Original Article

The role of biodegradable engineered random polycaprolactone nanofiber scaffolds seeded with nestin-positive hair follicle stem cells for tissue engineering

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Abstract

Background: Tissue engineering is a new approach to reconstruction and/or regeneration of lost or damaged tissue. The purpose of this study was to fabricate the polycaprolactone (PCL) random nanofiber scaffold as well as evaluation of the cell viability, adhesion, and proliferation of rat nestin-positive hair follicle stem cells (HFSCs) in the graft material using electrospun PCL nanofiber scaffold in regeneration medicine.

Materials and Methods: The bulge HFSCs was isolated from rat whiskers and cultivated in Dulbecco's modified Eagle's medium/F12. To evaluate the biological nature of the bulge stem cells, flow cytometry using nestin, CD34 and K15 antibodies was performed. Electrospinning was used for the production of PCL nanofiber scaffolds. Furthermore, scanning electron microscopy (SEM) for HFSCs attachment, infiltration, and morphology, 3-(4, 5-di-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay for cell viability and cytotoxicity, tensile strength of the scaffolds mesh, and histology analysis were used.

Results: Flow cytometry showed that HFSCs were nestin and CD34 positive but K15 negative. The results of the MTT assay showed cell viability and cell proliferation of the HFSCs on PCL nanofiber scaffolds. SEM microscopy photographs indicated that HFSCs are attached and spread on PCL nanofiber scaffolds. Furthermore, tensile strength of the scaffolds mesh was measured.

Conclusion: The results of this study revealed that modified PCL nanofiber scaffolds are suitable for HFSCs seeding, attachment, and proliferation. Furthermore, HFSCs are attached and proliferated on PCL nanofiber scaffolds.

Key Words: Hair follicle, nestin, polycaprolactone, scaffold, stem cell, tissue engineering

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INTRODUCTION

Tissue engineering is a new field that applies the principles and methods of bioengineering, material science, and life sciences toward the assembly of biologic substitutes that will restore, maintain, and improve

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tissue functions following damage either by disease or traumatic processes.[1-3] The general principles of tissue engineering involve combining living cells with a natural/synthetic support or scaffold (that is also biodegradable) to build a three-dimensional (3D) living structure that is functionally, structurally and mechanically equal to or better than the tissue that is to be replaced. The development of such a structure requires a careful selection of four key parameters: (1) 3D scaffold type, (2) growth factors, (3) extracellular matrix (ECM), and (4) cells.[4,5] The scaffold materials play an important role in tissue engineering by serving as a matrix that guides the organization, growth and differentiation of cells.[6] Currently, there are three techniques capable of generating nanofibrous scaffolding: Thermally induced phase separation, molecular self-assembly and electrospinning with an attractive feature of having simple and inexpensive setup.[7] The capacity to adjust fibers size is one of the strengths of electrospinning since fibers with diameters in the nanometer size range closely mimic the size scale of fibrous proteins found in the natural ECM, such as collagen. This ability of electrospun nanofibers to mimic the ECM is vital as previous studies have shown that both the size scale of the structure and the topography play important roles in cell proliferation and adhesion, respectively.[8,9] By varying the choice of materials and solution parameters of the fibers orientation (aligned vs. random) and porosity/pore size (cell infiltration) of the electrospun scaffold can be controlled and optimized for each individual application.[10] Polycaprolactone (PCL) is biodegradable and biocompatible aliphatic polyester that has been successfully electrospun.[11,12] This polymer is degraded by hydrolysis of its ester linkages in physiological conditions (e.g., in the human body) and; therefore, has received a great deal of attention for use as an implantable biomaterial in tissue engineering.[11] Scaffolds can be used to generate new tissue alone or seeded with cells. It is imperative to integrate basic polymer science with molecular biology and stem cell biology, in the design of new materials that perform very sophisticated signaling needed for integration and function.[13] One of the cell sources can be used for this intention is hair follicle stem cells (HFSCs), that located in the hair follicle bulge, possess stem cell characteristics, including multipotency, high proliferative potential, and ability to enter quiescence.[14] HFSCs are very proper for medicine intention because: (1) They are easily accessible, (2) they can easily culture, (3) they do not associate with ethical issues like embryonic and fetal stem cells, and (4) HFSCs don't express major histocompatibility complex I.[15] The bulge region of Hair follicle has various stem cells for hair and skin regeneration such as nestin-positive cells.[16] The goal of this study was to investigate the suitability of electrospun PCL random nanofibers for the nestin-positive HFSCs seeding, viability, and proliferation and using these nanofibers and cells for potential use in diabetic skin wounds healing applications.

