for 30 min and then rinsed in 0.1 m PBS and dehydrated in increasing concentrations of ethanol (25, 50, 75, 95 and 100%). Dehydrated specimens were immersed in hexamethyldisilazane (Fluka Chemical, Sigma, USA). After drying, the samples were mounted on aluminum stubs and coated with gold using sputter coating for the observation of cell morphology [16, 17].

3- (4,5-di-methylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay. Cell viability and cell proliferation on PCL nanofiber scaffolds and TCP were measured by MTT solution (5 mg/ml). The cells were placed in a 24-well plate with a density of 2×10^4 cell per ml and cultured with a medium as described. After 1 and 3 days of cell seeding in 24-well dish, the original medium was removed and 1 cc fresh medium and 100 µl MTT solution were added to each well. Cells were allowed to incubate in the dark at 37°C (5% CO_2) for 4 h. Then, the medium was removed, the scaffolds were gently aspirated, and the formazan reaction products were dissolved in dimethyl sulfoxide and the plates were shaken for 20 min. The solution was transferred to a 96-well plate for spectrophotometric analysis. The optical density of the formazan solution was read on an ELISA plate reader (Dynex MRX) at 570 nm.

Cell phenotype study by immunocytochemical staining. The cells seeded on collagen-coated coverslips and nanofiber scaffolds (1×10^5 cell per ml) were washed 3 times with PBS for 5 minutes and fixed in 4% paraformaldehyde for 10 minutes. The fixed cells were then washed with PBS for 3×5 minutes and incubated in a blocking buffer (10% goat serum [Invitrogen, USA]/0.3% Triton X-100 [Fluka, USA]) at room temperature for 30 minutes. They were then incubated at 4°C overnight with the following primary antibodies: mouse monoclonal βIII-tubulin (1:200, Sigma-Aldrich, USA) and mouse anti-nestin monoclonal antibody (1:200, Millipore, USA). The next day, the cells were rinsed for 3×5 minutes to remove unbound primary antibodies. Subsequently, they were incubated at room temperature for 2 hours with the following conjugate antibody: goat: anti-mouse IgG (1:1400, Abcam, UK). Cell nuclei were counterstained with 1 µg/ml 4,6-diamidino-2-phenyl-indole (Sigma-Aldrich, USA) in PBS in the dark at room temperature for 1 min. After washing, the samples were mounted on a slide with mounting media for visualization using a fluorescence microscope. To examine the specificity of the nestin antibody, 3T3 fibroblast-like cells (Pasteur Institute of Iran, Tehran) were used as negative control and PC12 cells (Pasteur Institute, Tehran) were used as positive controls for βIII-tubulin. Labeled cells were identified using fluorescent microscopy (Olympus Ax70).

Statistical analysis. All data were expressed as mean ± standard deviation. An ANOVA, followed by the Tukey and Student's *t*-test were conducted with SPSS version 16.0 software (SPSS, Chicago, IL, USA). $P<0.05$ was considered as statistically significant.

RESULTS

Morphology of electrospun nanofiber. SEM micrograph of electrospun PCL aligned nanofibers have been shown in Figure 2. Average fiber diameter was estimated to be 349.61 nm with diameter ranging from 100-1,000 nm (Fig. 5).

Cell morphology and distribution in scaffolds. After 1-day incubation of HFSC scaffolds with 4, 6-diamidino-2-phenylindole staining (Fig. 6E) and SEM examination showed that the seeded cells were adhered to scaffolds and covered the pores. This issue indicated that PCL scaffolds possessed biocompatibility for attachment of HFSC *ex vivo*. The SEM image also indicated that the cell body had an apparent bipolar elongated morphology with the outgrowing neuritis and revealed cell attachment on the nanofiber scaffold (Fig. 6A and 6B). The cells were proliferated on the nanofibrous network and covered the scaffold (Fig. 6A and 6B). The direction of HFSC elongation was exactly parallel to that of fiber (Fig. 6C and 6D).

