Comparing Behavior of Chondrocyte Cells on Different Polyhydroxybutyrate Scaffold Structure for Cartilage Tissue Engineering

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ABSTRACT

Introduction: As the ability to repair cartilage tissue in body is limited, finding a suitable method for cartilage regeneration has gained the attention of many scholars. For this purpose, scaffold structure and morphology, along with cell culture on it, can be a novel method to treat cartilage injuries, osteoarthritis.

Methods: In this study, polyhydroxybutyrate (PHB) is selected as the scaffold. Firstly, PHB (6% w/v) solution was prepared using chloroform solvent by employing solvent and electrospinning methods. With regard to phase studies, morphology, and specifying agent groups, we used specific characterization devices such as Fourier transform infrared spectroscopy (FTIR). To compare the behaviour of cellular scaffolds, they were divided into 2 groups of scaffolds, and the chondrocyte cells were cultured. To perform phase studies, analysis of MTT and trypan blue were carried out for measuring the viability and attachment on the surface of the scaffold, and the specification of scanning electron microscope (SEM) was employed to determine the morphology of the cells.

Results: Through performing MTT test on the first, third and seventh days, it was found that these types of scaffolds are significantly different from those in the control group ($P<0.05$). Scanning electron microscope (SEM) indicates good attachment of chondrocytes on all scaffolds. Results obtained from trypan blue exclusion test also indicated an increase in cell attachment on scaffolds.

Conclusion: Comparing cell behavior on two scaffolds indicates that cell attachment, cell growth and proliferation, and cell migration on the electrospun scaffold is better than the scaffold provided by using solvent casting approach.

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1. Introduction

Cartilage tissue has little ability for self-repair because it has no blood vessels and nerves. Injury to the cartilage, for any reason, leaves a permanent and chronic lesion, which can result in disability or deformation of damaged organ depending on the case. Currently, different types of cartilage implantations such as autograft, allograft, and xenograft are being used to treat massive cartilage damages. However, each method has some disadvantages, along with its advantages, that can make their use problematic. One of the treatment methods is using chondrocytes of the patient, but it has many limitations such as decline of proliferation capability of chondrocytes in old people. On the other hand, chondrocytes isolated from body more toward fibroblast phenotype after monolayer culture and expression of effective factors in cartilage-building would be changed in them.

Tissue engineering is a novel approach, which has been considered over the years to surpass the limitations and repair injured tissues [1, 2]. It includes growing special cells in an extracellular domain to create structures similar to natural organs or tissues. In this method, 3-D scaffold is usually applied to simulate natural extracellular matrix, initial cell attachment, and cell proliferation [3]. The main purpose of tissue engineering is making tissue structures that can reconstruct structure and functions of injured tissues after implantation. To achieve this goal, choosing a proper cell is an important issue as mentioned before. However, a cell cannot create functional structures by itself. The reality is that a cell can use its maximum potential to generate a tissue that is cultured on the scaffold. Applied scaffoldings in tissue engineering have been designed in a manner that provide the required 3-D environment to protect and conduct cell processes like migration, proliferation, and differentiation to generate functional tissues [4].

A lot of biodegradable polymers and ceramics have been used as biocompatible scaffolds in tissue engineering [5, 6]. The mechanism of degradation of biodegradable polymers in the body is different based on their molecular structure, although in most cases, degradation products of the polymers are also biodegradable and cause no damage to living tissues. Among the degradations, one can refer to bulk degradation and surface degradation. In the former, polymer begins to be degraded from inside, but in surface degradation, it is degraded layer by layer from the surface [7]. Among biodegradable polymers, polyhydroxyalkanoates or PHAs are considered because of their superior biodegradability and biocompatibility as well as suitable physical and mechanical properties [8, 9].

In our study, we used solvent castings and electrospinning methods to make PHB scaffolds. Some studies are conducted on the cartilage restoration using electrospinning scaffolds. Li et al. [10] provided a nanofiber scaffold of polycaprolactone and examined the ability of the scaffold in cartilage generation using mesenchymal stem cells. The scaffolds produced by them had uniform fibers with diameter of 700 nm running random directions in the scaffold. Results obtained from 21 days culture of mesenchymal stem cells on scaffolds in the presence of growth factor TGF-β1 indicated differentiation of these cells from chondrocyte phenotypes. In Thorvaldsson et al. [11] study, scaffolds were made from microfibers of polylactic acid covered by nanofibers of polycaprolactone. The study was conducted with the aim of controlling the size of porosity and cell infiltration and using advantages of nanofibers in cell growth. In this study, scaffolds with porosity of 95% and 97% were obtained.

To investigate cell behavior on scaffolds, human chondrocyte cells are seeded on the scaffold. Results obtained from the study indicate that pore size has a significant effect on cell infiltration inside the scaffold. Moreover, the presence of nanofibers in the structure increases the bioactivity of scaffolds. Zheng et al. [12] conducted a study on gelatin/polycaprolactone electrospinning scaffold in cartilage tissue engineering. Their aim was assessing the effect of different ratios of gelatin to caprolactone in the structure on the attachment and proliferation of chondrocytes.

