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Research Article

Strategic Design and Fabrication of Nanofibrous Scaffolds for Articular Cartilage Repair

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Abstract

The loss of articular cartilage is a common defect occurs by accidents, disease, sport injuries and gradual wears; however, it won't be spontaneously repaired as it is an avascular tissue. Recently, cartilage tissue engineering has suggested attractive strategy for regeneration of cartilage tissue. Here, aligned nanofibrous PCL/PLGA scaffolds are fabricated to mimic the aligned fibers of the extracellular matrix of the superficial zone. Bone marrow-derived mesenchymal stem cells (BMMSCs) along with transforming growth factor β1 (TGFβ1), insulin-transferrin-selenium (ITS), ascorbic acid, and dexamethasone are seeded on the scaffolds to investigate the chondrogenesis efficiency using histology and real-time PCR. The expression levels of collagen II and aggrecan are detected which reveal a higher level of type II collagen compared to aggrecan in the presence of differentiating medium. Moreover, the expression of collagen II and aggrecan is significantly higher in the presence of differentiation medium rather than the group without signaling factors. The surface morphology and signaling factors have dominant role in upregulated expression of aggrecan and collagen II, respectively. It is believed that simultaneous application of signaling molecules and aligned nanofibrous PCL/PLGA scaffold enhances the efficiency of chondrogenesis by recapitulating the conditions in the superficial zone of cartilage.

Keywords: Articular Cartilage; Cartilage Tissue Engineering; Aligned Nanofibrous Scaffolds; Signaling Factors; Chondrogenesis

Introduction

The articular cartilage is a connective tissue that consisting of extracellular matrix (ECM) and embedded chondrocytes. Loss of cartilage volume in weight-bearing areas can result from a variety of the causes such as accidents, disease, sport injuries and gradual wears. The defects of cartilage tissue cannot be spontaneously repaired because cartilage is an avascular tissue and there is no lymphatic and nervous systems [1]. Among different therapeutic strategies, recently the simultaneous application of biomaterials and cells in tissue engineering approaches is more attractive for researchers [2,3]. Tissue engineering depends on the use of two or more key elements including cells, signaling molecules or growth factor and supporting material as the scaffolds [4].

The main sources of cells in cartilage regeneration are adipose-, bone marrow- or Wharton's Jelly-derived mesenchymal stem cells (MSCs) and induced pluripotent stem cells [5,6]. The major element with functional relevance differentiation of human stem cells is signaling factors [7,8]. Researches showed that MSCs different sources require the different signaling molecules with various concentrations to induce chondrogenesis [9]. The common factors in the induction of proteoglycan and collagen type II synthesis in chondrogenesis are transforming growth factor β1 as single inducers, insulin- like- growth factors (IGF- 1) and fibroblast growth factor family (FGF) as promoters with TGF-β [10,11]. TGF-β is a multifunctional cytokine which could be added to chondrogenic medium as a classic growth factor. Among different isoforms of TGF, several studies showed that TGF-β1 includes transcription of different target genes that are involved in chondrogenic differentiation of MSCs and their proliferation [10].

The basal chondrogenic culture medium is often supplemented with different complements such as insulin- transferrin- selenium (ITS), dexamethasone and ascorbic acid. ITS regulates the proliferation and matrix synthesis of articular hyaline chondrocytes *in vitro* and efficiently enhances expression of collagen II/collagen I and aggrecan. This while ITS reduces expression of collagen X and MMP 13 which prevent chondrocyte dedifferentiation and hypertrophy of engineered cartilage [11,12]. The addition of 1% ITS to culture medium plus 10% FBS enhances cartilage organization, cell proliferation and extracellular matrix synthesis by chondrocytes [11,13].

Ascorbic acid (vitamin C) reduces iron to the ferrous state which can hydroxylate the proline and lysine involving in the formation of the mature collagen molecule. Several studies have shown ascorbic acid can enhance the synthesis of collagen and aggrecan in the level of transcription and post-transcription as a co-factor in an articular cartilage explant culture [14,15]. Dexamethasone (DEX), a synthetic glucocorticoid, in combination with the growth factors such as TGF-β1 could promote chondrogenic differentiation of MSCs from bone-marrow origin [16].

