PAPER DETAILS

TITLE: Investigation of the Interaction of Adipose-Derived Mesenchymal Stem Cells with

?-Polycaprolactone and Egg White Scaffolds

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Investigation of the Interaction of Adipose-Derived Mesenchymal Stem Cells with ϵ -Polycaprolactone and Egg White Scaffolds



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Highlights

- This paper focuses on 3D cell culture models used in tissue engineering applications.
- The importance of 3D mesenchymal stem cell culture on egg white scaffolds was emphasized.
- A model compatible with adipose tissue-derived mesenchymal stem cells has been proposed.

Article Info

Abstract

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Keywords

Adipose-derived mesenchymal stem cells, 3D cell culture, ɛ-Polycaprolactone, Egg white The development of three-dimensional (3D) cell culture models is becoming increasingly important due to their numerous advantages over conventional monolayer culture. This study aimed to examine the interaction of adipose tissue-derived mesenchymal stem cells (AD-MSCs) with scaffolds composed of ϵ -polycaprolactone (ϵ -PCL) and egg white. In our study, ϵ -PCL and egg white scaffolds were produced from their monomers by tin octoate catalyzed and heat polymerization, respectively. Characterization of ɛ-PCL was carried out by Gel Permeation Chromatography (GPC), Fourier Transform Infrared Spectrophotometry (FTIR), Proton Nuclear Magnetic Resonance (H-NMR), Differential Scanning Calorimetry (DSC) and Scanning Electron Microscopy (SEM). AD-MSCs labeled with red fluorescent CellTracker CM-Dil were cultured on egg white and ɛ-PCL scaffolds for 12 days. Cell viability was determined using 3-(4.5-Dimethylthiazol-2yl)-2.5-diphenyltetrazolium bromide (MTT) and nitric oxide (NO) level was evaluated for toxicity. The results showed that the number of AD-MSCs in the egg white scaffold increased periodically for 12 days compared to the other groups. Although the number of AD-MSCs in the ϵ -PCL scaffold increased until day 6 of the culture, the number of cells started to decrease after day 6. These results were associated with the toxic effect of lactic acid release on cells resulting from the decomposition of ε -PCL scaffolds through catabolic reactions. Therefore, these results indicated that the egg white scaffold enhanced and maintained cell adhesion and cell viability more than the ε-Polycaprolactone scaffold and could be used as a scaffold in tissue engineering studies involving stem cells.

1. INTRODUCTION

Tissue engineering products created using three-dimensional (3D) scaffolds, cells, synthetic or natural biocompatible and biodegradable materials, growth factors and state of art technologies are promising tools to mimic the physiology and functions of normal and/or cancerous tissue structures [1-3]. 2D cultures have many limitations and cannot represent real cell environments due to the disruption of interactions between cells and extracellular environments. Therefore, 3D cultures are closer to *in vivo* mechanisms in expressing *in vitro* cellular mechanisms in drug development studies [3,4].

To date, synthetic scaffolds [5-7] and natural scaffolds [8-12] have been widely used in 3D cell culture studies [13,14]. The parameters used in the characterization of scaffolds include degradation profiles, biomechanical properties, and biocompatibility [15]. Despite the advantages of closely resembling the natural extracellular matrix (ECM), animal-derived polymers are of limited use due to the limitations of the immune response, difficulty in obtaining, purity, and presence of disease agents. Consistency in biological

and chemical structure, mechanical properties, and degradation rates are other disadvantages of natural polymers. However, they are advantageous in cell adhesion and other physiological processes as they have natural biological signals and can be easily degraded and remodeled *in vivo*. Although most synthetic scaffolds have adjustable mechanical properties, controllable biodegradation, and batch-to-batch stability, they are thought to be weaker than natural polymers in their intercellular signaling and differentiation capabilities [16-18].

Eggs are a high-quality and low-cost animal product widely used in the food industry as well as in human nutrition. The white component 67% of the total egg consists of 87.8% water, 9.7-10.6% protein, carbohydrates, and trace lipids. The protein content of egg white includes 54% ovalbumin, 12% ovotransferrin, 11% ovomucoid, 3.5% ovomucin, 3.5% lysozyme, both 4% G2 and G3 globulins, 0.05% avidin, 0.05% cystatin, 0.5% ovoglobulin, 0.8% egg yolk protein, 1.0% egg glycoprotein and 1.5% egg inhibitor. Egg white has potential in the pharmaceutical industry due to its antioxidant, antibacterial, immunomodulatory, anti-inflammatory, anti-tumor, cell growth stimulant, ACE inhibitory effect and hypoglycemic properties, and amino acid content essential for the human body [19,20]. In addition to these properties, foaming, emulsification, adhesion, gelation, heat polymerization, n and biocompatibility make egg white a promising candidate for biomaterial technologies [21].

 ϵ -PCL is a highly hydrophobic nanofibrous synthetic scaffold used in biomedical applications owing to its high tensile strength, low cell toxicity, cost-effectiveness, and slow degradation rate. ϵ -PCL has support structures for the native ECM microenvironment and provides a 3D scaffold for mesenchymal stem cell (MCSCs) attachment, growth, migration, and differentiation [22, 23].