The obtained results indicated that scaffolds with more gelatin can increase attachment and proliferation of chondrocytes and formation of cartilage tissue in the first 3 weeks of implantation. However, after 12 weeks, new cartilage tissues were formed in all samples and no significant difference was observed among samples. In another study by Sombatmankhong et al. [13], 3-D fiber scaffolds were made of polyhydroxybutyrate/polyhydroxybutyrat-co-hydroxyvalerate (PHBV) alloy using electrospinning method. In this study, fibers with diameter of 2.3 to 4 μm were obtained. Produced fiber scaffolds had more hydrophilicity than the films from the same material prepared for comparison [14]. Moreover, tensile resistance failure of alloy fibers compared to pure fibers of PHB and PHBV was obvious. Indirect toxicity evaluation using rat fibroblast cells (L929) indicate that the fibers had no negative effects on cells [15].

This study mainly focuses on a scaffold design for cell evaluation of the seeded scaffolds. To optimize the seeding efficiency and observe the cell proliferation into the inner structure, we developed a special design for the scaffold. The objective of the design was to maximize its surface and facilitate the seeding process to enhance cell adhesion and good supply of the scaffold interior with medium. The cultivation of cells seeded onto the scaffolds was carried out under static and dynamic conditions. Cell evaluation was carried out on days 3 and 7. Cell adhesion on the designed structure was also analysed. Additionally, cell growth inside the bulk material and cell morphology were examined. Comparing cell behavior on the two scaffolds and using PHB alone (without combination with other materials as main scaffold to culture chondrocytes) can be regarded an innovation of our study. After making scaffolds, porosity percentage was determined. Finally, to investigate attachment of chondrocytes, cell culture and SEM imaging were employed [16].

2. Materials and Methods

Materials

We used materials listed in Table 1. In addition, the employed cell line was chondrocyte cell line manufactured by Tehran Pasteur Institute.

Scaffold preparation

In this study, we used Haji Ali applied method to prepare scaffold using solvent casting [17]. First, 6% w/v polyhydroxybutyrate solution was prepared, and then it was entirely solved in chloroform at 55°C-60°C for 12 h. As the study has considered salt/polymer weight proportion of 9 to 1 to create porosity, as per 0.6 g polymer, 5.4 g salt (particle size of 212-250 µm) was weighed and added to the solution. The salt in polymer solution was properly mixed by vertex to distribute salt particles, and then it was poured immediately in 8-cm Petri dish (here as a frame). The mixture was placed in outside environment for 48 h to bring out the solution uniformly and slowly. Next, a white trapped disk formed of polymer and salt particles remained. The sample was washed using deionized water. Washing samples and bringing out salt particles from them lasted 5 days. For final drying, samples were placed in vitro for 24 h, and then for 48 h in outside temperature under the pressure of 0.8 kPa. By the end of solvent casting process, washing particles and drying samples, scaffolds were prepared for evaluating and performing tests.

We used Heydar Khan Tehrani method to prepare scaffold using electrospinning method [18]. In this method, polymer solution was used with a similar mechanism to solvent casting method, but to provide PHB solution, in addition to chloroform solvent, deodorized methanol fraction (DEMEF) method (8:2) was also used. Produced PHB solution was placed on horizontal injection pump, and then attached to the tip of a 21-gauge syringe.

Physical properties

Porosity measurement

In order to determine the percentage of porosity, water replacement formula was applied as follows [19]:

$$P(\%)=\frac{M_w-M_d}{V_a}\times 100$$

where P, M_w, M_d, and V_a are "porosity percentage," "wet mass of scaffolding", "dry mass of scaffolding", and "apparent volume", respectively.

Fourier transforms infrared spectrometer

Infrared spectrometry is carried out based on radiation absorption and studying the vibrionic transitions of molecules and multi-atomic ions. This method is used as a powerful and developed method to determine the structure and amount of chemical compounds. It is mostly employed to identify organic compounds because their spectra are usually complicated and have large number of maximum and minimum peaks, which can be employed for comparative purposes. This test is appropriate for polymer samples [19].

Cell culture

Sterilization of scaffold

For sterilization, each group was placed in a 9-cm plate. Scaffolds were sterilized in 75% ethanol for 3 h under ultraviolet radiation for 1 h, and then they were immersed in DMEM (Dubecco's Modified Eagle Medium, Invitrogen, California, USA) overnight.