In cartilage tissue engineering, scaffolds could be manufactured from nanomaterials such as nanofibers that mimic ECM environments which makes the material more biocompatible and biodegradable [17]. The scaffolds for cartilage tissue engineering should be highly porous, non-cytotoxic, chondroinductive, and nonimmunogenic. Polycaprolactone (PCL) is considered as a biodegradable material for scaffold design which promote chondrocyte proliferation and maintain phenotype in tissue engineering. PCL has appropriate permeability and slow degradation rate, but it has a poor hydrophilicity effect [18]. PLGA is another biocompatible polymer including polylactic acid (PLA) and polyglycolic acid (PGA). PLGA can be degraded hydrolytically through de-esterification reactions to monomeric compounds which hydrolyze by natural pathway. Moreover, it has high degradation rate that in combination with other compounds can improve biological properties of scaffolds [19].

In this study, a novel hybrid PCL/PLGA nanofibrous scaffold is fabricated for investigation of chondrogenesis of bone marrow mesenchymal stem cell. The effect of signaling factors, including TGF-β1 and ITS supplemented with ascorbic acid and dexamethasone upon induction of mesenchymal stem cell chondrogenesis on PCL/PLGA nanofiberous scaffold is then investigated. It is assumed that a multifactorial tissue engineering approach to cartilage regeneration includes combining cells and scaffold for creation of a biologically active graft.

Materials and Methods

Isolation of human bone marrow- MSCs and cell culture

Isolation of human bone marrow- MSCs (BMMSCs) was performed as previously described [20]. In brief, to collect bone marrow stem cells, after aspiration of 100 ml bone marrow blood from the iliac bone of the patients, the samples were diluted with (1:1) phosphate-buffered saline/ethylene diamine tetracetic acid (PBS/EDTA, Gibco BRL, NY) and carefully layered over ficoll (1.077 g l-1, Sigma). Isolation of the mononuclear cell layer using Ficoll Histopaque is based on a density gradient centrifugation method at 400 g for 30 min.

Bone marrow mononuclear cells (BMMNCs) were plated in 75 cm2 tissue culture flasks containing high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (10,000 units/mL), streptomycin (10 µg/mL), and amphotericin B (250 µg/mL, Gibco BRL, Scotland). The medium was changed after the first 48 hours to remove non-adherent cells. When Cells were grown to 50% confluence, the cells were detached using 0.05% (w/v) Trypsin (Gibco)-0.53 mM EDTA

and the cells replated at a lower density (about 1000 cells/cm2). Cells were passaged twice or three times and then were used for cell culture experiments.

Fabrication of scaffolds

The PLGA/PCL nanofibrous membranes were fabricated via an electrospinning array. Electrospinning instrument consisting of two sets of distinct syringes and Dual Syringe Pump. The PCL solution was prepared by dissolving 1.2g PCL (Sigma) in chloroform/DMF with ratios of 9:1 v/v with optimum conditions (voltage = 24 kV, distance = 24 cm). The PLGA polymer solution was prepared by adding PLGA powder (0.5g, 0.28 mmol) to 5.2 ml chloroform as solvent. In the following, DMF (1.7 ml) was added to PLGA/chloroform solution after dissolving the granules. (Voltage = 20 kV, distance = 16cm). The mixture was vigorously stirred for 3.5 h at room temperature and loaded into a plastic syringe (22 gauge) with stainless steel needle through an extension tube with a syringe pump. Aligned PCL/PLGA nanofibrous scaffold was fabricated with 60:40 (w/w) % ratios at a rotation speed of 2800 rpm by DC high-voltage power supply (Stem cell Technology Research Center, Iran). Oxygen plasma treatment was enhanced surface hydrophilicity of the scaffolds at 100 W for 30 s (Diener electronics, Germany) to improve adhesion of cells to scaffolds.