Cell viability and cell proliferation assessment. MTT chromometry assay was used to determine cell viability and cell proliferation to compare the number of cells in the nanofiber scaffold and TCP. Our data showed the MTT reduction results of the HFSC on PCL nanofiber scaffolds (Fig. 7). The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazen crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazen crystals formed is proportional to the number of viable cells. Statistical analysis of data showed that the difference between

viable cells significant.

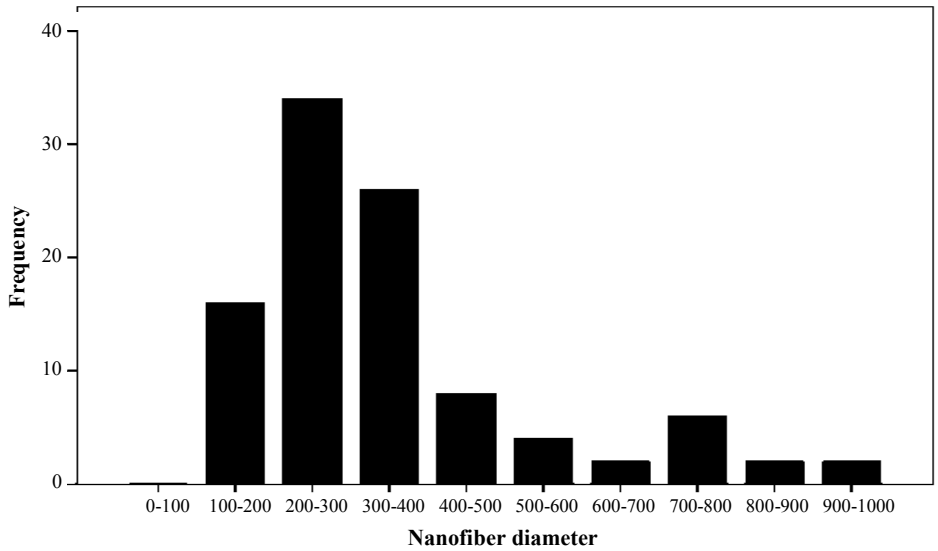

Fig. 5. Fiber diameter distribution was measured for 100 randomly selected fiber in scaffold ($P<0.05$).

Fig. 5. Fiber diameter distribution(P<0.05)

Cell phenotype assessment. Immunocytochemical staining showed expression of nestin and neural markers in bulge cells. Our results showed that the nestin antibody stained the bulge cells was seeded on coverslips and nanofiber scaffolds (Fig. 8A and 8B). Eleven days after differentiation, some cells showed neuronal morphology characteristics. Immuno-cytochemical staining showed that these cells expressed βIII-tubulin as a neuronal marker (Fig. 8C and 8D).

DISCUSSION

The use of synthetic polymers for cellular attachment and neurite extension has been focused recently. Nanofibrous scaffolds have many advantages that make them well-suited for tissue engineering applications. Besides, mimicking the architecture of natural extracellular matrix, higher surface area to volume ratio of nanofibers leads to more cellular attachment in comparison to larger fibers. In the