MATERIALS AND METHODS

Preparation of polycaprolactone solution

Polycaprolactone was used with an average molecular weight of about 80,000 (Mn) (Aldrich, 440744, USA). PCL solution was prepared by dissolving (14% w/v) in formic acid (Merck, Germany) through a magnetic stirrer. The dissolution process was carried out at room temperature.

Polycaprolactone electrospun mesh fabrication and surface modification

Nanofiber mesh was fabricated using electrospinning technique by a setup consisting of a polymer solution in plastic syringe and purge by syringe pumps through a capillary tube into a high electric field. Nanofiber size was controlled by processing parameters including a 14% solution viscosity, voltage equal to 20 kV, 12 cm distance between 18-G stainless steel needle from collector at an angle of 25° and 0.5 mL/h flow rate of solution. Finally, the nonwoven scaffold samples were collected on a thin aluminum foil. Figure 1 shows schematic illustration of electrospinning setup.

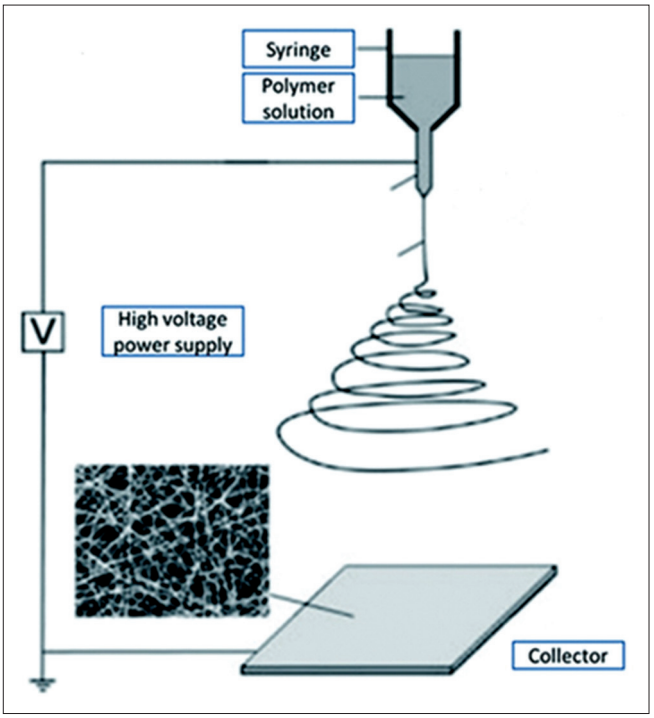

Figure 1: Schematic illustration of the electrospinning process. The mandrel can be rotated at various speeds to achieve different fiber orientations

Plasma treatment

Polycaprolactone was functionalized by plasma treatment. Plasma surface modification process was done by Diener device (Diener Electronic Co., Germany). Plasma treatment was done under high purity oxygen gas with 0.4 milibar pressure and 30 watt power. Plasma process performed about 5 min. Samples were exposed to air after treatment.

