

Evaluation of biocompatibility of random or aligned electrospun polyhydroxybutyrate scaffolds combined with human mesenchymal stem cells

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Abstract: Polyhydroxybutyrate (PHB) is a polymer used to restore tissues or regenerate bones. However, its compatibility with human bone marrow-derived mesenchymal stem cells (MSCs) has not been examined. PHB membranes of random (r-PHB) or aligned (a-PHB) electrospun nanofibers that generate bone and spinal axon scaffolds were combined with human MSCs. The adhesion and proliferation of cells on these membranes were examined. The orientation of cells on PHB membranes was analyzed by confocal microscopy and scanning electron microscopy (SEM). The MSCs maintained their characteristic properties on the membranes, adhered to the membranes, and preserved their viability. The cell morphology was different when they were grown on differentially designed matrices. Cells expanded on the a-PHB membrane and showed fibroid-like morphology. Conversely, cells interacting with the r-PHB membrane were located homogeneously and demonstrated polygonal morphology. The adhesion and proliferation of human MSCs was higher on the a-PHB membrane than on the randomly oriented one. Fiber orientation influenced the phenotype and biological behavior of human MSCs. This property may be useful in the selection of specifically designed scaffolds for the desired tasks. The results of this preliminary study indicated that PHB membranes designed for bone or nerve tissue engineering are compatible with human MSCs.

Key words: Polyhydroxybutyrate, electrospinning, bone marrow, mesenchymal stem cell, biocompatibility, tissue engineering

1. Introduction

Scaffolds are used to aid in tissue engineering and to provide appropriate micro- and macroenvironments for cell expansion and/or differentiation to enhance recovery. Many types of scaffolds have been fabricated and designed for tissue engineering, such as membranes, fabric, sponge-like scaffolds, and gels. Electrospinning is the simplest method to create a porous scaffold by random or aligned arrangement of nano- or microfibers (McLane et al., 2013; Yu et al., 2013). Several scaffolds were reported to have many outstanding properties (e.g., high porosity) that mimic those of the extracellular matrix (Takahashi and Tabata, 2003; Li et al., 2005). Modification of the surface properties of biomaterials is important for controlling the biological activities of cells such as cell adhesion, migration, proliferation, and differentiation (Palmquist et al., 2010). On the other hand, this process is highly regulated by the extracellular microenvironment and the cell type (Lutolf and Hubbell, 2005). Recently, there

has been increasing interest in constructing desirable extracellular microenvironments on the surface of biomaterials in order to obtain optimal cell and/or tissue responses. Studies with polyhydroxybutyrate (PHB) membranes and mesenchymal stem cells (MSCs), which have high differentiation capacity, showed that PHB membranes induced MSCs to osteogenic differentiation in vitro and in vivo (Wang et al., 2004; Wollenweber et al., 2006; Rentsch et al., 2010)

PHB is the most thoroughly investigated member of the PHA family and has shown good biocompatibility with different cell types, including mouse fibroblast cell lines (Yang et al., 2002), chondrocytes (Saito et al., 1991), osteoblasts (Köse et al., 2003a, 2003b), endothelial cells (Shishatskaya and Volova, 2004), and gastrointestinal cells in rats (Freier et al., 2002). Although PHB is inherently biodegradable and biocompatible, the use of PHB is significantly limited in biomedical applications due to several characteristics, such as brittleness, rigidity, and low

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mechanical properties (Engelberg and Kohn, 1991; Misra et al., 2006). Studies have shown that the degradation rate of PHB in vitro and in vivo is slow relative to that of biodegradable polymers such as poly(lactic acid), poly(glycolic acid), and polydioxanone (Yasin and Tighe, 1992). It has been evaluated as a suitable material in medicinal applications, including controlled release systems, wound dressings, surgical sutures, orthopedic devices, tissue engineering, and skin substitute materials (Galego et al., 2000; Chen and Wu, 2005; Wu et al., 2009).

In vitro studies with PHB scaffolds for nerve tissue engineering showed that PHB membranes induced neural progenitor cells and embryonal cell lines to neuronal differentiation (Xu et al., 2010; Khorasania et al., 2011). PHB scaffolds have been used as a guidance channel in in vivo studies. PHB scaffolds supported nerve survival and axonal regeneration in both spinal cord and peripheral nerves (Novikov et al., 2002; Young et al., 2002; Novikova et al., 2008).

Two studies focused on the compatibility of MSCs with PHB. These studies revealed that poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) stimulated MSC attachment and growth (Hu et al., 2009) and osteogenic differentiation (Wei et al., 2009). A recent study confirmed that peptide-functionalized and aligned PHB nanofibrous scaffolds may serve as a potential scaffold for Schwann cell regeneration (Masaeli et al., 2014). Fiber orientation is assumed to influence the phenotype and genotype of MSCs on PHA matrices. Aligning PHA nanofibers using electrospinning is a new approach, and its outcomes, when combined with MSCs, are less studied.