Scaffolds characterization

The water contact angles on the surface of scaffolds was assessed by a sessile drop method using an optical bench type contact angle measuring system (Rame- Hart instrument company, Model 100-0, USA) and the scaffold hydrophilicity was determined.

Mechanical properties of PCL/PLGA fabricated scaffolds were measured and analyzed using a 50 mm/min crosshead speed of the mechanical test machine (Instron 5565 A). Samples were cut in a rectangular test strip with 10 mm in width and 60 mm in length and the thickness of the nanofibrous membrane (NFM) were measured by digital micrometer. The stress-strain curve was recorded after using of 0.5 kN force to each specimen.

The tensile strength and elongation of specimens were calculated using the following formula

Tensile strength (MPs) = F/A ----------(1)

F = the maximum load of force (N), A (squared meter) = the cross-sectional area of samples

Elongation (%) = (L-L0) /L0 × 100----------(2)

L0 = the initial length of the samples, L = the elongated length of samples at the break point [21].

The porosity of fabricated electrospun nanofibrous membranes was calculated by Following equation [22].

Porosity = (1 -ρ/ρ0) × 100----------(3)

ρ = density of each hybrid NFM, ρ0 = density of bulk polymer

The reported value was the average of three measurements for five NFM samples.

PCL, PLGA, and PCL/PLGA were analyzed using Fourier transform infrared spectroscopy (FTIR) at the wavelength range of 500-4000 cm-1. The infrared spectra were obtained on a Perkin Elmer Spectrum 100 FT-IR system (Perkin Elmer, USA) to find the chemical state of fabricated nanofibrous membranes.

Chondrogenic differentiation

The PCL/PLGA hybrid nanofibrous scaffolds were punched to fit in a 24- well tissue culture plate with the thickness of approximately 30 µm. The samples were sterilized by incubation in fresh 70% ethanol for 2 h followed by exposure to UV for 40 minutes. The ethanol was aspirated and scaffolds were washed twice with phosphate buffered saline solution (PBS), (2 x 1 ml). Then PBS was removed and replaced with 1 ml cell culture medium for 24h prior to cell seeding. The passage 3 human BMMSCs were seeded with a density of 3×105 cells/cm2 in 30 µl culture medium on the nanofiberous scaffolds. Cell attachment was occurred after 1 h incubation at 37˚C, 95% humidity and 5% CO2. Then, 800 µl chondrogenic differentiation medium was added to the each well (DMEM supplemented with 100 nM dexamethasone (Sigma), 50 µg/mL ascorbic acid 2-phosphate (Sigma), ITS + (6.25 µg/mL insulin, 6.25 µg/mL transferrin, and 6.25 µg/mL selenous acid (Gibco)) with 10 ng/mL TGFβ1 (Peprotech, Rocky Hill, NJ)). The culture media were changed every 3 days.

Cell morphology

Scanning electron microscopy (SEM) was used to assess cell adhesion and morphology of hBMMSC on the hybrid scaffolds after 1 and 21 days of incubation. Each sample was lightly rinsed three times with PBS and fixed with glutaraldehyde (2.5% v/v) at 4˚C for 40 minutes. The hybrid scaffolds were dehydrated in a graded ethanol series (50%, 60%, 70%, 80%, 90% and 100%). The fabricated scaffolds were air dried, gold spatter coated and observed by SEM.

MTT assay

The viability of hBMMSCs seeded on the scaffolds was identified by MTT assay. Briefly, cells were seeded into a 96-well culture plate with a density of 3000 cells/cm2 in 200 µl cell culture medium and grown for 1, 4, 7 and 14 days. After each time point, MTT solution (20 µL of 5 µg/mL MTT in PBS) was added into each well and incubated for another 4 hours. The living cells reduce the tetrazolium dye into purple formazan crystal, mainly by mitochondrial dehydrogenases activities. Formazan crystals were dissolved in DMSO and the optical density of each well was read at 570 nm using a microplate reader.