Tensile strength of the scaffolds mesh

Mechanical behavior of the nanofibrous PCL webs was tested using SANTAM machine (SPM20 model, ASTM D882-02 from F2150-02, Load Cell, Korea, SPM20). Prepared scaffolds were cut into 10 mm × 30 mm, and the tensile test was conducted at 10 mm/min crosshead speed at room temperature. The test has been repeated thrice and mechanical properties such as Young modulus (E) were determined.[17]

Preparation of nanofiber scaffolds for cell culture

The PCL nanofiber scaffolds were sterilized by an immersion in a 70% ethanol solution for a period of 30 min and then exposed to UV lamp (Thermo Scientific™, 30 W, 25 nm) radiation for 90 min. Thereafter, the scaffolds were soaked in a culture medium Dulbecco's modified Eagle's medium (DMEM)/F12 24 h (37°C and 5% CO_2) prior to cell seeding in order to facilitate protein adsorption and cell attachment on the nanofiber surface.

Structural morphology of polycaprolactone scaffolds

The nanostructural morphology of nanofibers was studied by scanning electron microscopy (SEM) (Hitachi, [Japan], S-4160, SEM). Before the observation, the scaffolds were coated with gold using a sputter coater (Polaran SC7620 sputter coater) and imaged at 20 kV. The average diameter of the fibers was measured using the SEM electromicrographs by measuring at least 100 fibers using Microstructure Characterizer Software version 3.0. (TCR Engineering Services, India). The porosity of scaffold was calculated by the following formula: $P = (1-\rho/\rho_0) \times 100$.

Where P is porosity, ρ is the density of electrospun scaffold and ρ_0 is the density of the bulk polymer.

Hair follicle isolation and cultivation

Male Wistar rats (weighing 160–180 g) in the animal center of Iran University of Medical Science, Tehran, Iran, were used. All animal experiments were carried out according to the guidelines of the Iranian Council for Use and Care of Animals and were approved by the Animal Research Ethical Committee of Tehran University of Medical Sciences. The rats were permitted free access to food and water at all times and were maintained under light – dark cycles.[18] The rats were sacrificed, and the whisker follicles were dissected as described by Amoh *et al*.[19,20] with a slight modification. After disinfection with betadine and 70% ethanol, the upper lip was completely shaved. To isolate the whole intact follicle of vibrissa, the upper lip containing the vibrissae was cut and its inner surface was exposed. The lip tissues were trimmed into small pieces, and the samples were incubated in 3 mg/mL collagenase I/dispase II solution (Sigma-Aldrich) in an incubator for 7 min. Then, most of the connective tissue and dermis around the follicles was removed, and the whisker follicles were plucked by the neck with a forceps. The bulge region was then detached from the follicles by making two transversal cuts at the site of enlargement spots of outer root sheath with a fine needle. About 15–20 isolated bulges from every lip immersed in amphotericin B for 5 min. Then, the follicles were cut into smaller pieces, plated into tissue culture flasks (TCFs) precoated with collagen type I (Sigma-Aldrich) and immersed in a 3:1 supplemented mixture of DMEM/F12 containing 15% fetal bovine serum, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin), 10 ng/mL epidermal growth factor (Sigma-Aldrich), 0.5 mg/mL hydrocortisone, and 0.1 U/mL insulin. All surgical procedures and cultivation were performed under sterile conditions and incubation was at 37°C and 5% CO_2. The bulges were allowed to attach to the TCFs for 4–6 days. At the 12th cultivation days (after two cell passage), HFSCs incubated with scaffolds for cell seeding for 1, 2, and 4 days.

Flow cytometry

To determine the percentage of cell expressing special markers (Nestin, CD34, and K15; Sigma-Aldrich) at indicated times, cells were detached from the culture flasks by trypsin-ethylenediaminetetraacetic acid 0.25% and incubated in the same primary antibodies for 1 h at room temperature. After washing with PBS (0.2 M), they were incubated for 1 h with secondary antibody conjugated-fluorescein isothiocyanate (Sigma-Aldrich, 1:1,400) at room temperature in the dark. Labeled cells were analyzed using the flow cytometry technique.