The continuous proliferative capacity of MSCs, their differentiation potential, and their cell surface molecule changes have not been previously investigated. The present study investigated whether human MSCs grown on nanoscaled PHB scaffolds would retain their morphological characteristics and regenerative features. Furthermore, we explored whether random (r-PHB) and aligned (a-PHB) nanofibrous PHB membranes would change the phenotype and features of these cells. The aim of the present study was to characterize the interactions of r-PHB and a-PHB electrospun nanofibers with human MSCs by evaluating their viability, adhesion, continuous proliferation, differentiation, and apoptosis capacity.

2. Materials and methods

2.1. Preparation and characterization of PHB scaffolds

Commercially available PHB (Sigma-Aldrich, Catalog number: 29435-48-1) was dissolved in chloroform at a concentration of 5% (w/v). Using a syringe pump, the PHB solution was fed at a rate of 0.1 mL/h through a syringe. The distance between the tip of the syringe and the rotating collector was 20 cm. A high voltage power supply

(Spellman CZE1000R) was used to apply a positive voltage of 11 kV DC to produce PHB nanofibers.

The PHB solution was fed into the center of the rotary concentrator, where it was randomly distributed, resulting in randomly oriented fibers. This central area was surrounded by a circular area of approximately 3 cm, which consisted of both random and aligned fibers and was not used in the study. The outer border of the collector contained aligned fibers only and was used for comparison. Samples from the central area (with random configuration) and the outer border (with aligned fibers) were selected by scanning electron microscopy (SEM).

The conditions for electrospinning were optimized in order to obtain a continuous process and reproducible mesh morphology. A nanoscaled regular fibrillar surface property was obtained on the membranes. The morphology of electrospun PHB nanofibers was assessed and their diameter was measured under an SEM (ZEISS EVO 50 EP and ZEISS SUPRA 50 VP).

2.2. Isolation and characterization of MSCs

Human MSCs were obtained from three healthy bone marrow transplantation donors with an average age of 32. The use of human MSCs in the present study was approved by the Ethics Committee of Hacettepe University (Ankara/Turkey, TBK 09/15-194). Informed consent was obtained from all donors. Mononuclear cells (MNCs) were isolated from human bone marrow by density gradient centrifugation using a Ficoll solution (Biocoll; density 1.077 g/mL, Biochrom AG). The harvested MNCs were transferred into tissue culture flasks containing Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% (v/v) fetal bovine serum (FCS; Gibco) and 1% (v/v) antibiotics (10,000 U/mL penicillin and 10,000 µg/mL streptomycin; Biochrom AG) and incubated at 37 °C under an atmosphere of 5% CO₂. The culture medium was changed every 3 to 4 days until the primary adherent cells reached subconfluency. The confluent cells were routinely subcultured by trypsinization. Only passage 3–4 cells were used in the present study.

Third passage human MSCs were characterized with respect to the expression of surface antigens and differentiation assays. Experiments were performed in duplicate. For fluorescence-activated cell sorting (FACS), cells were trypsinized and incubated for 20 min in dark at room temperature with 5 µL of fluorochrome-conjugated monoclonal antibody (BD Pharmingen and eBioscience). Cells were analyzed for negative expression of hematopoietic (CD 34 and CD 45) and positive expression of mesenchymal (CD 90, CD 105, CD 73, CD 44, and CD 49e) cell surface markers. Cells were analyzed with a FACSaria flow cytometer (Becton Dickinson) using v6.1.2 software with 10,000 events recorded for each sample. For differentiation analysis, osteogenic and adipogenic

differentiations of human MSCs were induced by culturing the cells in an osteogenic (DMEM-LG, 10% FCS, 100 nM dexamethasone, 10 mM beta glycerophosphate (AppliChem), and 0.2 mM L-ascorbic acid (Sigma)) or an adipogenic differentiation reagent (DMEM-LG, 10% FCS, 1 μ M dexamethasone (Sigma), 60 μ M indomethacin (Sigma), 0.5 mM isobutylmethylxanthine (Sigma), and 50 μ M indomethacin). The media were refreshed every 3 to 4 days.

2.3. Cell viability and proliferation on PHB scaffolds

2.3.1. MTT assay

The tetrazolium dye (MTT) colorimetric assay was used to examine the number of attached cells on the PHB scaffolds. Membranes were cut into small, 0.31-cm² pieces and specimens were placed in wells of 96-well culture plates. MSCs were seeded at a concentration of 1×10^4 cells/scaffold and cultured for 2, 5, 7, 9, 11, and 13 days. The scaffolds were transferred to a fresh 96-well culture plate and 5 mg/mL MTT (Sigma-Aldrich) solution was used in the MTT assay to test cell viability. Absorbance was read at 620 nm (Tecan).