Cell seeding

The human osteoblast cell lines C28/I2 (Species: human; Tissue: cartilage) were purchased from Pasteur Institute of Iran (IPI) (Tehran, Iran). C28/I2 cells were cultured in RPMI1640 (Roswell Park Memorial Institute) supplemented with 10% fetal bovine serum (FBS), and 100 mg/mL streptomycin (Jinke Biotech). Cells were incubated in a 5% CO_2 incubator at 37°C, and were harvested by trypsinization followed by addi-

tion of fresh culture medium to create a new single cell suspension. When the cells reached the plateau phase of growth, they were harvested by trypsinization, followed by addition of fresh culture medium to create a new single cell suspension with desired seeding cell number per 100 mg [20]. Inoculation was performed in polystyrene 24-well flat-bottom culture plates. Scaffolds were placed in the center of the wells added with 1 mL cell suspension to allow full attachment of cells to scaffolds. Cultivation was conducted for 3, 7, 14, and 28 days. Culture media were changed every 4 days [21].

Cell viability

For attachment study, C28/I2 was allowed to attach on scaffold and all of the scaffold specimens for 8 and 24 h, respectively. At each specified seeding time, the viability of the attached cells was quantified by MTT assay [22]. Each sample was rinsed with phosphate buffer saline (PBS; Sigma–Aldrich, USA) to remove unattached cells prior to MTT assay. Since no studies were carried out on the expression of attachment proteins or the strength of the attached cells, this evaluation only served as a qualitative measure of the cell attachment study. The viability of the cells was again quantified by MTT assay [23].

Quantification of viable cells (MTT assay)

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 the formed purple formazan crystals is proportional to the number of viable cells. First, each sample was incubated at 37° C for 1 h with 250(10) µL/well of MTT solution at 0.5(5) mg/mL without phenol red for a 24-well (or 96-well) scaffold. After incubation, MTT solution was removed. A buffer solution containing dimethyl sulfoxide (DMSO; Carlo Erba, Italy) of about 900(100) µL/well and glycine buffer (pH=10) of about 125(5) µL/well was added into the wells to dissolve the formazan crystals. After 10 min of rotary agitation, the solutions were transferred into a cuvette and were placed in a thermo Spectronic Genesis10 UV–vis spectrophotometer, from which the absorbance at 540 nm representing the number of viable cells was measured [24].

Cell attachment

Trypan blue

Chondrocyte cells from line of C28/I2 were used for the assays. Briefly, osteoblast cells were released from nearly confluent cultures with trypsin (Sigma-Aldrich Chemie GmbH, Germany), and were washed with a solution containing trypsin inhibitor (Sigma-Aldrich Chemie GmbH, Germany). After 30 min, non-bound cells were washed away, and attached cells were fixed, stained, and counted.

Scanning electron microscopy examination

It was tested in accordance with the ISO 10993-5 standards and carried out directly. To study the cell morphology and adhesion, a cell suspension containing 20000 cells with UV light was seeded on the surface of sterile scaffold. The specimens were washed twice by phosphate buffered saline (PBS) and immersed in PBS containing 3% glutaraldehyde (pH=7.4) for 4 h. Then, they were dehydrated in increasing concentrations of ethanol (from 30%, 50%, 70%, 90%, and 95% to 100%), followed by lyophilization. They were then mounted on aluminum stumps, coated with gold in a sputtering device for 1.5 min at 15 mA and examined under a scanning electron microscope (KYKY-2800, Tehran University-Iran) [25-26].

Statistical analysis

Significant differences were determined with ANOVA test (1-way analysis of variance) to compare the means of different data sets. Statistical significance was accepted at 0.05 confidence level. The results were reported as mean ±MTT and trypan blue.

3. Results

Assessment of density and porosity of scaffolds

As mentioned before, porosity is an important parameter for cell scaffolds. To measure density and porosity of scaffolds in solvent casting method, thickness of samples was determined using a thickness gauge device (Table 2). Mass density for PHB polymer was considered to be 1.24 g/mL [27]. It should be mentioned that two iterations were considered for each sample and measured values were reported as average. According to Table 2, porosity of PHB samples made by both methods is higher than 80%, indicating that samples have high and suitable porosity for cell attachment and proliferation. Porosity percentage of produced scaffolds through electrospinning method was measured using MATLAB software.

FTIR of PHB

This test is appropriate for polymer samples. In Figures 1-4, 1555cm^{-1}, 1128-1 and 1176^{-1} peaks are resulted from

