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In Vitro and *In Vivo* investigations on fibronectin coated and hydroxyapatite incorporated scaffolds

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Abstract

Topological and biochemical aspects of the matrices are essential factors to be extensively studied for more successful tissue engineering. Other characteristics including biodegradability and biocompatibility should be also considered. Nanofibrous structure mimics topography of the natural matrix. Previous *in vitro* studies reported the favorable effects of nanohydroxyapatite (nHA) and fibronectin (Fn) on biodegradability and biocompatibility of scaffold. Herein, the synergistic outcome of co-application of Fn and nHA incorporation into aligned electrospun polycaprolactone (PCL) seeded by mouse mesenchymal stem cells (MSC) was investigated both *in vitro* and *in vivo*. Scanning Electron Microscopy (SEM), contact angle measurement and tensile test were applied for scaffold characterization. *In vitro* evaluation of the seeded cells was performed by MTT, SEM and cell-cycle analyses. In congruence with *in vitro* findings, *in vivo* assessment of four weeks fibronectin coated PCL/ nHA scaffold transplanted mice illustrated the suitable compact surrounding tissue with the most penetrated cells generation. Furthermore, Fn coating resulted in cell infiltration enhancement while nHA addition led to more scaffold biodegradation. In conclusion, fabrication of nanofiberous scaffold with this combination of biochemical composition and surface stimulation caused improved biodegradability and biocompatibility of the scaffold which are desirable in more effective tissue regeneration.

Key words: Polycaprolactone Nanofibers, Fibronectin, Hydroxyapatite, Proliferation, Tissue Engineering, in vivo.

Introduction

Current methods mainly apply auto- and allograft or synthetic implants to repair organ defects. However, many limitations such as disease transfer, potent immune response, restricted availability of tissue providers, donor morbidity or insufficient bioactivity have directed researchers toward tissue engineering as an appropriate approach (1, 2). In this regard, remarkable properties of bone marrow derived mesenchymal stem cells (MSCs) make them suitable candidates for tissue regeneration approaches (3).

Combination of suitable cell transplantation with supportive matrices has been emerged as an operative strategy in tissue engineering (4). Adherent cells convert signals from sensing matrix topography into morphological alterations (5). The role of nano-sized structure of extracellular matrix (ECM) in the regulation of cell functions such as cell adhesion and proliferation makes the nanofibrous scaffolds suitable to mimic the tissue architecture for tissue engineering (6). Nanofibrous scaffolds, as mechanical supports, with unique features such as high porosity, large surface area, proper interconnections and enhanced cell recruitment provide appropriate environments to the seeded cells (6). Electrospinning is a common method that processes polymeric biomaterials into nanofibers (7). It has been applied in some industrial applications such as water filtration, biotechnology and sensors because of its cost-saving scaling up (6).

Poly-ε-caprolactone (PCL) is a synthetic semi-crystalline biodegradable polymer with slow degradation rate in the homo-polymeric form (8). It has been employed for various applications, such as skin tissue engineering and bone regeneration (9, 10).

Hydroxyapatite (HA) nanocomposites have received a lot of attention for bone re-establishment because of excellent biodegradability, osteoconductivity, biocompatibility and direct bone-binding competency (2, 11). Some *in vitro* studies reported the accelerated degradation of the matrix interfaces by HA supplementation (12).

Several approaches have been followed to increase initial cell-adhesion. Integration of adhesion proteins such as Fibronectin (Fn) into the matrices is an effective strategy. Fibronectin as a glycoprotein stimulates initial cell-adhesion due to its functional RGD domains which is specialized for binding of integrin receptors of cell surface (13). In addition, Integrin and its Fn ligand played critical roles not only in cell adhesion, but also in adhesion between stem and niche cells (14). In this regard, fibronectin should be immobilized in a biological active conformation (15). To overcome the potential masking of nHA or fibronectin inductive properties due to probable embedding inside the polymer, electrospinning technique has been strongly suggested (16).