This study aims to comparatively examine the interaction between AD-MSCs with ϵ -PCL and egg white scaffolds and propose a stem cell three-dimensional scaffold model.

2. MATERIAL METHOD

2.1. Culture and Characterization of AD-MSCs

AD-MSCs were taken from cryobank which was used in the previous study. (24). In passage 2, the cryopreserved AD-MSCs were thawed and transferred into 25 cm² flasks containing DMEM [1.0 g/L D-Glucose (1X), Invitrogen 11880] supplemented with 10% fetal bovine serum (Biochrom AG 1038K), 1% Antibiotic Antimycotic Solution [(100×) penicillin 100 U/ml, streptomycin 0.1 mg/ml Invitrogen 15240-062] and 1% GlutaMaxTM (Invitrogen, 35050-079 Cells were incubated in a humidified atmosphere. Fresh culture medium was replaced every 72 hours until the adherent cells reached ~70% confluency.

Adipogenic differentiation and immunophenotypic analysis were performed as characterization tests. For adipogenic differentiation, AD-MSCs cultures were induced with the differentiation medium for two weeks and stained with Oil Red-O in our previous study [24]. Immunophenotypic analysis was performed by using positive (FITC-CD90, PerCP-cy5.5-CD105, and PE-CD73) and negative (PE-CD34) surface markers (Becton Dickinson, San Jose, CA).

2.2. CM-DiI Fluorescence of AD-MSCs

The culture medium of AD-MSCs reaching 70% confluence was removed, and the cells were washed with PBS (Gibco, 10010023). Cell TrackerTM CM-DiI (Invitrogen Life Technologies) was prepared in PBS at 2 mg/L concentration. Then the CM-Dil solution was added, and incubation periods were carried out at 37°C for 4 minutes and 4°C for 15 minutes respectively. After staining was completed, cells were washed with PBS, cells were incubated at 37°C with fresh culture medium. The dye became visible within 4 hours. Labeled cells were used in scaffold assays.

2.3. Scaffold Preparation Using Egg White

The egg-white scaffolds were prepared from organic chicken eggs as previously described by Kaipparettu et.al. [25]. Briefly; the eggshell was cleaned with 70% ethanol under sterile conditions, and a small hole was made in the shell using sterile forceps. The semi-transparent egg white was transferred into a 50 ml tube. A cutting tip of $1000 \ \mu$ l pipette tip was used to pour the egg white into the middle of a 6-well chamber slide. To polymerize egg white, the 6-well chamber slide was closed and incubated for 45 minutes in the heat block set to 60°C. The egg white became half-solid and stuck to the bottom of the well after 45 minutes. The non-adhesive egg parts were removed by pipette-wash using a 500 μ l DMEM medium.

2.4. Preparation, Characterization, and Sterilization of ϵ -PCL Scaffolds

Biodegradable, low–molecular–weight (10 kDa), and high-molecular-weight (85 kDa) ε-PCLs, approved by the Food and Drug Administration (FDA), have been synthesized from ε-caprolactone monomers under tin octoate catalyst following earlier studies [26, 27]. The synthesized ε-PCL scaffolds were prepared by mixing low-molecular-weight (80%) and high-molecular-weight polymers (20%). Webless, nanofiber membranes containing micropores were produced by electrospinning using polymers. The molecular weight of the produced membrane was examined by GPC, chemical and structural analyzes of it were carried out by the FTIR (Thermo Scientific Nicolet 6700 FT-IR Spectrometer), macromonomer formation and morphological structures were examined by H-NMR and SEM respectively, and thermal behavior was observed using DSC (Perkin Elmer, USA). Electrospun, microporous membranes are created to fit 8mm in diameter and 1mm in thickness. Following the characterization step, sterilization was performed by using ethylene oxide gas [27].

2.5. 3D Cell Culture Preparation and Microscopic Examination

Prepared and pre-sterilized ε -PCL and egg white scaffolds were equally divided into 4-well plates with four pieces each. CM-DiI-labeled AD-MSCs were trypsinized, and cell count and viability experiments were performed by hemocytometer. 500.000 cells with 50µL culture medium per well were added. For cell attachment, incubation times of 4 hours and 15 minutes in 37°C incubatör were applied for ε -PCL and egg white scaffold, respectively. After the incubation period, 1 ml of culture medium was added to each well, and the 3D cultures were allowed to incubate. Due to the hydrophobic nature of ε -PCL, it takes time for MSCs to adhere to the ε -PCL scaffold [28-30]. Contrarily, egg white is a hydrophilic biomaterial due to its rich protein content. Therefore, adhesion occurs faster in egg-white than in ε -PCL. All media were changed every 72 hours and microscopic examinations were performed with an inverted microscope (Olympus CKX41) on days 3, 6, and 12 of incubation to compare the growth rates of AD-MSCs in 2D and 3D cultures.