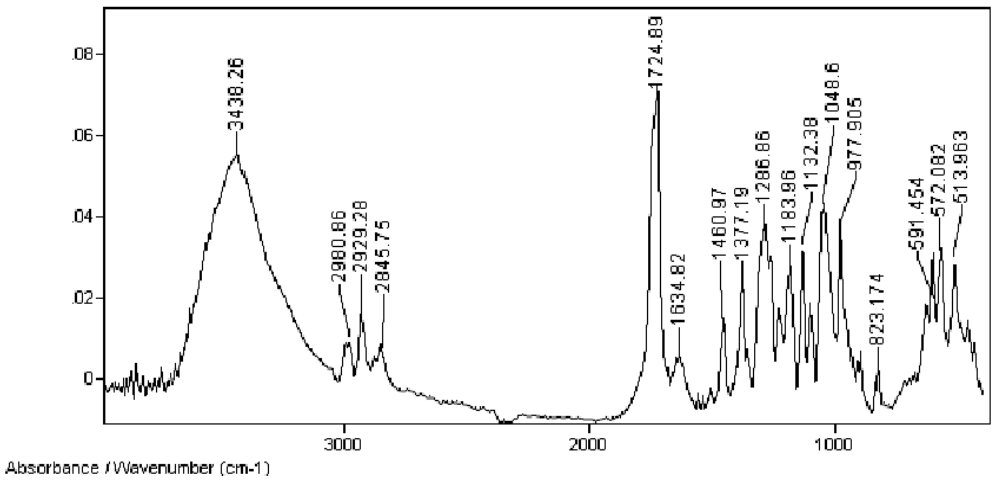

Figure 1. FTIR absorption spectrum of PHB.

the C-O bond in PHB; and 1128cm^{-1} and 1176^{-1} peaks are related to the asynchronous and synchronous stretching vibration of C-O bond, respectively. CH3 groups create an acute peak at 1377cm^{-1}, which is the result of the stretching vibration of this bond. At wave number of 1724cm^{-1}, a very clear and acute peak, which is the result of the stretching vibration of carbonyl groups, is noticed.

Since the nature of the scaffold and the method of its creation, and on the whole, its morphology are effective on cellular behaviour, the selection of polyhydroxybutyrate polymer using two manufacturing methods; namely, electrospinning and solvent casting in this research shows very good results with regard to cell culture and chondrocyte cells.

Trypan blue test results for cell attachment

Total number of attached cells to surface of scaffolds was estimated using Equation 2 and then analyzed in Excel software. As illustrated in Figures 1 and 2, the number of cells attached to scaffolds is approximately 70% to 80% of total cells poured on scaffold surface. This indicates that the scaffolds are good beds for cell attachment (CONT=control sample without the presence of scaffold). By comparing Figures 1 and 2, we can say that cell attachment on day 7 is more than that on day 3 for both scaffolds. This indicates suitability of the scaffolds for cell attachment and performing cell culture tests.

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MTT test results for cell proliferation

Metabolic activity of cells on scaffolds was determined using MTT analysis. Obtained results have been

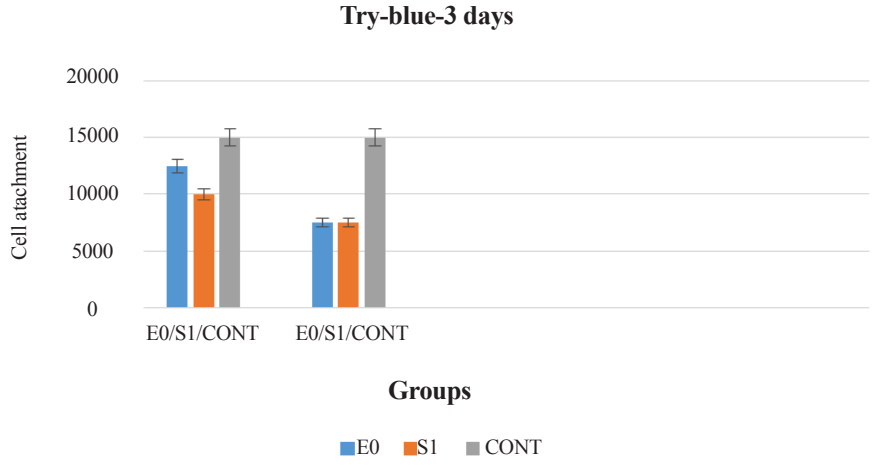

Figure 2. Trypan blue diagram for day 3.
E0: cell+scaffolds made by electrospinning, S1: cell+scaffold made by solvent casting.

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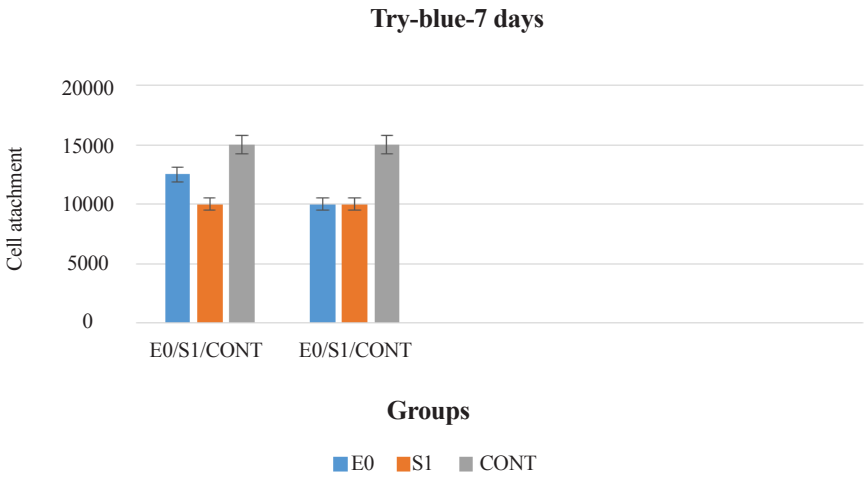

Figure 3. TB diagram for day 7.
E0: cell+scaffolds made by electrospinning, S1: cell+scaffold made by solvent casting.