Real-time PCR assay

Real-time PCR analysis was carried out for assaying the degree of chondrogenic differentiation and hBMMSC maturation on PCL/PLGA scaffolds after 21 days. Expression of cartilage marker genes, including collagen type II, aggrecan, collagen type I and versican was analyzed. Also β-2 microglobulin as internal control gene was used for normalizing the data. The primer sequences used are listed in Table 1. Total RNA extraction was performed by TRIzol reagent (Sigma). cDNA was synthesized based on the manufacturer's instructions (Fermentas) using random hexamer primers and M-MuLV reverse transcriptase (RT).

Real-Time PCR reactions were carried out in triplicate for each synthesized cDNA through MaximaTM SYBR Green/ROX real-time PCR Master Mix (Fermentas, Canada). The expression level of the genes was normalized to β-2 microglobolin. The real-time PCR data was analyzed by the comparative Ct method and the fold changes of expression were calculated as previously described [23].

Histological study and immunostaining

During the cryosection procedure, the cells that have been cultured for 21 days were embedded in an OCT and cut with a microtome at a thickness of 4-5 µm. After staining the sections with hematoxylin and eosin (H and E) and Alcian blue (pH 1.0), histological analysis was performd. Also, cartilage-specific ECM was analyzed by immunohistochemical staining. For this evaluation, sections were incubated with a mouse anti-human Col-II antibody (Abcam) and a horseradish peroxidase (HRP) -labeled secondary antibody (Abcam) for collagen II.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Turkey's test was utilized for comparing differences between means. A probability value of $p < 0.05$ was determined to be statistically significant. Each experiment was repeated at least three times and the results were represented as a mean ± standard deviation (SD).

Results and Discussion

Scaffold characterization

The aligned PCL/PLGA hybrid NFM was fabricated with 60:40 ratio of polymers, respectively. For determining the chemical state of electrospun nanofibrous scaffold FT-IR spectroscopic analysis was performed and the characteristic peaks were demonstrated that hybrid PCL/PLGA was successfully fabricated. As indicated in Figure 1, the bands at 2924 and 2855 cm^{-1} are attributed to C-H stretching vibrations, the two bands at 1730 (corresponding to PCL) and 1760 (corresponding to PLGA) are related to carbonyl (C = O) stretching vibrations whereas the bands at 1480 and 1390 cm^{-1} are assigned to $-CH_2$ bending and C-H bending vibrations, respectively. The bands between 1000-1300 cm^{-1} are related to C-O stretching vibration. The FTIR spectrum shows that no chemical reaction carried out between two biocompatible polymers PCL and PLGA in the hybrid scaffolds.

The contact angles for aligned PCL/PLGA with 60:40 ratio was approximately 125 degree. It is found that porosity of this scaffold was in the range of 66 to 70 percent. Electrospun PCL/PLGA NFM showed tensile strength with values of 24.36 ± 0.28 MPa and 0.613±0.04 and elongation at break of %76.34 ± 2 and %29.44 ± 3 in length and tension, respectively (Figure 2). The results showed that the mechanical properties of the scaffolds are suitable for cartilage differentiation and in the range of articular cartilage tissue [24].

The electrospun nanofibrous membranes possess high porosity with micron-sized pores. Figure 3 shows the SEM images of PCL/PLGA hybrid scaffolds prepared with the ratio of 60:40 of polymers. The fiber diameter of nanofibrous membrane measured by ImageJ software which was in the range of 300-1100 nm. (Figure 3). The SEM images showed that in the hybrid membranes, PCL nanofiber diameters were thicker than PLGA fibers and there was a micrometric distance between the fibers. SEM images also re-

vealed porous structure of nanofibrous scaffold which was interconnected pores that form a three-dimensional network resulted to a high filtration flux.

MTT assay

Figure 4 shows percentage of cell viability of the hBMMSCs cultured on fabricated aligned hybrid nanofibrous scaffolds (mean ± standard deviation) after 1, 3, 5, 9 and 14 days of cell culture. The results showed that the cell viability on PCL/PLGA aligned nanofibrous scaffolds was higher than culture plates used as control, however, the difference was not significant. This indicates that the fabricated scaffolds are fairly biocompatible and could increase cell proliferation which is in agreement with previous reports [4]. The PCL/PLGA nanofibrous scaffolds could most likely mimic the ECM for the cells hence provoke cell proliferation.