2.3.2. Real time cell proliferation analysis

Using the xCELLigence system (Roche Applied Science and ACEA Biosciences) we analyzed cell proliferation using label-free and real-time monitoring. For this experiment, human MSCs were seeded at a concentration of 2×10^5 cells per scaffold and were cultured for 14 days for confluency. Then cells were removed from the membranes and seeded on an e-plate. Cell impedance was monitored for 10 days and impedance was measured every hour.

2.3.3. Growth kinetics experiment

PHB membranes were cut into 3.8-cm pieces and placed in individual wells of 12-well culture plates in order to generate a growth curve of human MSCs. MSCs were seeded at 5×10^4 cells per membrane and were cultured. The scaffolds were placed in a fresh 6-well culture plate for 3, 5, 7, and 10 days and cells were harvested and counted using trypan blue dye.

2.3.4. SEM

SEM was performed 5 and 10 days after cultivation. Cells were washed with PBS buffer and fixed with 2% glutaraldehyde solution (Sigma Aldrich) at 4 °C for 1 h. The samples were washed with PBS and dehydrated in a series of ethanol (25%, 50%, 75%, 95%, and 100%). The membranes were sputter-coated with platinum and examined using an SEM (ZEISS EVO 50 EP).

2.3.5. Confocal laser scanning microscopy (CLSM)

Cells were incubated with 5 μ L/mL lipophilic dye (Vybrant DiO dye, Invitrogen) and seeded at 7×10^5 per scaffold. CLSM was performed on day 5 and day 10. Viable human MSCs retained the lipophilic label. Following cultivation, the membranes were placed in a confocal microscopy plate

(MatTek Corporation). Fluorescent-labeled cells were observed under a confocal microscope, LSM 510 (LSM Pascal, Zeiss), and analysis was performed with Zeiss LSM Image Browser software.

2.4. Evaluation of MSC characteristics on PHB scaffolds

2.4.1. Differentiation capacity

MSCs were seeded at a concentration of 5×10^3 cells/scaffold and cultured for 10 days. The differentiation analysis was performed after 21 days. For adipogenic differentiation, the cells were fixed with 10% buffered formalin; 0.3% Oil Red O (Sigma Aldrich) was used to detect intracellular lipid accumulation. For quantitative analysis, the stains were solubilized using 2% IGEPAL in isopropanol (v/v); the resulting absorbance was measured at 492 nm. For osteogenic differentiation, 0.6% M HCl was added to the cells. The amount of calcium was quantitatively measured with QuantiChrom Calcium Assay Kit (Bioassay Systems) and absorbance was measured at 620 nm.

2.4.2. Cell surface markers

MSCs were seeded at a concentration of 1×10^5 cells/scaffold and were cultured for 10 days. The membranes were placed to a fresh 6-well culture plate for the indicated time intervals (3, 5, 7, and 10 days) and cells were harvested and counted. Cells were trypsinized in order to obtain a cell suspension, and then incubated for 20 min in the dark at room temperature with 5 μ L of fluorochrome-conjugated monoclonal antibody (BD Pharmingen and eBioscience). Cells were analyzed with different surface markers, including CD 105, CD 271, CD 90, ALP, CD 73, CD 29, and CD 49e. Following incubation with the specific antibody, cells were washed and analyzed with a FACSaria flow cytometer (Becton Dickinson) and BD FACSDiva using v6.1.2 software with 10,000 events recorded for each sample.

2.5. Statistical analysis

All statistical analyses were performed using SPSS 21 (IBM SPSS 21.0 for Windows). Multiple comparisons were performed using the Kruskal–Wallis test. The Mann–Whitney U test was used for pairs. Descriptive statistics were presented as median and minimum and maximum values. The confidence interval was 95%.

3. Results

3.1. Characterization of PHB scaffolds

The electrospun PHB membranes were analyzed for surface topology. The electrospun surfaces ultrastructurally exhibited regularly arranged aligned nanofibers following a parallel direction. Figure 1 shows that the fiber diameter was about 700–900 nm. As shown in Figure 2, fibers were aligned in the outer periphery of the rotary concentrator and randomly distributed in the center. Between 80% and 90% of the a-PHB electrospun nanofibers were aligned