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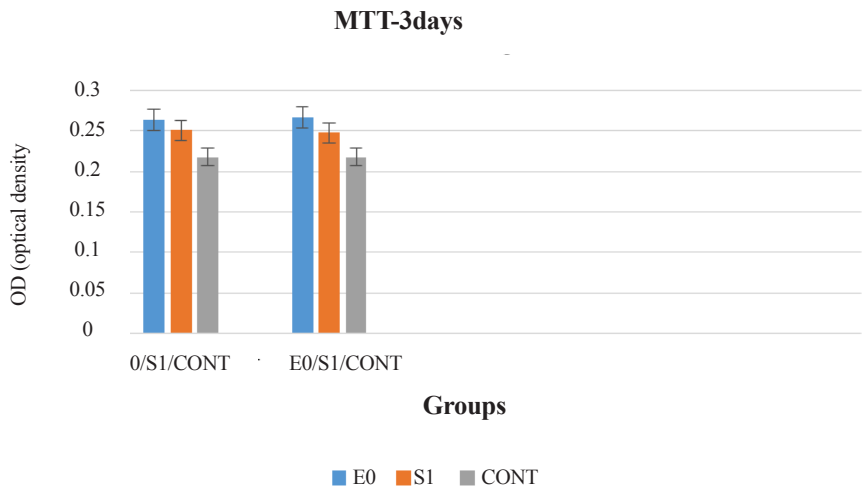

Figure 4. MTT diagram for day 3.
E0: cell+scaffolds made by electrospinning, S1: cell+scaffold made by solvent casting.

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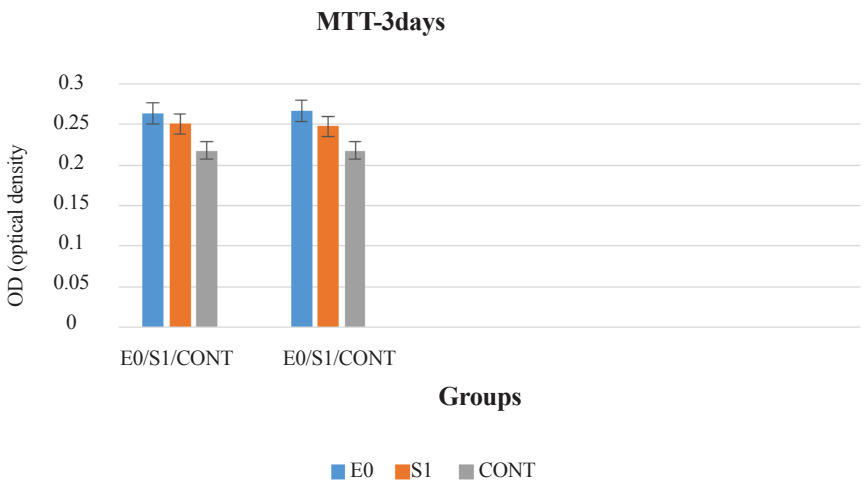

Figure 5. MTT diagram for day 7.
E0: cell+scaffolds made by electrospinning, S1: cell+scaffold made by solvent casting.

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Table 1. The materials used in the study.

Company	Country	Material
Sigma Aldrich	Germany	Natural origin polyhydroxybutyrate (molecular weight of 300000)
Scharlau	Spain	Chloroform ($CHCl_3$) (Purity: 99.47%)
Merck	Germany	Sodium chloride (NaCl) (Purity: 99.5%)
Merck	Germany	96% ethanol (C_2H_5OH)
Gibco	USA	RPMI 1640 Medium
Gibco	USA	Phosphate buffered saline (PBS)
Gibco	USA	Fetal bovine serum (FBS)
Gibco	USA	Trypsin/EDTA
Gibco	USA	Penicillin/Streptomycin
Promega, Madison	USA	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)
Merck	Germany	Glutaraldehyde
Merck	Germany	Dimethyl sulfoxide (DMSO)
Sigma Aldrich	USA	Trypan Blue
Sina Gen	Iran	Distilled water
Sina Gen	Iran	Deionized water

presented in Figures 3 and 4. As it is obvious, increase in culture time has resulted in increased chondrocytes proliferation on scaffolds. Moreover, percentage of survived cells on the electrospinned scaffolds is more than scaffold made by solvent casting. In addition, obtained values have been analysed using SPSS. The results indicate that the relationship between survival of cells on scaffold surface has correlation coefficient of 75% with P value under 0.05, i.e. this correlation is not random. Certainly, $P\text{-value}_{E0}$ for samples made by electrospinning method is 0.023 and $P\text{-value}_{s1}$ for samples made by solvent casting is 0.033. Through comparing P-values obtained from samples with control group, it could be found that there was a significant difference between the groups and control group (P-value<0.05).