Cell morphology on polycaprolactone nanofibers scaffold

The morphology and adhesion of HFSCs on the PCL scaffolds was observed by SEM cells (4×10^4) per 100 μl of culture medium DMEM/F12 were cultured on a circle sample (d = 6 mm) and incubated at 37°C (5% CO_2). After 3 h, the culture medium was added to cover the sample surface. The scaffolds were taken out after 1-day of cell seeding and treated with fixation procedure. Samples were fixed in 4% paraformaldehyde at room temperature for 30 min. After washing with PBS (0.2 M),

samples were dehydrated with a graded concentration of ethanol for 50 min. Dehydrated samples were immersed in hexamethyldisilazane (Fluka Chemical, Sigma, USA), a specimen drying agent. After drying, the samples were mounted on aluminum stubs and coated with gold using sputter coating for the observation of cell morphology.[21,22]

4',6-diamidino-2-phenylindole staining

Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and then permeabilized with Triton X-100 (0.3%) for 15 min. After washing with PBS (0.2 M), cells were incubated with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, DAPI; 1:1,400) in the dark for 15 min for nuclear staining.

3-(4, 5-dimethylthiazol-yl) 2, 5-diphenyltetrazolium bromide assay

To evaluate the viability of HFSCs seeded on PCL random nanofiber scaffolds, 3-(4, 5-dimethylthiazol-yl) 2, 5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) assay was performed. The cells were placed in a 24-well plate with a density of 3×10^4 cells/mL and cultured with a medium as described. After 1, 2, and 4 days of cell seeding in 24-well dish, the culture medium of the cells was removed and 1 mL fresh medium and 100 μL MTT solution were added to each well. Cells were incubated in the dark at 37°C (5% CO_2) for 4 h. Then, the MTT solution was removed, the scaffolds were gently squeezed, and the purple formazan reaction products, produced by active mitochondria were dissolved by addition of 1 mL 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 at 570 nm.

Statistical analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed by Student's *t*-test and one-way ANOVA, followed by Tukey's *post-hoc* test to evaluate the statistical significance between groups. $P < 0.05$ was considered statistically significant.

RESULTS

Hair follicle isolation and cell culture

In this study, bulge HFSCs from dissected rat were successfully isolated and cultured with a slightly modified method. One isolated follicle and bulge region is shown in Figure 2a and b. Within 3–4th cultivation days, stem cells started to an outgrowth from the isolated bulges [Figure 3a]. According to rapid proliferation after 7–8 days, the bulge cells compacted around the bulge segment and formed dome-like cell layers [Figure 3b]. Finally, the cells began to migrate out of the dome-shaped colonies edge [Figure 3c].

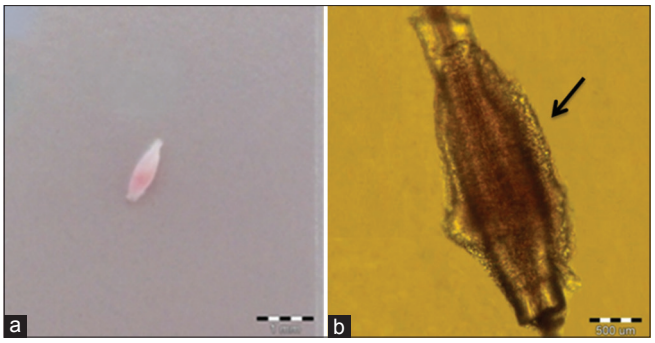

Figure 2: Dissection of hair follicle bulge from adult rat whisker follicle. (a) Hair follicle surrounded by connective tissue; (b) hair follicle bulge rolled in capsule (arrow shows the bulge region). Scale bars = 1000 (a), 500 μm (b)

Flow cytometry

To confirm that these cells were primitive stem cells, the flow cytometry was performed and the results indicate that bulge cells were nestin (70.96%) and CD34 (93.03%) positive and K15 (6.88%) negative [Figure 4].

Structural morphology of electrospun nanofiber

Scanning electron microscopy electromicrograph of PCL nanofibers have been shown in Figure 5a and b. Average fibers diameter was estimated to be 116.30 nm with a diameter ranging from 50 to 250 nm. Most of the fibers had a diameter ranged from 101 to 150 nm (54% nanofibers), by using image analysis software (ImageJ, National Institutes of Health, Bethesda, MD, USA), and is shown in Figure 6.