In this study, nano-hydroxyapatite (nHA) and fibronectin were selected as supplementary additives to PCL

nanofibers in order to investigate the biocompatibility and biodegradability of the matrices. It was postulated that electrospinning of PCL with nHA followed by fibronectin coating created *an in vitro* natural tissue-resembling environment. The effect of HA or fibronectin presence on the functional and morphological properties of PCL nanofibrous scaffolds were investigated. In addition, the performance of PCL/nHA, PCL/Fn and PCL/nHA/Fn nanofibers for proliferation and adhesion of mouse MSCs were assessed *in vitro* and *in vivo* and finally the biocompatibility and biodegradability of the biocomposite scaffolds were evaluated *in vivo*.

Materials and methods

Cell Culture, Differentiation and Specific Staining

Mouse MSCs (from Stem Cells Technology Research Centre) were expanded in growth medium containing high glucose DMEM (Gibco), supplemented with 10% (v/v) fetal bovine serum (FBS) and a 1% (v/v) penicillin/streptomycin. Cells were cultured in 24-well tissue culture polystyrene (TCP) with initial cell density of 10^4 cm^{-2} under humidified atmosphere of 5% CO_2 at 37 ºC. For osteogenic, adipogenic and chondrogenic differentiations, the induction media were prepared according to our previous reports (3). Oil Red dye was used for detection of accumulated oil droplets, calcium mineralization were stained with Alizarin Red and the presence of glycosaminoglycans was confirmed by Alcian Blue dye (3).

Scaffold Fabrication and Scaffold Surface Modification

Electrospinning was used to construct PCL and PCL/nHA nanofibers. First solution for electrospinning were prepared by dissolving PCL (8% w/v) in a mixed solvent of chloroform and DMF (8:2 v/v) and then homogenized by stirrer. For PCL/nHA scaffolds, HA nanoparticles (10% w/w of polymer mass) were dispersed in chloroform/DMF and homogenized by ultrasonic homogenizer to form a suspension for dissolving polymer. nHA average size was less than 200 nm. Electrospinning was performed by two nozzles and high voltage potential of 22 kV was applied. The collector was located in a fixed distance (23 cm) from the 21-gauge needle. A cylindrical stainless steel collector at 2300 rpm rotation collected the fibers as mat. After the electrospinning, the fibrous scaffold was placed in the vacuum for solvent removal. O_2 Plasma treatment was used in order to make the scaffolds hydrophilic; using a plasma generator with a cylindrical quartz reactor set on 44 kHz (Diener Electronics, Germany). For fibronectin coating, plasma-treated scaffolds were immersed in NHS and EDC (5 g/l) for 24h. Then supernatant was removed and mats were rinsed with PBS and placed in fibronectin (1% v/v) for 24h at room temperature.

Scaffold Characterization

Tensile Properties

The tensile properties of the nanofibers were evaluated before and after plasma treatment. The scaffolds were cut into 10 mm × 60 mm × 0.12 mm pieces. Tensile test was performed in parallel direction at 20 mm/min using tensile testing equipment (SANTAM, Iran).

Contact Angle Measurement

Contact angles of water droplets were measured at room temperature and pressure. Evaluations were performed by placing 4 µl deionized water on dry scaffold surface for 10 s and visualization by CCD camera (G10, Kruss) before and after plasma treatment.

Fourier Transform Infrared (FTIR) Spectroscopy

To investigate the incorporation of Fn into the electrospun nanofibers, FTIR spectroscopy was applied. FTIR spectra were gotten with an Equinox 55 spectrometer (Bruker Optics, Germany) having a resolution of 2 cm^{-1}.

Cell and Fiber Morphology

To investigate morphology of nanofibers, nHA involvement and Fn coating, the mats were coated with gold by sputter coater. Then gold coated scaffolds scanned using scanning electron microscopy (SEM, LEO 1455VP, Cambridge). Prior to gold coating, the cell-loaded mats after 7 days of culture were fixed in 2.5% v/v glutaraldehyde for 45 min and dehydrated with immersing in gradients of alcohol concentration.

Cell Seeding and Biocompatibility Studies

Cells with initial density of 10^4 cells per cm^2, were cultured in five groups described in Table 1. Prior to cell seeding, scaffolds were sterilized in 70% ethanol in 24-well tissue culture polystyrene. In order to facilitate cell attachment, the scaffolds were incubated with growth medium overnight at 37ºC. At different time intervals, the medium of cells cultured on tissue culture polystyrene (TCP) was removed and replaced with culture medium containing 500 µl of sterile MTT dye (5 mg/ml). The cells were then incubated for 4h at 37ºC. After incubation, for dissolution of the dark-blue intracellular formazan, the supernatant was removed, 500 µl of MTT solublizer solution was added and thoroughly mixed for 10 min followed by optical density measurement at 570 nm by Biotek Synergy microplate reader.