Morphology of scaffolds

As it is obvious in SEM images, all scaffolds have had good biocompatibility for cell culture (Figures 5-12). Cells have a tendency for colon formation in day 1, and as time goes by, they began to discharge redundancies. Therefore, PHB creates a good interaction between scaffold and cells with better attachment and growth of cell redundancies, and finally more migration and expansion of cells. As mentioned before, lack of cell penetration and migration deep inside the scaffolds are basic problems with tissue engineering. Through comparing obtained SEM images, it could be found that cell attachment, migration, and penetration on electrospun scaffold is better than solvent casting-based scaffold

Table 2. Density and porosity of scaffolds made by electrospinning (E0) and solvent casting (S1).

Sample	Sample Weight (g)	Thickness (mm)	Area (mm^2)	Mass Density (g/mL)	Scaffold Density (g/mL)	Porosity Percentage (%)
PHB/E0	2.384	0.51	20	1.24	0.234	82.48
PHB/S1	3.458	0.87	20	1.93	0.437	89.67

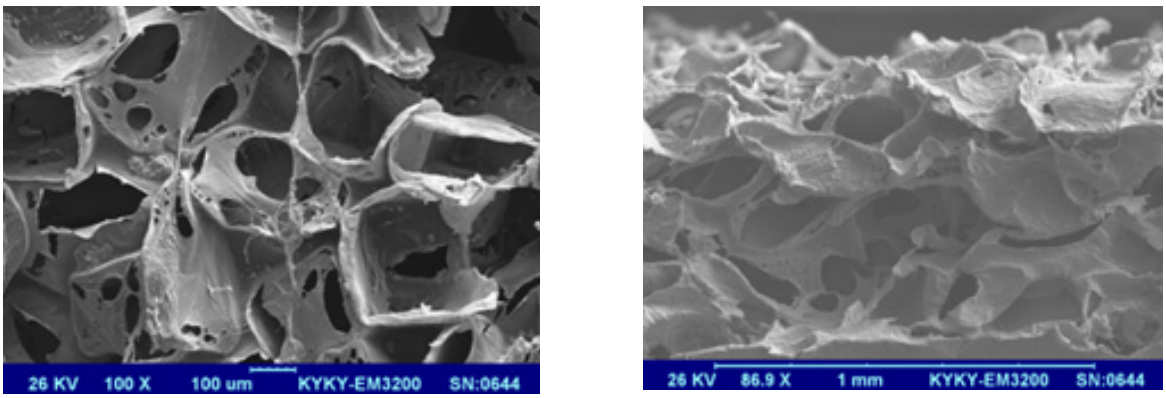

Figure 6. SEM images of scaffold surface made by solvent casting.

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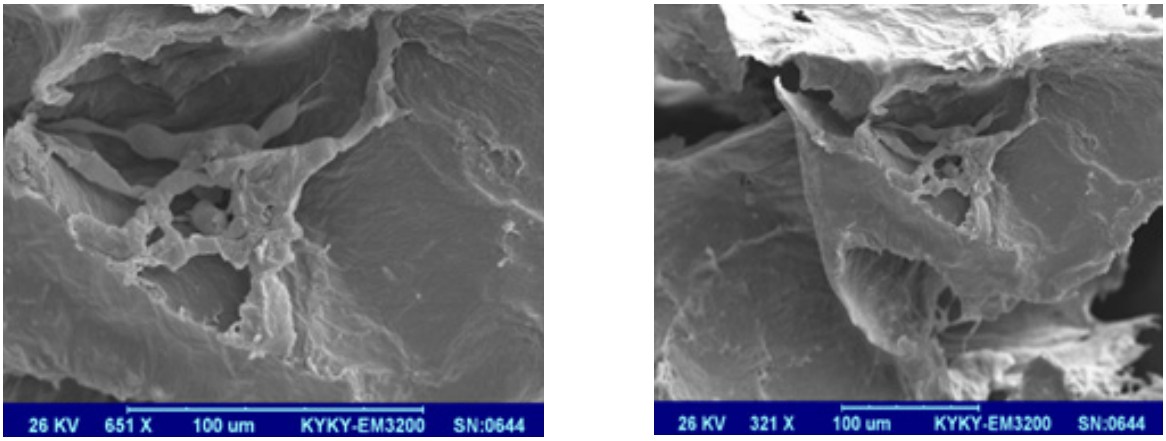

Figure 7. SEM images of cell attachment to scaffold surface made by solvent casting.