Cell morphology

SEM micrographs of electrospun nanofibrous scaffolds along with cellular morphology are presented in figure 3. Analysis of SEM images illustrated that the hBMMSCs are attached to the aligned scaffolds and proliferated after 1 and 21 days of cell seeding. Also cell morphology observations exhibited their phenotypic morphology on nanofibrous aligned scaffolds and cell orientation was along the fibers.

Histological and immunohistochemical evaluations

The cartilage tissue is mainly composed of collagen II and proteoglycan having negative charge which allow the absorption of water molecules and resist against compressive loading. The progenitor of chondrocytes could secret and produce proteoglycan. In the late chondrogenesis, chondrocyte colonies appear and produce and secret collagen type II. In the current study, aligned nanofibrous PCL/PLGA scaffolds which recapitulate the ECM of articular cartilage and signaling factors, including TGF-β1 and ITS supplemented with ascorbic acid and dexamethasone was used. It has been shown that the alignment of nanofibers of scaffold could positively effect chondrogenesis even more than scaffold composition [17]. The scaffold alignment activate the expression of some cartilage specific genes through signaling pathways [25]. Moreover, continuous administration of TGFß1 and ITS increase expression of collagen II, collagen I, aggrecan and reduces expression of collagen X and MMP 13, which could block the expression of specific cartilage genes [11,12,26,27]. Here, microscopic analysis of histological and immunohistochemical sections were performed after 21 days of cell seeding on the scaffolds. Figure 5 shows the morphology of chondrocytes and their ability to synthesize aggrecan and collagen II. The H and E staining showed that the cells in which the nuclei of the cells became blue-purple whereas the cytoplasm become pinkish and the region with a high proteoglycan content became pinkish with bluish aspect. Alcian blue stain sulfated proteoglycan-rich ECM whereas Safranin O, a cationic dye, stain proteoglycans and glycosaminoglycans. The proteoglycans are typically considered as structural components of the cartilage ECM. H and E staining showed spherical shaped cells on the scaffolds in the presence of chondrogenic medium (scaffold + d Medium group). Moreover, Alcian blue staining showed that the proteoglycans secreted from scaffold + d medium group were higher than the scaffold group (cells cultured on aligned PLGA/PCL nanofibrous scaffolds in the presence of DMEM+10% FBS). Safranin O staining also showed the intense distribution of proteoglycans and collagen type II in scaffold + d medium group as compared to scaffold group. Overall it was verified that niche structure of the cells and signaling factors has positive effects on chondrogenesis.

Transcriptional evaluations

Chondrogenic differentiation of hBMMSC was evaluated by measuring the expression levels of related genes after 21 days of cell seeding on the scaffolds in chondrogenic medium using RT-PCR and quantitative real-time PCR. Figure 6 illustrates the values of cartilage-specific gene expression including Aggrecan and Collagen II. The experimental groups consisted of a scaffold group, a scaffold + d medium group, and hBMMSCs cultured in DMEM+10% FBS used as a negative control group. Collagen type II in the matrix of cartilage provides mechanical strength for the tissue. Aggrecan molecules, the other ECM component of cartilage, with their negative charges absorb water molecules, thereby resist compressive loading to cartilage. Real time-PCR results showed an increased expression of aggrecan and type II collagen as the most important proteins of cartilage extracellular matrix in the cells cultured on the PCL/PLGA scaffolds compared to the negative control group. Moreover, the scaffold + d medium group expressed aggrecan and collagen type II even more than scaffold group which verified the synergistic effect of the niche structure of the cells and chemical composition on chondrogenesis. The results showed that the difference in expression of collagen II and aggrecan in the scaffold + d

medium group was more than the scaffold group. This may be due to the important role of the chemical component in the expression of collagen II. The studies have shown that mechanical properties and surface structure of extracellular matrix affects aggrecan expression [28]. Furthermore, it is known that the continuous administration of TGFß would cause the Smad2/3- signaling blockage and a decline in Sox9 expression. This events could lead to an increment in collagen expression and decline in aggrecan expression [26,29]. Moreover, the ITS regulates matrix synthesis of articular hyaline chondrocytes *in vitro* and efficiently increase expression of collagen II, collagen I, aggrecan while reducing the expression of collagen X and MMP 13, which prevent chondrocyte dedifferentiation and hypertrophy of engineered cartilage [11,12]. These are in agreement with the results of the present study.