Table 1. Descriptions for different groups.

GROUP	NAME	MATRIX
1	TCP	tissue culture polystyrene plates
2	PCL	Nanofibrous scaffold
3	PCL/nHA	Nanohydroxy apatite incorporated scaffold
4	PCL/Fn	Fibronectin-coated scaffold
5	PCL/nHA/Fn	Fibronectin-coated PCL/nHA

Cell Cycle Analysis Using Propidium Iodide (PI) DNA Staining and Flowcytometry

Cell cycle analysis was performed by PI flow cytometry of DNA content. For this purpose 10^6 cells were seeded on the scaffolds and TCP. After 7 days, cells were detached by Trypsin and centrifuged twice at 1200g in PBS. Then cells were fixed for 30 min at 40ºC and washed in PBS twice. After spinning at 850g, cells were treated with ribonuclease (100 µg/ml) to omit RNA contamination. Finally, 200 µl PI (50 µg/ml) was added. Samples were analyzed using FACS Calibur cytometer (Becton Dickinson) and CellQuest software. Win MDI 2.8 software was used to create the histograms.

Subcutaneous Transplantation

Bulb C mice (25-30 g weight, Stem Cell Research Center, Iran) were maintained in standard condition at 20ºC under 12h /12h periods of darkness/ lighting. Preoperative preparation involved determination of the health status of the Bulb C mice, removing food from the animal's cage, and organizing what will be needed for surgery and for the postoperative period. Animals were fasted a minimum of 12 hours before being anesthetized. Decisions concerning the choice of surgical instruments, suture material and intravenous fluids for surgery as well as the need for antibiotics and analgesics administration postoperatively were made upon the IACUC protocol. The experiments were performed based on by law ethics of experimentation of Stem Cell Technology Research Center that is in accordance with guidelines of NIH in the USA on the Care and Use of Animals for Scientific Purposes. Mice were fainted using ketamine (20 mg/kg weight of mouse) and xylene (2 mg/kg weight of mouse) intra-peritoneal injection. After hair cutting and skin disinfection, skin of mouse was slivered and a proper pocket was created for scaffold transplantation. The scaffolds of all 4 groups were implanted and surgery sites were stitched and disinfected.

Histopathology

Four weeks after transplantation, mice were fainted; implanted scaffolds with surrounding tissue were isolated, fixed in 10% formalin and placed in paraffin. Sections with 2 to 5 µm thickness were prepared, stained with Hematoxylin and Eosin (H&E) and investigated using light microscopy.

Statistical Analysis

Student's t-test was used to find significant difference between the groups by SPSS program. The statistically significant difference was considered at $P<0.05$. All experiments were performed in triplicate and results are shown as mean ± standard deviation (SD).

Results

Cell Expansion and Characterization

Isolated mouse MSCs were cultured and expanded without any alteration in morphology till fifth passage (Figure 1A). Their differentiation potential was investigated after 21-day cultures in induction media. Alizarin red staining visualized the calcium precipitation during osteogenic differentiation (Figure 1B). Moreover, these cells showed the potential to differentiate into adipocytes and chondrocytes in favorable conditions. Red cytoplasmic oil vacuoles, as indicators of adipogenic differentiation, were observed also Alcian Blue staining confirmed the strong expression and accumulation of acidic proteoglycans as shown in Figures 1C and D.

Scaffold characterization

According to SEM micrograph in Figure 1E, aligned morphology of nano-scaled, porous and beadless fibrous structures confirmed scaffold formation under controlled conditions. Moreover, Figures 1F, G and H indicate that nHA and fibronectin were dispersed homogenously all over the scaffolds. nHA is shown by arrows in Figure 1F. The average diameter for the PCL nanofibers was measured as 472nm ± 90. FTIR spectra of PCL nanofibers, with or without fibronectin, are shown in Figure 2. Strong characteristic peaks of PCL was detected at 1723.43 cm^{-1} for C=O group and at 1046 for C-O stretching. Peaks at1364-1467 and 3436 cm^{-1} are referred to the vibrations in hydrophilic groups of OH and COOH. Existence of fibronectin was confirmed through the amide I and II bands which were detected at 1661 and 1550 cm^{-1}.