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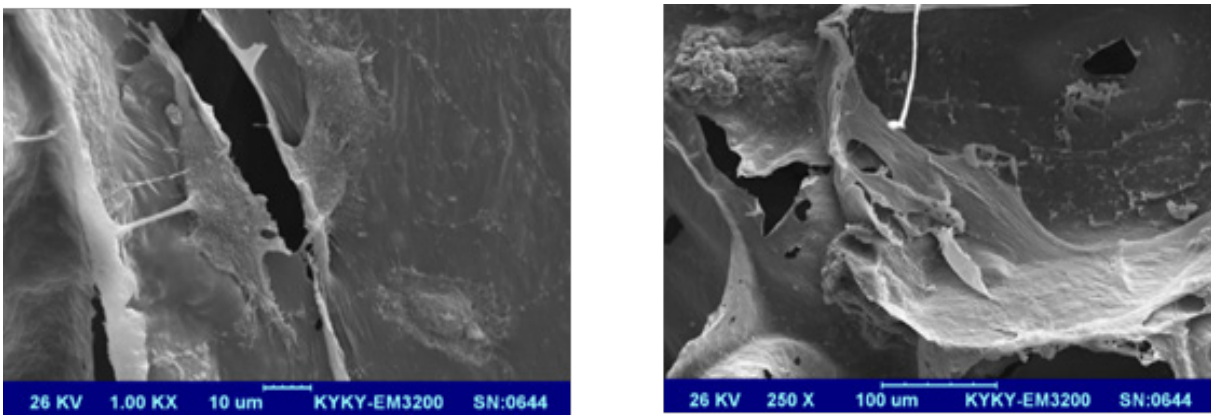

Figure 8. SEM images of cell penetration inside structure of scaffold surface made by solvent casting.

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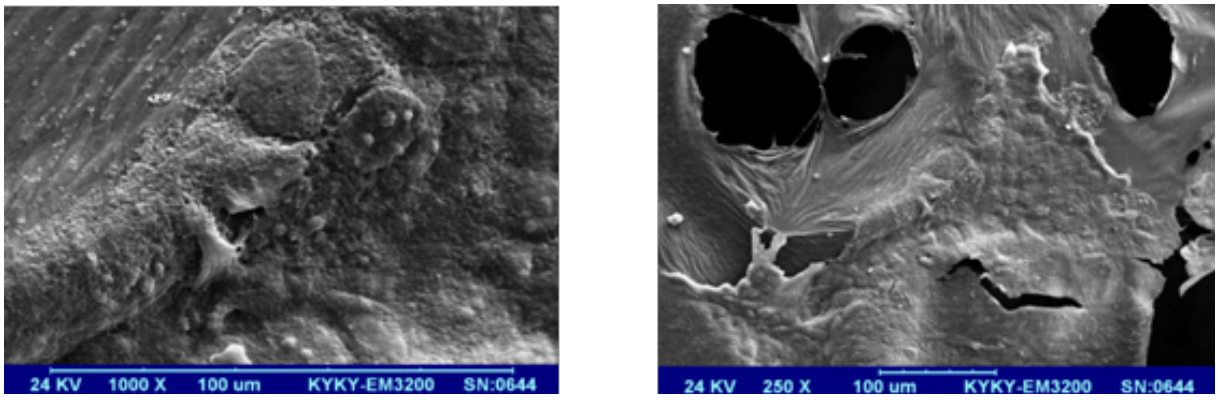

Figure 9. SEM images of cell penetration inside structure of scaffold surface made by solvent casting. ANATOMICAL SCIENCES

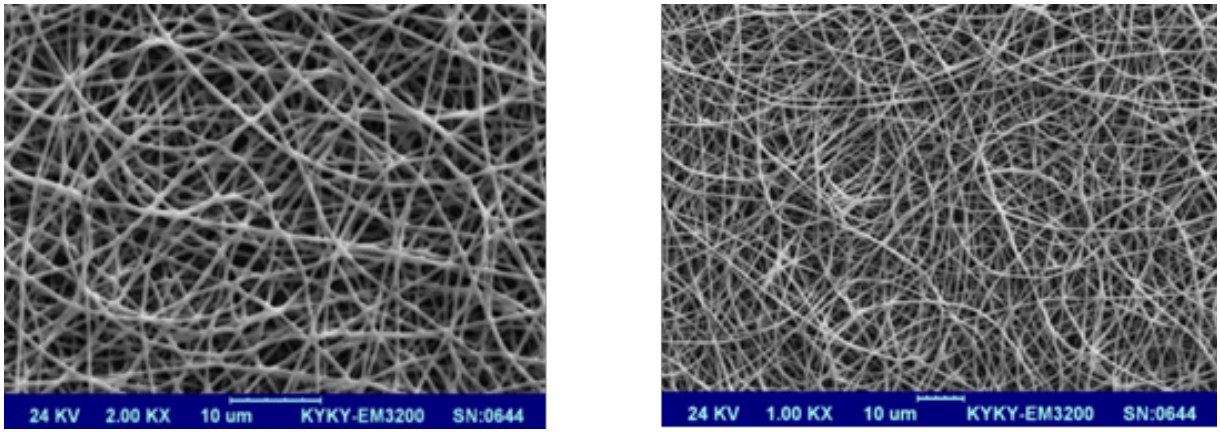

Figure 10. SEM images of scaffold surface made by electrospinning method. ANATOMICAL SCIENCES