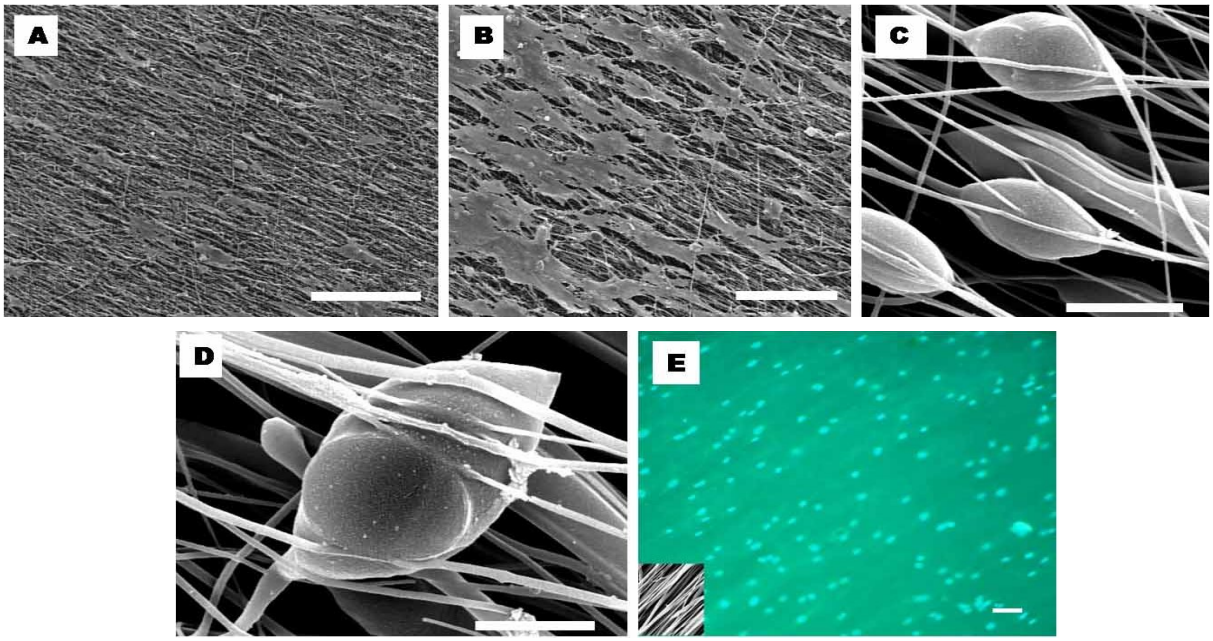

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Fig. 6. The scanning electron microscopy micrographs of hair follicle stem cells seeded on PCL nanofiber scaffolds after 1-day culture.(**A**, **B**, **C** and **D**). The interaction between cells and electrospun PCL aligned nanofibers.**(E)** 4, 6-diamidino-2-phenylindole staining image . Scale bars represent 300 (A), 150 (B), 6 (C), 3.75 (D) and 20 μm (E).

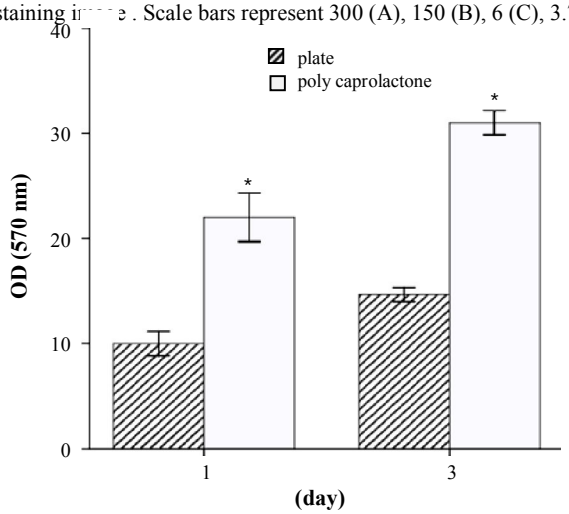

Fig. 7. 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) results of hair follicle stem cell on the nanofiber scaffolds and tissue culture plate ($^{*}P<0.05$).

present study, a nanofibrous matrix of PCL was produced via electrospinning. Recently, electro-spinning has attracted great interest due to its simplicity and effectiveness in producing nanofibers.

TCP due to nanoscale dimension and 3-dimensional structure of nanofibrous scaffolds. MTT assay of cell viability has been currently the main *in vitro* method to test the biocompatibility and cytotoxicity of biomaterials [18, 19]. Here, the nanofiber scaffolds were cultured with HFSC to investigate whether nanofiber scaffolds have cytotoxicity to tissues. The MTT assay results showed that nanofiber scaffold had no cytotoxicity to HFSC and did not cause inhibition of proliferation or differentiation of HFSC (Fig. 5). The results of MTT assay also showed that the difference between proliferation of cells in TCP and nanofibrous scaffold is statistically significant.