The water contact angles of PCL and PCL/nHA scaffolds were found to be 115.6º ± 0.6º and 132.8º ± 0.6º respectively. After plasma treatment, the drop spread on both scaffolds surfaces in less than 10 sec, representing

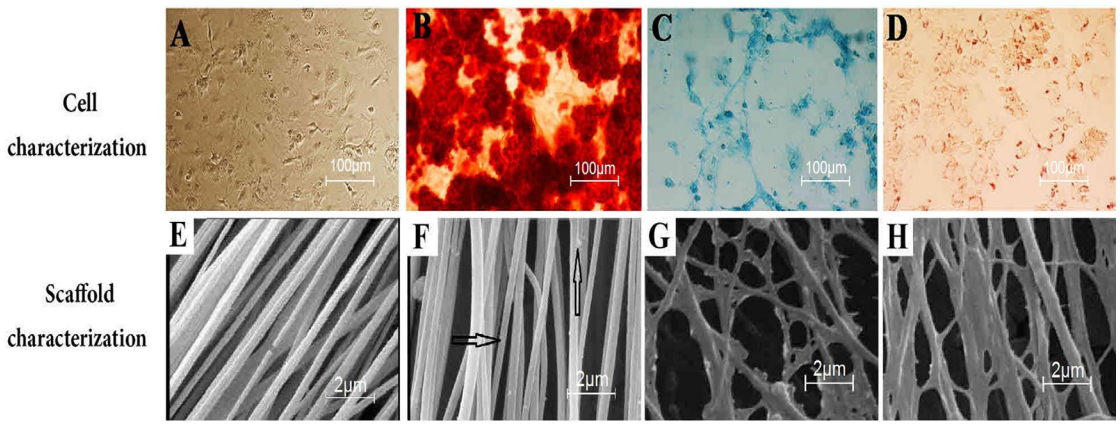

Figure 1. Mouse MSCs characterization: (A) phase-contrast microscopy of the cells, (B) alizarin red staining of calcium deposition caused by 21-days osteogenic induction, (C) Alcian Blue staining of proteoglycans produced by chondrogenesis and (D) oil red staining of vacuoles from adipogenic differentiation. Scaffold characterization by SEM: (E) PCL scaffold, (F) PCL/nHA, (G) PCL/Fn scaffold and (H) PCL/nHA/Fn scaffold. Scale bars are given in the figures.

Table 2. Stress–strain measurements of aligned nanofibrous scaffolds along the fiber axis.

Scaffold	Break Strain (%)	Tensile modulus (MPa) ± SD
PCL	72.95	47.40 ± 0.04
Plasma-treated PCL	47.39	44.48 ± 0.03
PCL/nHA	56.92	52.37 ± 0.71
Plasma-treated PCL/nHA	32.32	46.35 ± 0.53

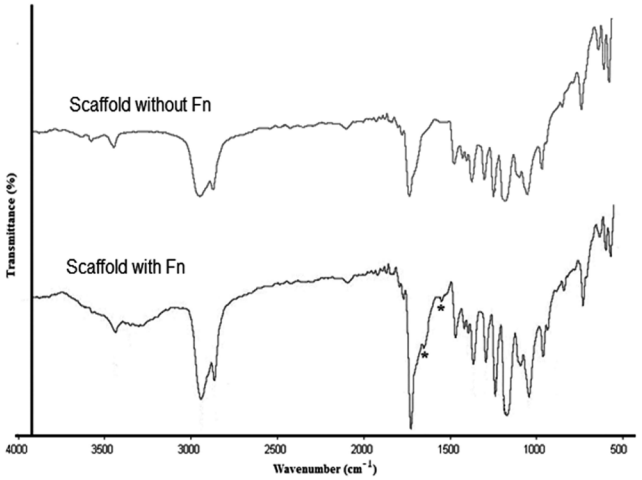

Figure 2. ATR-FTIR spectra of PCL with and without fibronectin. Asterisk shows picks of amide I and II at 1661 and 1550 cm-1.

a highly hydrophilic surface. Mechanical properties of hybrid aligned scaffolds are given in Table 2. As shown in this table, incorporation of nHA in PCL scaffold did not change the tensile modulus considerably but decreased elongation at break. Plasma treated PCL and PCL/nHA scaffolds showed the same trends as untreated scaffolds.