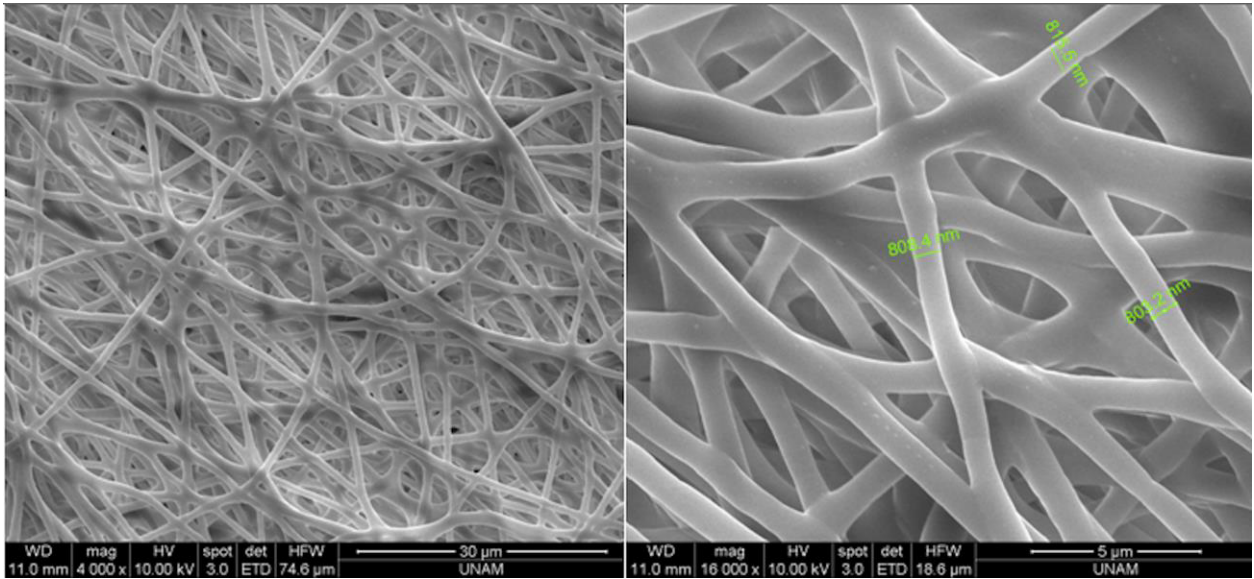


Figure 1. SEM images of PHB fibers.

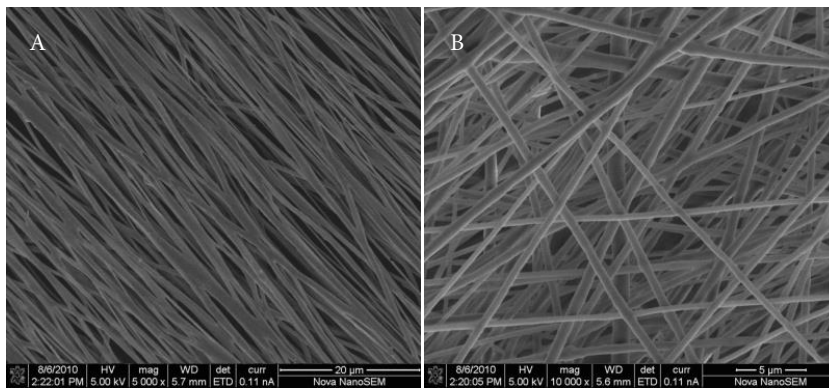


Figure 2. SEM images of aligned (A) and random PHB membranes (B).

the same way (Figure 1). This structure was similar to the natural extracellular matrix of most human regular connective tissues. Additionally, bead formation was not observed (Figures 1 and 2)

3.2. Characterization of human MSCs

The criteria proposed by the International Society for Cellular Therapy for MSC characterization were taken into consideration for the characterization of human MSCs (Dominici et al., 2006). In vitro culture-expanded human bone marrow MSCs showed plastic adherent and fibroblastic morphology (data not shown). The immunophenotyping analysis of the MSCs revealed positive immunolabeling for CD 105, CD 44, CD 73, CD 49e, and CD 90 surface antigens, and negative labeling for hematopoietic markers, including CD 45 and CD 34 (data not shown). These morphological and immunophenotypic characteristics confirmed the stromal and multipotential nature of our cells.

3.3. Cell viability and proliferation on PHB scaffolds

The number of cells on the a-PHB membrane was higher than that on the r-PHB membrane on all days of the MTT cell viability assay analysis (Figure 3). The proliferation rate of MSCs was higher on the aligned membrane at the beginning of culture, but the viable cell number was higher on the r-PHB membrane at the end of the culture period. Population doubling analysis of human MSCs at the end of days 3, 5, 7, and 10 of culture on the two types of membranes demonstrated lower human MSC proliferation on both membranes as compared with that of the control cells ($P < 0.05$) (Figure 4).

Real time cell proliferation analysis using the xCELLigence system showed that the growth curves of the a-PHB and r-PHB membrane-incubated human MSCs were slightly lower than the growth curve of the controls, but they were close to each other for 10 days (Figure 5). Those results were confirmed by the MTT and population doubling assays.