Furthermore, the scaffolds were revealed to be highly porous with an average porosity of 78%.

Cell morphology and distribution in scaffolds

4',6-diamidino-2-phenylindole staining [Figure 7a] and SEM examination showed that the seeded cells were adhered to scaffolds and covered the pores (after 1-day incubation). This issue indicated that PCL random scaffolds possessed biocompatibility for attachment of HFSCs *ex vivo*. The SEM electromicrograph also indicated that the cell body had an apparent bipolar elongated morphology and demonstrate cell attachment on the nanofiber scaffold [Figure 7b and c]. The cells were infiltrated into the nanofibrous network and enveloped the scaffold [Figure 7b and c]. HFSCs orientation was haphazard on the randomly oriented fibers [Figure 7b and c].

3-(4, 5-dimethylthiazol-yl) 2, 5-diphenyltetrazolium bromide assay

3-(4, 5-dimethylthiazol-yl) 2, 5-diphenyltetrazolium bromide chromometry assay was used to determine cell viability to compare the number of viable cells in the nanofiber scaffolds and control group. The MTT assay

is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. Our data showed the MTT increment results of the HFSCs seeded on PCL nanofiber scaffolds after 2 and 4 days compared with control group ($P < 0.05$) [Figure 8].

Tensile strength of the scaffold mesh

Tensile strength of the random nanofibrous PCL mesh was estimated for three times and mechanical properties such as Young modulus (E) were determined [Figure 9 and Table 1].

DISCUSSION

The search for and use of an appropriate multipotent or pluripotent stem cell in tissue engineering is an emerging concept. Certainly, many areas of stem cell research and their potential clinical applications are associated with controversies; therefore, it is important to address the ethical, legal, accessible, and social issues early. Somatic stem cells (also called adult stem cells) exist naturally in the body.[23] The investigation has demonstrated that stem cells can be found in various tissues of the body, including the adipose tissue, musculoskeletal, liver, nervous system, hematopoietic system, and epidermal system.[24] HFSCs, situated in the hair follicle bulge, possess stem cell characteristics, including multipotency, high proliferative potential and ability to enter quiescence. In this study, HFSCs were isolated and specified and shown to express the stem cell marker CD34 and nestin, but negative for keratinocyte marker keratin 15 [Figure 4]. According to the results of both present and previous studies, proliferated cells derived from the bulge region of the rat hair follicle can also be nestin-positive.[15,25-27] These stem cells can differentiate into various neural cells, keratinocytes, blood vessels, smooth muscle cells and melanocytes *in vitro*.[15] So, it can be a good candidate for using in tissue engineering to produce a variety of tissues. Several studies have been reported the important role of synthetic polymers in tissue engineering to the improvement of many disease treatments. Nanofibers are widely being used for tissue engineering and have advantages over traditional scaffolds because of increased surface area-to-volume ratio, which increases cell to scaffold interactions and cell proliferation, which is confirmed by SEM and MTT results.[28] In the present study, a nanofibrous mesh of PCL was produced by electrospinning. MTT assay of cell viability and proliferation has been currently the main *in vitro* method to test the biocompatibility and cytotoxicity of biomaterials. Here, the PCL nanofiber scaffolds were cultured with HFSCs to check whether nanofiber scaffolds have cytotoxicity to tissues. The MTT cell viability and cytotoxicity assay results showed that nanofiber scaffold had no cytotoxicity to HFSCs and did not cause inhibition of proliferation of HFSCs [Figure 5]. The results of MTT cell viability and cytotoxicity assay also showed that the difference between the proliferation of cells in control and scaffold groups are statistically significant. Similarly, in the 2 and 4th cultivation days, the results of MTT assay