In vitro Evaluations

Morphological Evaluation

As shown in Figure 3 (A-D), the morphological monitoring by SEM indicated that cells covered the scaffold surface with appropriate adhesion. In addition the cells demonstrated oriented growth along the fiber direction.

MTT Assay

Evaluation of normalized activity of cellular enzymes by MTT assay, as an indicator of cell proliferation, is shown in Figure 3E. The increasing trend of activity during 7day cultures can be considered as biocompatibility of the scaffolds. Considering the same cell numbers in the initial day of seeding, it can be concluded that all scaffolds have the similar potential for cell adhesion. Noteworthy, the rate of cell proliferation in TCP and Fn-coated scaffolds was higher than the others. In contrast, normalized cellular enzymatic activity in PCL and PCL/nHA groups did not change considerably.

Cell Cycle Analysis

Investigation of the PI-stained population by flow cytometry indicates the portions of the cells in G0/G1, S or G2 phases of cell cycle (17). Portion of the S phase cells in the population is a good indicator for proliferation (18). Flow cytometry results in Figure 4 show that the percentage of S phase cells in each population was 5.48% for TCPS group, 8.73% for PCL group, 9.11% for PCL/nHA group, 11.22% for PCL/Fn group and 11.40% for PCL/nHA/Fn group. It is clear that the presence of Fn in the scaffold significantly increased the percentage of S phase cells or cell proliferation.

In vivo Evaluations

Since the initial number of seeded cells in all groups of this study was the same, density of H&E stained nuclei could be considered as an indicator of in vivo cell proliferation and migration. As illustrated in Figure 5, the surrounding tissue around scaffold with nHA and

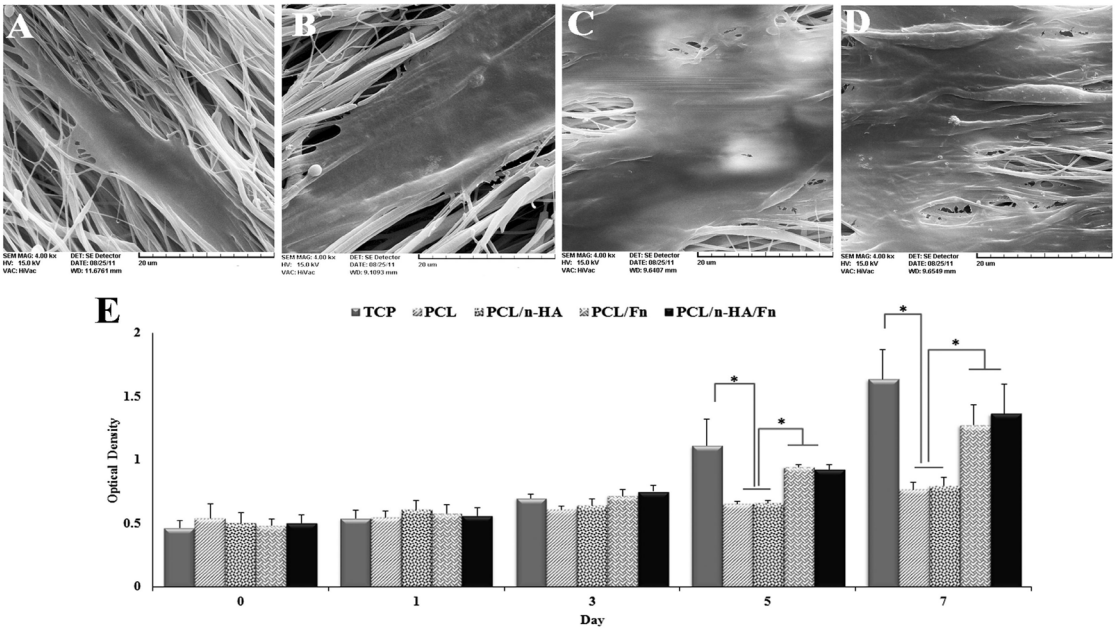

Figure 3. Investigation of MSC adhesion and proliferation by SEM on (A) PCL, (B) PCL/nHA, (C) PCL/Fn and (D) PCL/nHA/Fn scaffolds or by MTT assay on (E) scaffolds and TCPS during a 7-day culture period. Scale bars with their magnifications are given in the figures. Asterisk shows significant difference with $p < 0.05$.