Likewise, during the 1-3 days of culture period, the results of MTT assay showed that the difference between proliferation of cells in nanofibrous scaffold is statistically higher (one-way t-test, $P<0.05$). Mignone *et al.* [20] demonstrated that nestin-expressing cells in the bulge region of the human hair follicle have stem cell-like properties, are multipotent, and can effectively generate cells of neural lineage *in vitro* and *in vivo*. Neural stem cells are marked by the expression of an intermediate filament of nestin. The expression of the unique protein, nestin, in both neural stem cells and HFSC suggests their possible relation [21]. These cells differentiated *in vitro*, lost their primitive biological characteristics and obtained new properties. These morphological and biological results support the idea that bulge cells have the ability to differentiate and express β-III tubulin, a neural marker [20-22].

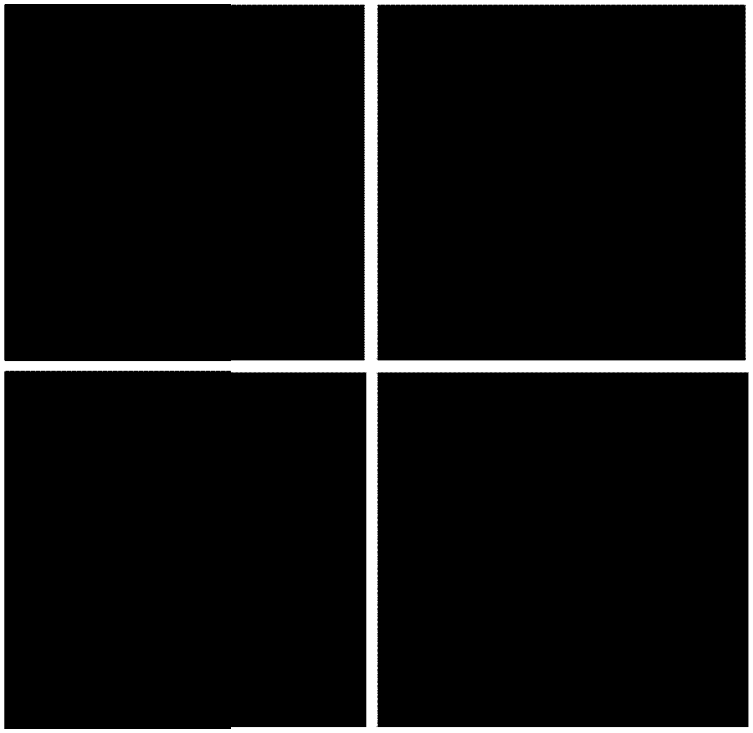

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Fig. 8. Immunocytochemical staining showed expression of Nestin and βIII-Tubulin in bulge cells: Immunostaining with Nestin antibody on the cells seeded on the coverslips and nanofiber scaffolds (**A** and **B**) Immunostaining with βIII-Tubulin antibody on the cells seeded on the coverslips and Nanofiber scaffolds after 11days (**C** and **D**). Scale bars represent 50 µm (A, C and D), 10 µm (B).

Recently, several studies have focused on the factors that influence proliferation and differentiation of neural stem cells. These factors, including diffusible factors from the medium [23] and the complicated interactions among cells [24] may enhance or inhibit proliferation and differentiation of neural stem cells. For example, NT-3 can stimulate neural stem cells to differentiate into neurons and oligodendrocytes [25]. It has also been shown that some substrates guide neural cell bodies or processes to extend toward the targets [18, 26]. Similarly, chitosan membrane has been shown to enhance differentiation of neural stem cells [17, 18].