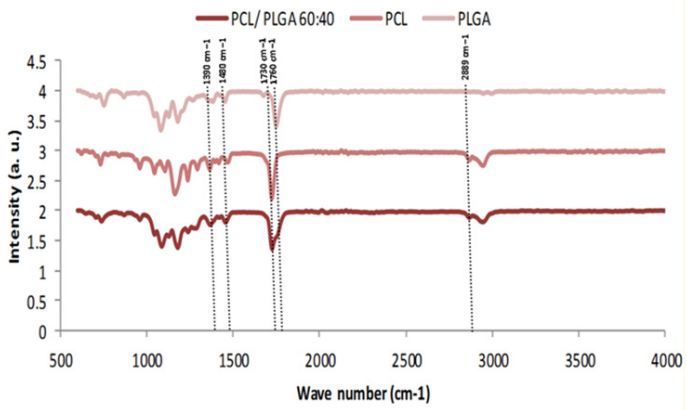

Figure 1: ATR-FTIR spectra of PLGA, PCL, and PCL/PLGA nanofibrous scaffold.

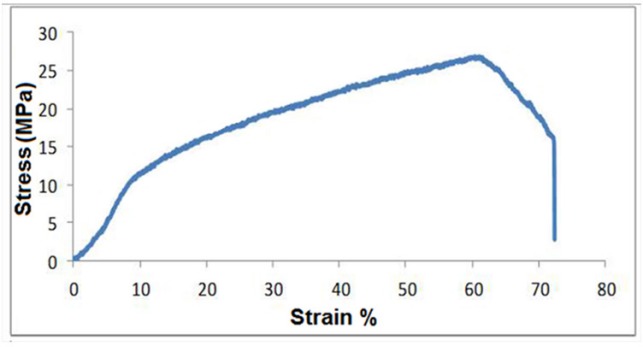

Figure 2: The stress-strain curve of the aligned PCL/PLGA nanofibrous scaffold.

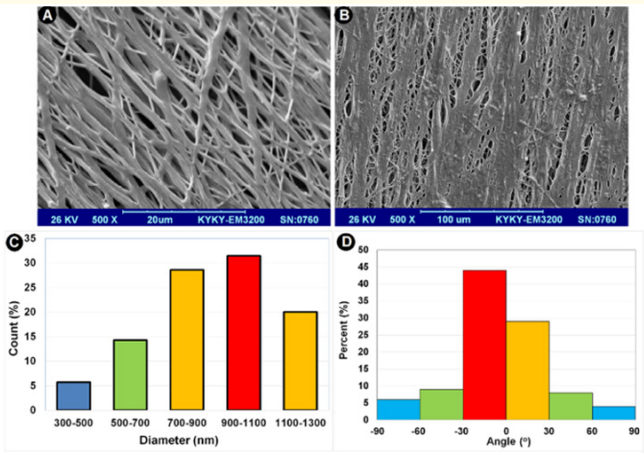

Figure 3: SEM images of PCL/PLGA scaffold with aligned nanofibers fabricated using electrospinning (A), MSCs after 21 days of incubation on the scaffold (B). The distribution of nanofibers of the scaffold having different diameters (C), and the distribution of the fiber angles in aligned fibrous scaffolds compared to their major axis (D).