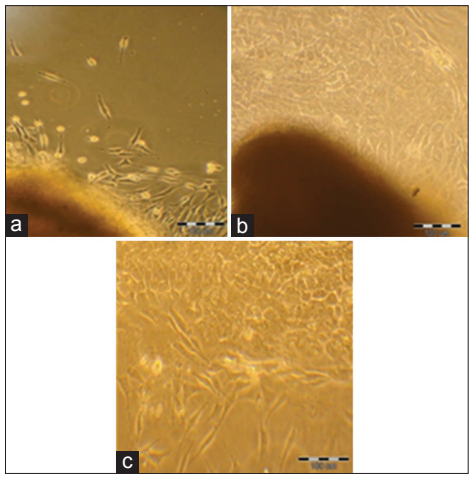

Figure 3: The primary cultivation of bulge cells from rat hair follicles. (a) 3 or 4 days after cultivation stem cells surrounded the bulge region; (b) Stem cells make a dome-shaped and gradually start to migrate after 8–10 days; (c) Cells start to migrate from colonies. Scale bars = 100 μm (a-c)

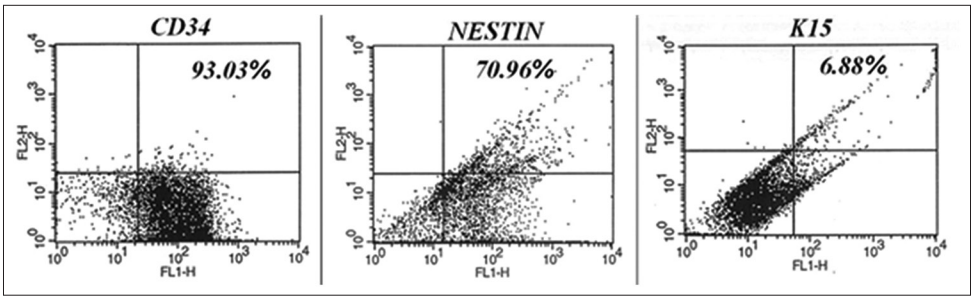

Figure 4: Flow cytometry results show the percentage of CD34 (93.03) and nestin (70.96) positive that are stem cells markers, but negative for K15 (6.88) that is keratinocyte marker

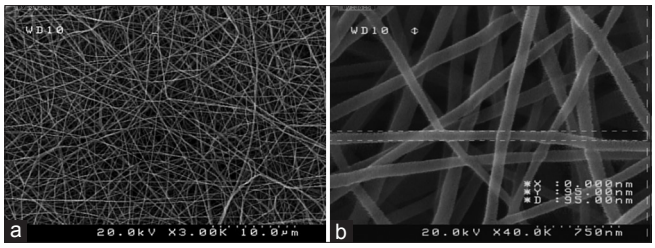

Figure 5: Electromicrogragh by scanning electron microscopy shows the polycaprolactone random nanofibers. Diameter of a nanofiber is seen in B (95 nm). (a) Scale bar = 10 μm and (b) scale bar = 750 nm

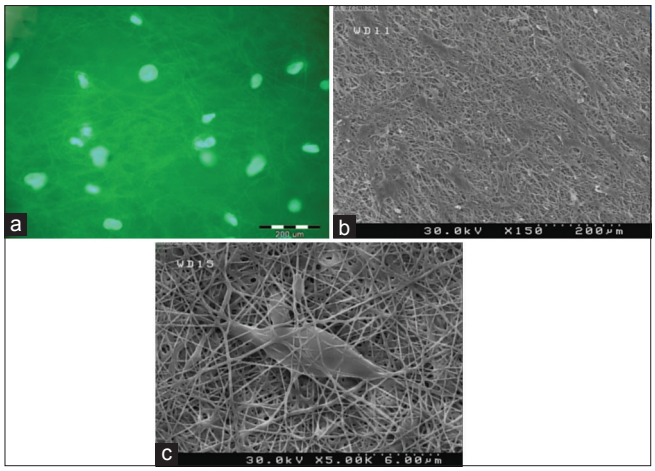

Figure 7: Nuclear staining with 4',6-diamidino-2-phenylindole that shows direction of hair follicle stem cells (HFSCs) seeded on polycaprolactone (PCL) scaffolds (a). The scanning electron microscopy electromicrographs of HFSCs seeded on PCL scaffolds after 1-day of culture shows the interaction between cells and PCL nanofibers (b and c). Scale bars represent 200 (a), 20 (b) and 6 μm (c)