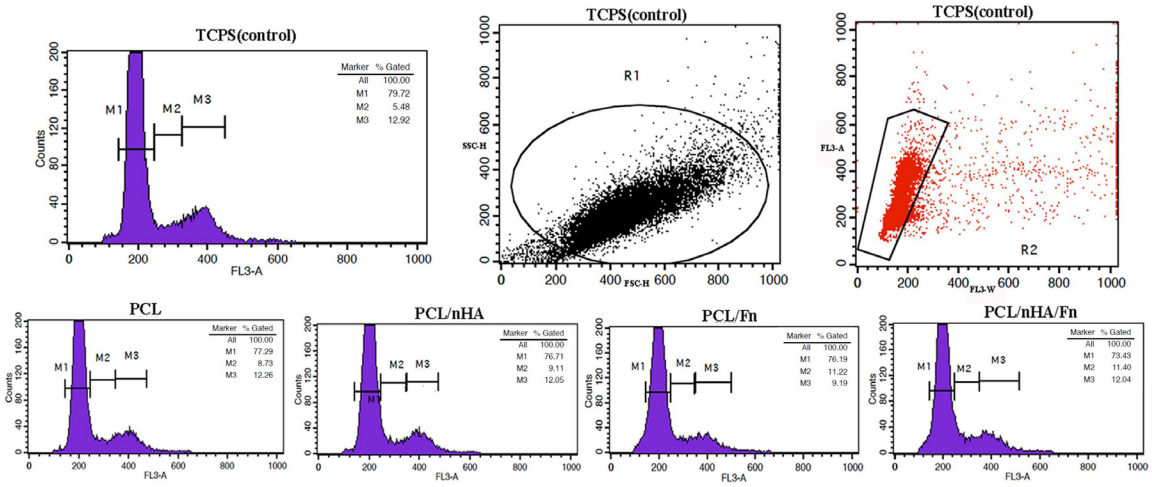

Figure 4. Cell-cycle analysis of the seeded cells on the investigated scaffolds by PI flowcytometry. The portions of the population in the different phases of the cell cycle are illustrated for each scaffold (M1: G0/G1 phase, M2: S phase and M3: G2 phase).

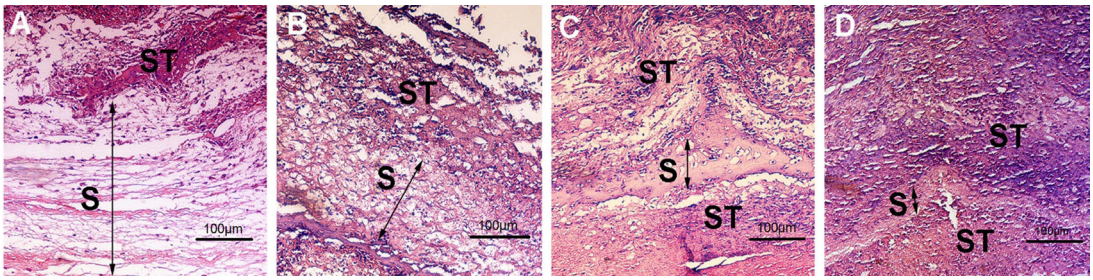

Figure 5. The histopathology of the cell-seeded scaffolds after 4weeks implantation by H&E staining; (A) control PCL scaffold, (B) PCL/nHA scaffold, (C) PCL/Fn scaffold and (D) PCL/nHA/Fn scaffold. S: scaffold and ST: surrounding tissue. Scale bars are given in the figures.

fibronectin was the most condensed. In addition, the degradation of mentioned scaffold was more rather than the others and remarkable cell penetration in nHA/Fn scaffold and Fn scaffold was observed. The least cell penetration and surrounding tissue were obtained with control group (Scaffold without nHA and Fn).