Research interest in developing an electrospun-based conduit for neural repair has been gradually increased. The majority of these studies have focused on evaluating neural proliferation and differentiation. Consequently, various synthetic and natural polymers have been investigated for fabricating scaffolds for neural applications. The polyesters, poly glycolic acid; and poly ε-PCL, are the most commonly used synthetic. They are biodegradable and biocompatible polymers for neural repair. Studies have evaluated these compositions as electrospun mats having aligned and/or random fiber arrangements. Mouse embryonic stem cell differentiation into neurons, astrocytes, and oligodendrocytes was enhanced when cultured on aligned and random PCL fibers [27]. U373 (a human glioblastoma-astrocytoma epithelial-like cell line) cells and human neural progenitor-astrocyte committed cells had similar astrocyte process alignment and extension on PCL and PCL/collagen aligned nanofibers. The U373 cells demonstrated higher proliferation than the human neural progenitor-astrocyte committed cells on both scaffolds [28]. For this reason, we also used PCL nanofibers for the culture and differentiation of bulge cells. The results of immunocytochemical staining showed that the differentiation of cells in PCL nanofibers is observable. Li [29] demonstrated that SEM observations and immunohistochemical detection of lineage-specific marker molecules developed the formation of three-dimensional constructs containing cells differentiated into the specified cell types. As a result, the PCL-based nanofibrous scaffold is a promising candidate scaffold for cell-based and tissue engineering.

Our aim was to obtain the experimental basis that would provide a more efficient and economical method for differentiation of neural stem cells into neurons, which would then be transplanted to repair the injured nervous system. Plasma treatment generally is performed on the nanofibrous scaffold to create a more hydrophilic surface. Previous literature suggests that cellular adhesion improves with hydrophilicity [30], with regards to the improvement of cellular behavior on the hydrophilic surface. It is surprising that the cell proliferation on the TCP is comparable to nanofibrous scaffolds. The results of SEM images of HFSC on the nanofiber scaffold and MTT assay showed that the cells tend to spread on the nanofiber scaffold and nanofiber scaffold supports cell adhesion and proliferation as well as TCP.

The results of other researches show that the cells cannot penetrate into the nanofiber scaffold due to small size of pores of nanofiber scaffold [31, 32]. To overcome this problem, methods such as cell electrospinning and fiber leaching have been proposed [33, 34]. Therefore, it is concluded that for those applications where cells must remain on the surface of nanofibers mat, the prepared PCL nanofiber mat is a suitable candidate. For example, in tissue engineering vascular grafts, thrombus formation is a major problem. One of the effective approaches to prevent thrombus includes endothelial cell seeding on synthetic materials to render the surface anti-thrombogenic, because endothelial cells can release factors to control thrombogenesis or fibrinolysis [33].

The increase in the expression of P0, a myelin-specific gene, was only observed on the aligned PCL scaffolds, suggesting that promotion of schwan cell maturation is more favored on aligned fibrous scaffolds compared to other compositions [35]. Fiber diameter can also influence the cell adhesion, proliferation, migration, and differentiation. Yang *et al.* [16] demonstrated that electrospun nano-sized fibers (300 nm) of polyesters could enhance neural differentiation of neonatal mouse cerebellum C17.2 stem cells compared to micron-sized fibers (1.25 µm). In addition, Yoshimoto *et al.* [17] demonstrated that electrospun PCL, a biodegradable nanofiber scaffold, was a potential candidate scaffold for bone tissue engineering. The results of SEM micrographs, MTT assay and immunocytochemical staining suggest that electrospun PCL nanofibrous scaffold is a promising candidate for tissue engineering application. Due to its advantage for cell attachment, it is believed that this electrospun nerve conduit could find more applications in cell therapy for nerve regeneration in the future and to further improve functional regeneration outcome especially for longer nerve defect restoration. Previous study showed that PCL is capable of supporting a wide variety of cell types such as muscle cells, mesenchymal stem cell, glia, chondrocyte etc. [8]. The results of this study confirm the suitability of PCL nanofiber scaffolds and HFSC for tissue engineering application.

In this study, electrospinning was used for the production of PCL nanofibrous scaffold. PCL aligned

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nanofiber scaffold was used accompanying with the HFSC culture. The results of the MTT assay, SEM images and immunocytochemical staining showed that electrospun PCL nanofibrous scaffold is suitable for HFSC culture. Accordingly, rat bulge HFSC showed the ability to express βIII-tubulin and differentiate into neural lineages on PCL nanofibrous scaffold. This study would be a guide for further development of electrospinning techniques for optimizing PCL and design of nanofibers for tissue engineering applications.

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