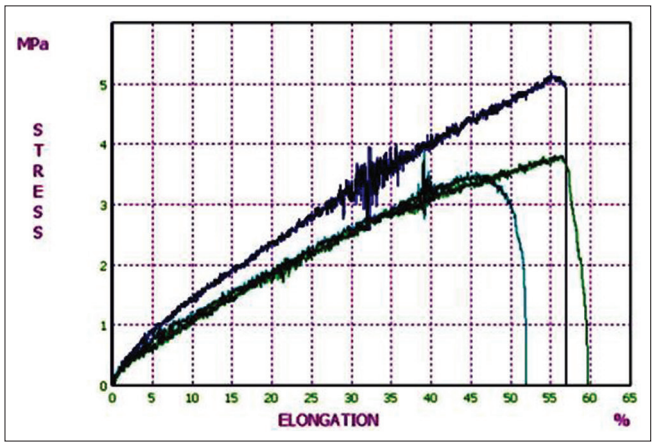

Figure 9: Stress-strain curves for scaffolds mesh produced by electrospinning methods (repeat 3 times)

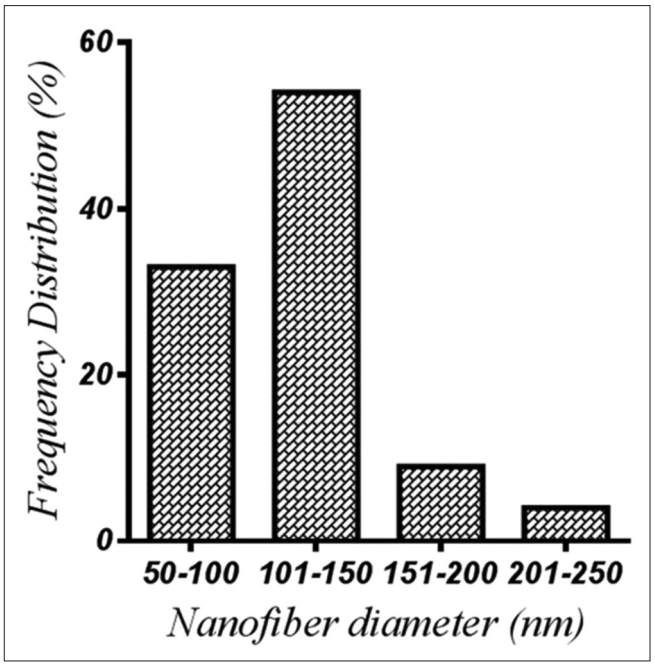

Figure 6: The average diameter of the fibers was measured for 100 randomly selected fibers per scaffold. As the diagram shows, most of the fibers had a diameter ranged from 101 to 150 nm

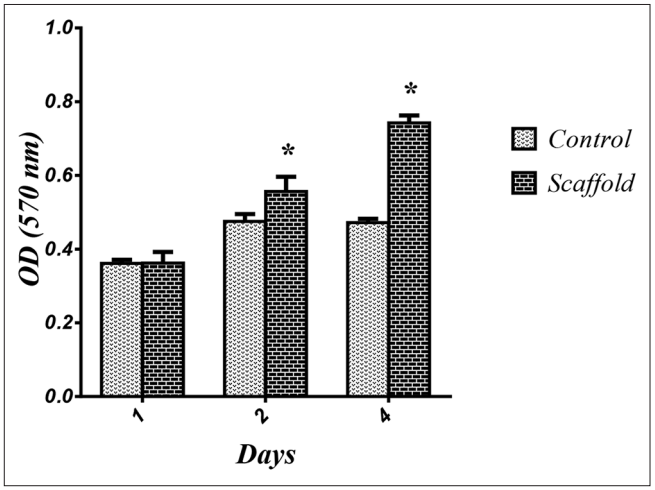

Figure 8: 1, 2, and 4 days after co-culture of polycaprolactone, cell viability in the control and scaffold groups were assayed. Asterisks demonstrate that cell viability of scaffold groups (2 and 4 day) was significantly higher than the control groups (*P < 0.05). Error bars represent means ± standard deviation