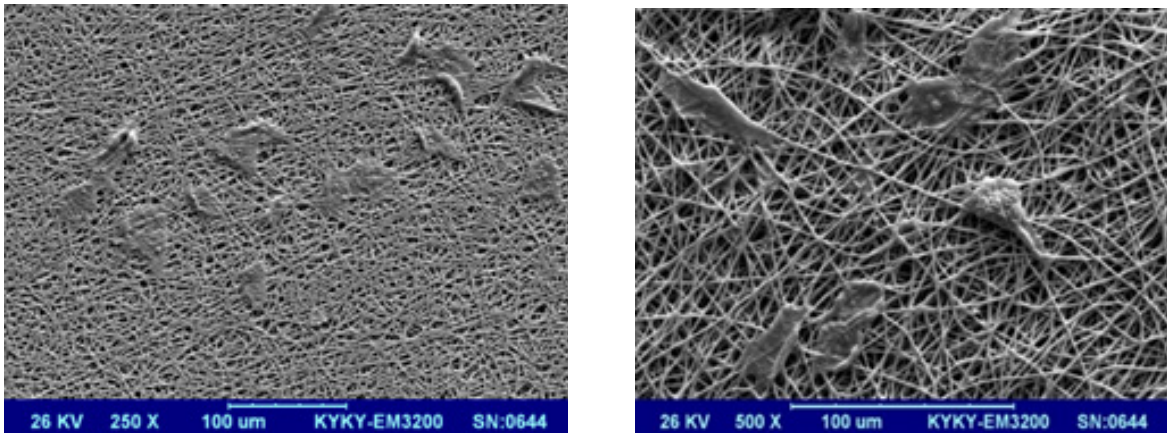

Figure 11. SEM images of cell attachment on scaffold surface made by electrospinning method. ANATOMICAL SCIENCES

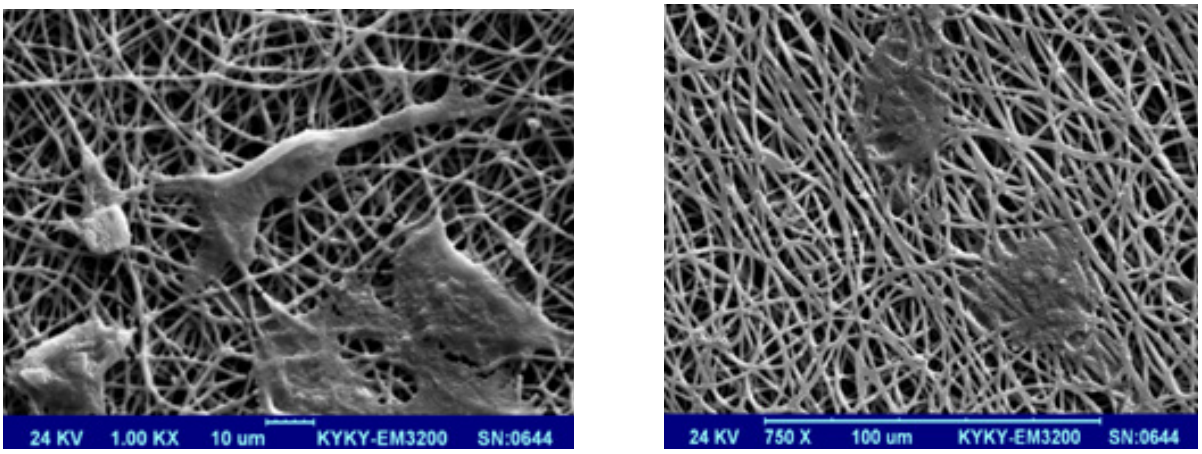

Figure 12. SEM images of cell penetration inside scaffold structure made by electrospinning method. ANATOMICAL SCIENCES

because of suitable surface topography, its nanoscale, open porosity, pore continuity, and link to each other, that makes the surface to volume ratio high.

4. Discussion

The study results were obtained from two fields of construction and assessment of PHB using two methods of solvent casting and electrospinning and through optimization of factors affecting the process. The scaffolds have been evaluated with regard to porosity and attachment behavior of chondrocytes on them by trypan blue, MTT, and SEM tests. By analyzing the results, it was found out that attachment of chondrocytes on scaffold made by electrospinning method is better than solvent casting method because of its nanoscale, higher surface energy, and good topography. Moreover, because of continuity of porosities in electrospun scaffold, migration and proliferation of chondrocytes on this scaffold are more than that on the scaffold made by solvent casting method. Because of high percentage of porosities created on scaffold by solvent casting and good growth and proliferation of chondrocytes on the scaffold, it can be considered as a good bed for extracellular matrix of cartilage. In addition, chondrocytes show good attachment on the scaffolds.

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Conflicts of Interests

The authors declared no conflict of interest.

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