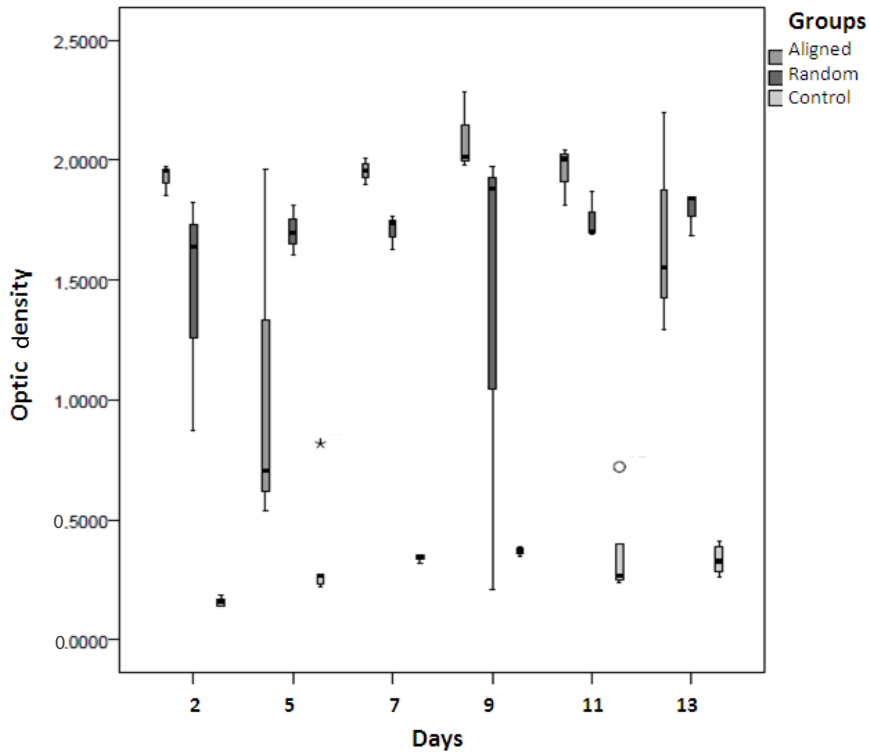


Figure 3. MTT assay of human MSCs reveals a higher cellular proliferation rate on the aligned PHB membrane than on the random PHB membrane.

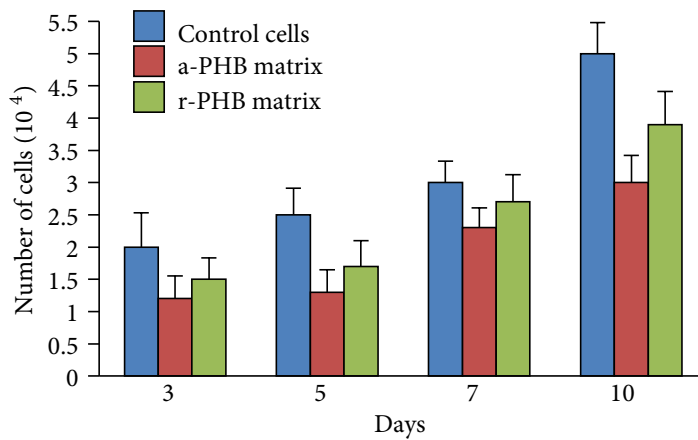


Figure 4. Population doubling assay of human MSCs on the aligned PHB membrane (red bars) and the random PHB membrane (green bars) as compared with control cells (blue bars).

3.4. Cell morphology on PHB scaffolds

DiO-labelled live cells proliferated homogeneously on the whole surface by making aligned rows on aligned fibers (Figures 6 and 7). The cells exhibited spindle-shaped morphology on the a-PHB surface, but had polygonal appearance and expanded at unequal intervals and

nonhomogeneously on the r-PHB surface on both day 5 and day 10 (Figures 6 and 7). Similar results were obtained by SEM analysis. Human MSCs expanded for 5 days on the a-PHB membrane were ultrastructurally aligned parallel to the direction of oriented nanofibers and were spindle-shaped. On day 10 of analysis, increased numbers