showed that the difference between proliferation of cells in PCL nanofibrous scaffold is statistically higher than control group (one-way t-test, $P < 0.05$). Researchers demonstrated that the viability and proliferation rate of fibroblast cells increased in PCL nanofibrous scaffold compare with tissue culture plate (TCP).[28] In a study, PCL nanofiber scaffolds were also used for cultivation of HFSCs. The results of MTT assay showed, which cell viability increasing in scaffold group was statistically significant compared with a control group that confirms our findings.[29] Another study carried out by Ghoroghi *et al*.[30] showed that the poly-L-lactic acid scaffold is also a suitable candidate for tissue engineering. In general, the hydrophilic/hydrophobic characteristic of scaffold is important in tissue culture and can influence the ability of cell adhesion, proliferation, and differentiation. Plasma treatment generally is performed on the nanofibrous

Table 1: Results of tensile strength for PCL nanofiber scaffolds mesh (repeat 3 times)

Variables	Peak stress (MPa)	Peak strain (%)	Peak energy (J)	Break stress (MPa)	Break strain (%)	Break energy (J)
1	5.1714	55.0583	33.64706	5.1714	55.0583	33.64706
2	3.8143	56.47	26.80245	0.3857	59.5947	28.49791
3	4	39.074	14.90814	0.6429	51.8417	23.46495
Delta	1.3571	17.396	18.73892	4.7857	7.752998	10.18211
SD	0.6007757	7.888892	7.742167	2.197888	3.180398	4.156919
Mean	4.328567	50.20077	25.11922	2.066667	55.49823	28.53664

PCL: Polycaprolactone, SD: Standard deviation

scaffold to create a more hydrophilic surface.[28] In recent study, the results of SEM electromicrographs of HFSC on the PCL nanofiber scaffold and MTT cell viability and cytotoxicity assay showed that the cells tend to expand on the nanofiber scaffold and nanofiber scaffold supports cell adhesion and proliferation as well as control group (TCP). The recent study results showed that plasma treatment improves the hydrophilicity of PCL nanofibrous scaffolds, increase human fibroblast cell attachment, and proliferation.[28] Researches showed 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 electrospinning and fiber leaching have been proposed.[33,34] The SEM results of our recent study demonstrated that the modified PCL scaffold fabricated with high porosity (78%) can be suitable for cell penetration. Electrospun nanofiber matrix has morphological similarities to the natural ECM. Their interconnected highly porous structures increase cell migration and nutrients and metabolic wastes transport. Fiber diameter is the other characteristic of scaffold can also influence the cell adhesion, migration, and proliferation by increasing of surface area-to-volume ratio and porous. The high surface area-to-volume ratio and the porous structure of nanofibers might increase the contact area between cells and the fibers. This property facilitates growth factor uptake by cells which lead to faster proliferation, migration, and differentiation,[35,36] which is confirmed by SEM electromicrographs and MTT cell viability and cytotoxicity results. Tensile strength and elongation of the scaffolds are important for their application in tissue engineering. Soft scaffolds are suitable for brain cells, and stiff scaffolds are suitable for skin cells.[37] According to the results of the tensile strength in recent study [Figure 9 and Table 1] we can suggest that scaffold designed in this study (stiff scaffold) could be useful for the treatment of skin ulcers. In this study, electrospinning was used for the production of modified PCL nanofibrous scaffold. PCL random nanofiber scaffold was used cultured with the HFSC culture. The results of the MTT cell viability and cytotoxicity assay and SEM electromicrographs showed that electrospun PCL nanofibrous scaffold is suitable for HFSC culture. 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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Nil.

Conflicts of interest

There are no conflicts of interest.

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