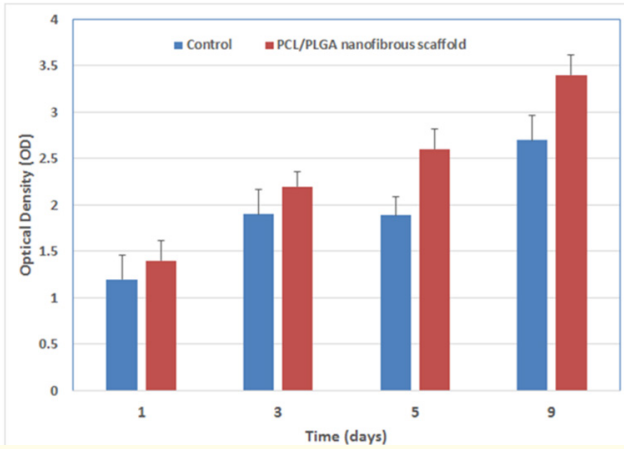

Figure 4: MTT cell proliferation of MSCs on the plasma-treated aligned PCL/PLGA scaffold. Culture plates used as a control.

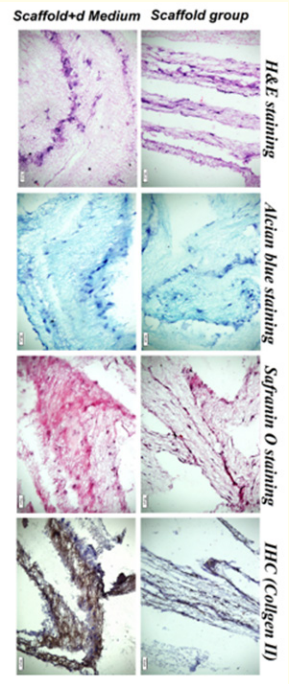

Figure 5: H and E, alcian blue, safranin O, and IHC staining of the cells cultured on the scaffolds with basal medium (scaffold group) and with the chondrogenic differentiating medium (scaffold + d medium group).

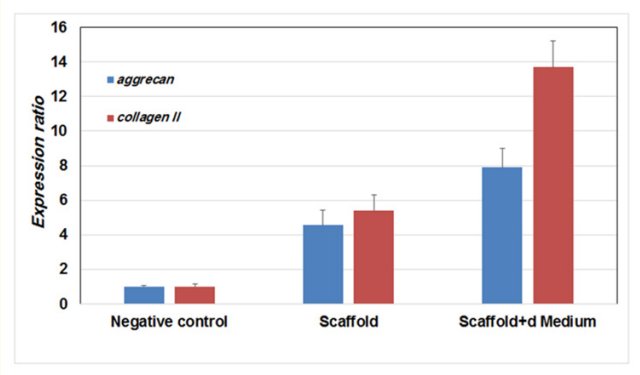

Figure 6: The result of Real-Time PCR presenting relative expression of cartilage-specific genes consist of Aggrecan and Collagen II. Cells cultured in tissue culture plate without differentiating medium or with basal medium were used as negative control (*P < 0.05).

Genes	Forward sequence 5'--> 3'	Reverse sequence 5'--> 3'	Product size (bp)	Tm
aggrecan	TGT CAG ATA CCC CAT CCA C	CAT AAA AGA CCT CAC CCT CC	85	60°C
collagen II	GGT CTT GGT GGA AAC TTT GCT	GGT CCT TGC ATT ACT CCC AAC	79	60°C
h-β-2 M	ATG CCT GCC GTG TGA AC	ATC TTC AAA CCT CCA TGA TG	90	60°C

Table 1: The list of primers used for real-time PCR analysis.

Conclusion

In this study, aligned nanofibrous PCL/PLGA scaffolds were fabricated to mimic the aligned fibers in the extracellular matrix of the superficial zone in cartilage tissue. To provoke chondrogenesis the hBMMSCs were cultured on the aligned fabricated scaffolds in the presence of signaling factors TGFβ1, ITS, ascorbic acid, and dexamethasone for 21 days. The results showed that the chemical components had a dominant impact on the collagen II expression. The application of signaling factors on the aligned scaffolds also showed that chondrogenesis and cartilage-related ECM secretion were improved. The results could be employed to find the effective parameters for enhanced chondrogenesis for cartilage regeneration therapy.

Acknowledgments

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Conflict of Interest

We state that no conflict of interest exists for this work.

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