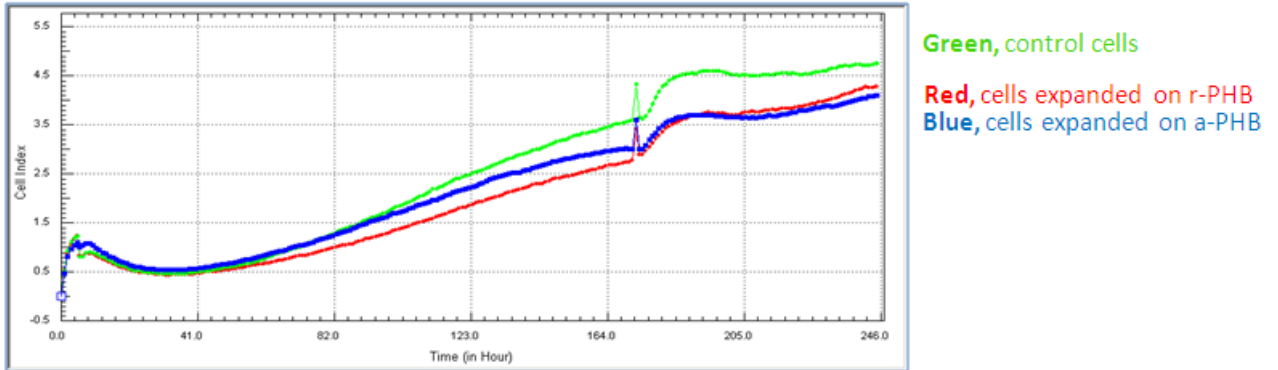


Figure 5. The green line shows the growth curve (cell index) of control cells; the red line shows the human MSCs expanded on the a-PHB membrane; and the blue line shows the human MSCs expanded on the r-PHB membrane.

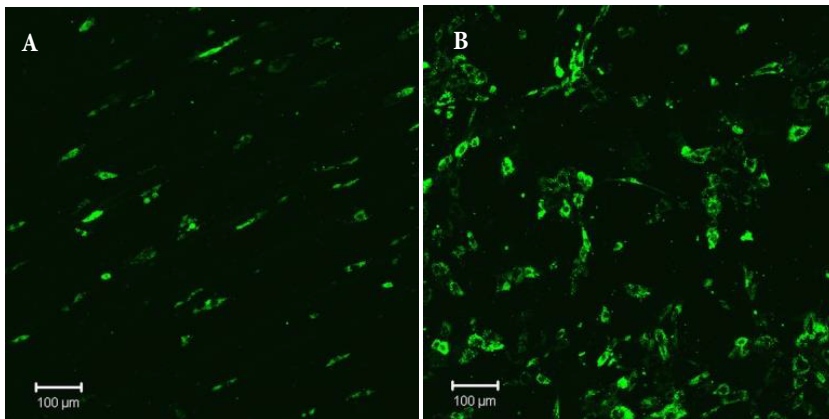


Figure 6. Confocal microscope analysis reveals spindle-shaped morphology of aligned organization of human MSCs on the a-PHB membrane (A) and their polygonal shape and clustered organization on the r-PHB membrane (B) on day 10.

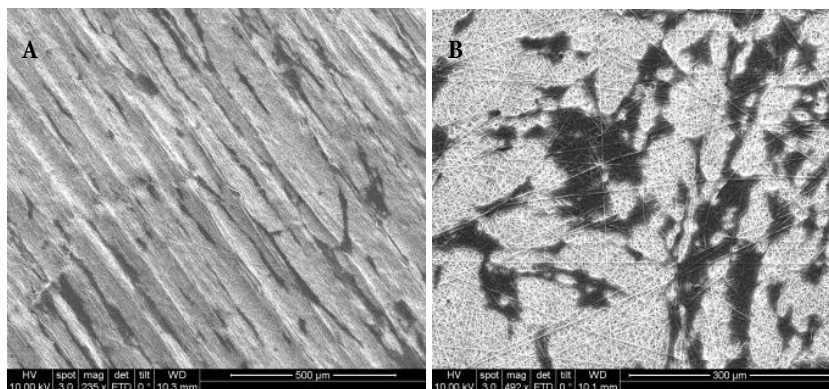


Figure 7. SEM analysis of aligned organization of human MSCs on the a-PHB membrane (A) and their polygonal shape organization on the r-PHB membrane (B).

of aligned human MSCs making intercellular connections were observed. Human MSCs expanded from 5 to 10 days on the r-PHB membrane made clusters or spread

homogeneously and exhibited polygonal morphology (Figures 6 and 7).

3.5. Cell characters and differentiation capacity on PHB scaffolds

3.5.1. Adipogenic and osteogenic differentiation capacity

MSCs differentiated into adipogenic and osteogenic cells on both PHB membranes. Figure 8 shows the morphological analysis of human MCS adipogenic cell differentiation on the a-PHB fabric. Quantitative analysis of adipogenic differentiation revealed that the differentiation rate of cells on the a-PHB membrane was 63.6%, which was higher than that of cells on the r-PHB membrane (9.8%) (Figure

9). Quantitative analysis of osteogenic differentiation revealed that human MSCs expanded on the a-PHB membrane displayed increased osteogenic differentiation capacity (156.2%) in comparison with those expanded on the r-PHB membrane (132.2%) (Figure 9).