Discussion

An important challenge in tissue engineering is the appropriate supply of the defect with suitable cells. Stem cells are under the focus of interest in the field of regenerative medicine both for their differentiation potential and the ability to serve as the live part of tissue (19, 20). Herein, mouse MSCs were isolated, expanded and characterized as shown in Figure 1. Osteogenic, chondrogenic and adipogenic differentiations of mouse MSCs, confirmed by specific staining, supported the stemness characteristic of these cells.

Electrospun nanofibers have gained an up-trust in recent years because of biomimicking the nanoscale natural features such as large surface area, interconnected pores and high porosity (21). Because of relatively small pores between the fibers, inadequate cell infiltration into electrospun nanofibrous scaffolds is considerable. Alignment provides relatively appropriate pore size; therefore cell infiltration into the aligned nanofibers could be enhanced significantly. Some researchers investigated the effect of aligned electrospun nanofibers on promoting astrocyte alignment, Schwann cell maturation, oriented axonal outgrowth, distribution of mouse fibroblast and improvement of endothelial cell infiltration into the scaffold (22-24) Hence in this study, the proliferation and adhesion of mouse MSCs seeded on different nanofibrous scaffolds were investigated.

In addition to geometry and physical properties of the scaffold, biochemical characteristics and composition of scaffolds play critical roles in successful tissue engineering. In order to integrate tissue implants into defected location, suitable cells must attach to the matrices and form a new tissue with favorable differentiation (25). For this reason the most important characteristic of the matrix is its potential for cell adhesion and proliferation.

High mechanical strength and elasticity of PCL make it appropriate for tissue engineering (26). Previous reports have been suggested the Fn and HA for improvement of cell adhesion and enhancement of biodegradability respectively (7, 12, 15, 27). In this study, aligned electrospun PCL nanofibrous scaffolds in combination with nHA or fibronectin were successfully fabricated.

As illustrated in SEM micrographs of Figure 1, electrospun scaffolds were composed of uniformed and aligned nanofibers with interconnected pores and nanometric distance between fibers which could facilitate nutrient transport and cell in-growth. Remarkably, fibronectin coating did not disturb these pores. FTIR analysis of PCL nanofibers supported successful fibronectin coating of the nanofibers (Figure 2). Moreover, effective presentation of nHA and Fn on the surface helped to attain rough appearance which is ideal for cell adhesion and proliferation. The SEM results in Figure 3 showed the coverage of the scaffolds by the cells which indicated biocompatibility of the scaffolds.

Hydrophilicity is a key property of biomaterials affecting the attachment and viability of cells. PCL nanofibers are very hydrophobic; Oxygen plasma treatment provides a hydrophilic surface and more biocompatible

scaffolds for the tissue engineering field (28). Using this approach, the water contact angle of oxygen plasma treated PCL nanofibers decreased to 0˚ and introduced a water-like surface because the water contact angle of a strong hydrophilic surface is <10˚. Cell proliferation was evaluated through normalized cellular enzymatic activity measurement of cultured MSCs by MTT assay. The normalized cellular enzymatic activity of all groups increased during the assay time as shown in Figure 3E. Compared to TCP, the PCL/nHA/Fn scaffold showed the potential to support the proliferation of MSCs but with slightly slower rate. This insignificant difference can be attributed to the surface characteristic of the scaffold which needs further improvement in future studies.

Cell cycle analysis by flow cytometry revealed that in the percentage of the S-phase cells (an indicator of population proliferation) increased in fibronection coated scaffolds (figure 4).

In accordance with *in vitro* findings, *in vivo* evaluation of four weeks transplanted mice revealed the most compact surrounding tissue with the most penetrated cells in nHA/Fn scaffold (Figure 5). Furthermore, supplementation of Fn in the scaffold resulted more cell adhesion and penetration while nHA addition led to more scaffold degradation. Altogether, based on these data, the fibronectin coated PCL scaffold enhanced proliferation in MSCs more effectively than nHA scaffold. As a conclusion, the *in vitro* and *in vivo* findings confirmed the appropriate biocompatibility and the capability of this biocomposite scaffold to support the attachment and proliferation of MSCs with more rapid biodegradation; as a suggestion, fibronectin coated PCL/ nHA scaffold could be a promising candidate for more successful regeneration of bone tissues.

Acknowledgments

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