3.5.2. Cell surface markers

MSCs expanded on both PHB membranes and their surface molecule expression patterns were similar to those of the control human MSCs. The Table shows the human MSC surface markers analyzed by flow cytometry. CD

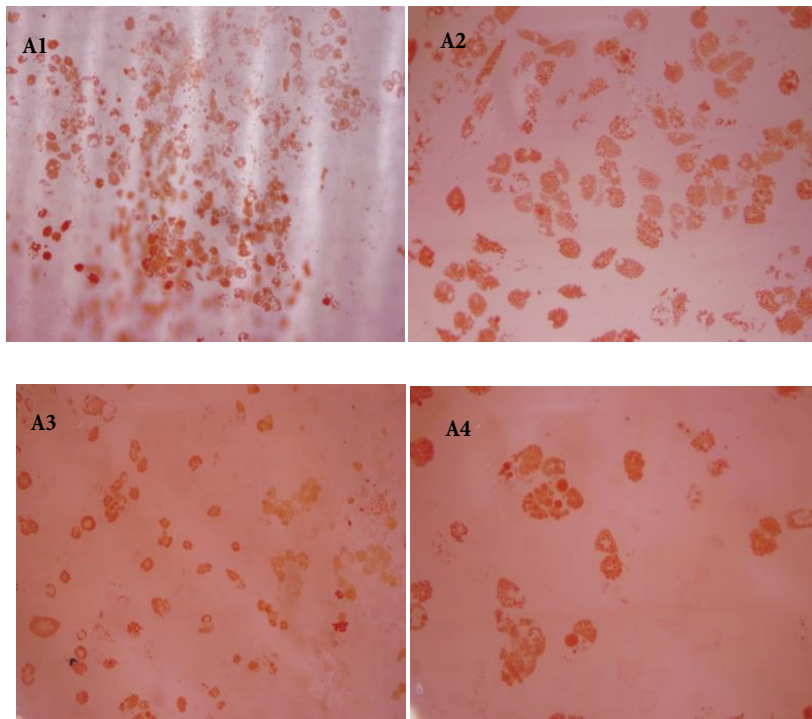


Figure 8. Morphologic analysis of human MCSs adipogenic cell differentiation on the a-PHB membrane (10×, A1; 20×, A2) and the r-PHB membrane (10×, A3; 20×, A4).

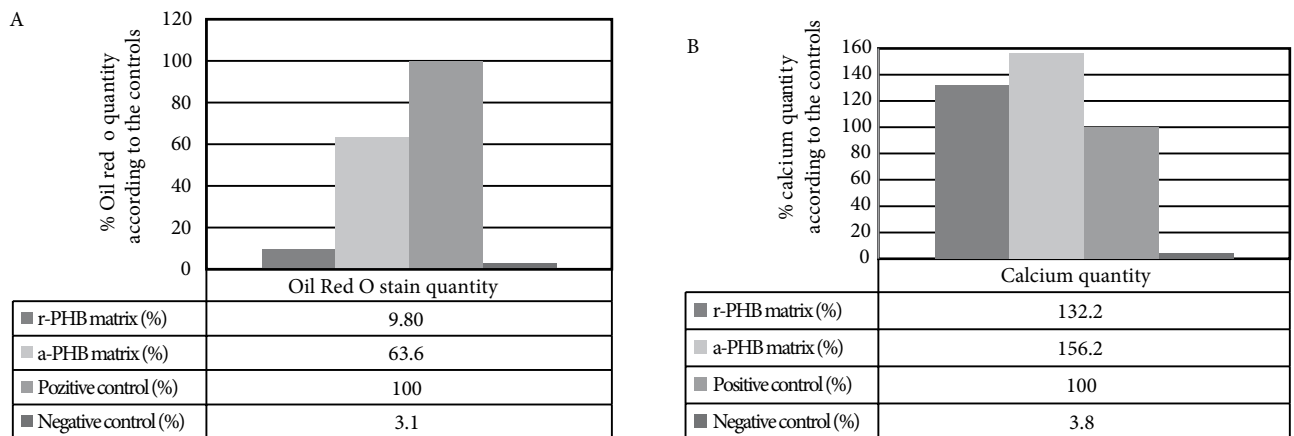


Figure 9. Quantitative human MSCs adipogenic (A) and osteogenic cell (B) differentiation on PHB membranes.

Table. Cell surface marker percentages of control cells and cells expanded on the a-PHB and r-PHB membranes.

| Surface marker | Control cells | Cells on the a-PHB membrane | Cells on the r-PHB membrane |
|----------------|---------------|-----------------------------|-----------------------------|
| HLA-DR | 0.7% | 3.2% | 3.4% |
| CD271 | 0.7% | 0.5% | 0.5% |
| ALP | 0.2% | 0.2% | 0.3% |
| CD 106 | 1.0% | 1.1% | 0.8% |
| CD 105 | 87.9% | 91.2% | 88.6% |
| CD 90 | 99.4% | 99.6% | 99.3% |
| CD 49e | 80.4% | 86.9% | 84.2% |
| CD 73 | 62.6% | 62.8% | 69.6% |
| CD 29 | 95.4% | 97.1% | 96.7% |
| CD 44 | 72.2% | 86.8% | 72.4% |

105, CD 44, CD 73, CD 49e, and CD 90 surface antigens were positive, while hematopoietic markers, including CD 45 and CD 34, were negative, as expected. This analysis showed that the PHB membranes did not affect the stem cell surface marker expression of human MSCs.

4. Discussion

Here we demonstrated the biocompatibility of PHB membranes with human bone marrow-derived MSCs using viability, proliferation, characterization, and morphology with confocal microscopy and SEM. Through a series of morphological, biochemical, and semiquantitative analyses, we demonstrated that nanofibrous PHB membranes with two different topographies have positive interactions with human MSCs. To evaluate a new type of scaffold for nerve and bone regeneration, the evaluation of physical and cell growth properties is important and in vitro cell culture studies are required to carry a new material to the stage of animal testing. This PHB scaffold has promising properties (e.g., biocompatibility, biodegradability, excellent physical properties, and excellent cell survival and growth responses).

The PHB membranes were produced using the electrospinning method. Viability assays (MTT, population doubling, and confocal microscopy) showed that cells on the PHB membranes had lower adhesion, but similar viability when compared with control cells. Proliferation was analyzed directly with the xCELLigence system. The results showed that the scaffold cells had a proliferation rate similar to that of the control cells. The characteristic features of the cells were also studied. The scaffold cells showed less adipogenic differentiation and more osteogenic differentiation than the control cells. The surface markers of the scaffold cells were found to be similar

to those of the control cells. Morphological examinations by confocal microscopy showed good communication between the human MSCs and the PHB scaffolds and a high rate of cell expansion on day 10. Histological analysis revealed that human MSCs on the a-PHB scaffold had spindle-shaped morphology and were located parallel to the fibrils; however, cells on the r-PHB scaffold showed polygonal morphology in the spaces.

This in vitro study, which examined different aspects of the interaction between bone marrow-derived human MSCs and PHB scaffolds, can be improved with immunohistochemical analysis to check the MSC expression profile of the extracellular matrix production. Further examinations need to be performed, including evaluation of the in vivo biocompatibility of PHB membranes with nerve and bone tissues at the molecular level. However, prior to in vivo administration, the in vitro immunogenic and inflammatory properties of MSCs should be examined in detail.

In previous studies, PHB nanofiber membrane diameters were obtained in the range of 100–680 nm by the electrospinning method (Sangsanoh et al., 2007; Suwantong et al., 2007; Li et al 2008; Asran et al., 2010). Here we found that the PHB nanofiber diameter was about 700–900 nm, which is an average value according to the literature. In the literature, the viability of different cell types on PHB scaffolds is analyzed. As mentioned before, limited analyses of the interaction between PHB and MSCs have been undertaken. Yu et al. (2010) investigated the viability and metabolic activity of human MSCs grown on various PHA films, including PHB, and found that the metabolic activity of human MSCs on PHB membranes was similar to that of the control cells. In another study, the vitality of fibroblasts grown on PHB scaffolds was

evaluated by MTT assay; although cell toxicity was found to be about 10%, cell adhesion was very low (Suwantong et al., 2007). Wollenweber (2006) demonstrated that osteogenic differentiation of human MSCs interacting with PHB membranes increased as compared with that of control cells, and exhibited good viability after differentiation. In a similar study consisting of both in vitro and in vivo experiments, human MSCs presented excellent adhesion and expansion on PHB membranes. In in vivo experiments, 6 months after subcutaneous implantation in rats, human MSCs implanted with PHB membranes exhibited increased alkaline phosphatase activity and calcium content as compared with control cells (Rentsch

et al., 2010). In our study, we obtained better adhesion and viability results for human MSCs with both types of PHB scaffolds in different qualitative and quantitative proliferation assays.

PHB membranes were successfully produced using the wet electrospinning method with a nanosize fiber diameter. The PHB membrane provided a suitable surface for the adhesion, proliferation, expansion, and differentiation of human MSCs. These results suggest that the PHB membrane has good potential as a scaffold for spinal cord and bone tissue engineering of MSCs. However, in vivo animal model studies are needed to assess the suitability of nanofibrous PHB membranes.

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