2.6. MTT Analysis

MTT assay was performed to assess the cell viability of AD-MSCs in 2D and 3D cell cultures. These analyzes were performed in 4 separate 96-well plates according to the 3rd, 6th, 9th, and 12th days of culture. Cells were added as 10,000 cells per well in 5 wells. The cell medium was changed every 72 hours, and the discarded medium was collected and stored for NO analysis. MTT analyzes were performed according to Topuzoğulları et al. (2013) [31] and the results were analyzed with Multiskan EX at 570 nm (Thermo Scientific, Rockford, IL, USA).

2.7. NO Analysis in 3D Cell Culture Medium

Nitric oxide plays a significant role in various physiological events such as catabolic reactions and immune responses by acting as a signaling molecule in mammalian cells [32]. NO production was determined by the amount of nitrite accumulation in the medium of 2D and 3D cultures using the colorimetric Griess test as previously described [33]. Briefly, equal volumes of cell culture medium were mixed with Griess reagents. After incubation for 10 minutes at room temperature, the wavelength was measured absorbance

of 540 nm using a Microplate-Reader (Bio-Rad). Results were expressed as μ M of NO based on a standard curve generated by known concentrations of sodium nitrite dissolved in the culture medium.

2.8. Statistical Analysis

Quantitative datas were showed as mean \pm standard deviation (SD). Statistical analysis were performed by using GraphPad Prism 6 software. Datas were examined by ANOVA with Tukey's multiple comparison tests. Group comparison statistics were made with two-way ANOVA. The significance level for all statistical analyzes was set at p <0.05, p <0.01, and p <0.001.

3. RESULT

3.1. Cell Culture Maintenance and Characterization Tests of AD-MSCs

Microscopic examination, immuno-phenotypic analysis, and differentiation assay for stem cell characterization revealed that the characteristics of the cells were within reference values. Fibroblast-like morphology of the cells was determined according to microscopic examination (Figure 1A), the presence of intracellular lipid droplets (Figure 1B), and Oil Red-O staining proves the multipotent properties of these cells (Figure 1C). Positive expression of FITC-CD90, PerCP-cy5.5-CD105, PE-CD73, and negative expression of cell surface markers PE-CD34 were detected using flow cytometry and showed the immunophenotype of MSCs (data not shown).



Figure 1. (*A*) *Cultured AD-MSCs cells (10X), (B) Presence of intracellular lipid droplets in differentiated AD-MSCs (40X), (C) Oil Red O staining of differentiated AD-MSCs (10X)*

3.2. Investigation of Polymerized Egg White Scaffold

The polymerized egg white was examined with the naked eye and under a microscope. It was observed that the surface of the structure was not flat. At this rate, a precise thickness could not be determined for this scaffold, but it was estimated to be about 0.3 cm thick.

3.3. Preparation and Characterization of the Polycaprolactone Scaffold

Meshless, microporous, nanofiber membranes synthesized from ϵ -PCLs have been examined. The 1750-1710 cm-1 peak in the FTIR graphic refers to the tension in the structure of the carbonyl group of -C=O ester. The peaks between 2950 and 2860 cm⁻¹ represent the tension in the structure of CH groups in the polymer (Figure 2A). The chemical analysis made with H-NMR shows individual functional groups of the produced polymers (Figure 2B). With the thermoanalytical technique, the DSC thermogram has shown as 59.84°C (Figure 2C), and this result is consistent with the literature [34]. SEM imaging has been done with 50 randomly selected fibers. It has been determined that the fibers were homogeneous, uninterrupted, and random. No bead structure has been found. The average fiber diameter and pore sizes were 517±198 (Figure 2D) and 1076±494 nm, respectively (Figure 2E).



Figure 2. (A) FTIR spectra of ε -PCL scaffolds at approximately 40.000 to 400 cm⁻¹ indicated by black lines, (B) H-NMR spectrum of ε -PCL, (C) DSC thermogram of ε -PCL, SEM images of micropores and macro channels of ε -PCL (D; X1000), (E; X5000)

3.4. Microscopic Examination of Interactions of AD-MSCs with Egg White and ϵ -PCL Scaffolds

The proliferation rate of AD-MSCs in scaffolds was analyzed by determining the intensity of red-light emission. Microscopic examination of CM-DiI-labeled AD-MSCs revealed that AD-MSCs proliferated in all groups. However, the proliferation rate in the scaffold which consisted of egg-white increased periodically over 12 days of culture compared to the other groups. Interestingly, the highest red light emission intensity of AD-MSCs was observed on day six on the ϵ -PCL scaffold. In addition, connections between AD-MSCs in the egg-white scaffold were visible from days 1 to 12, while connections between cells in the ϵ -PCL scaffold were not clearly defined (Figures 3-5).



Figure 3. Inverted microscope images. (A1) Egg white scaffold. (B1) ε-PCL scaffold. CM-DiI labeled AD-MSCs in incandescent (C1) and fluorescent light (A2). CM-DiI labeled AD-MSCs in the egg-white scaffold in incandescent (D1) and fluorescent light (B2). CM-DiI labeled AD-MSCs in the ε-PCL scaffold in incandescent (E1) and fluorescent light (C2) (10X) on 1st day



Figure 4. Inverted microscope images. (A1) Egg white scaffold, (B1) ε-PCL scaffold, CM-DiI labeled AD-MSCs in incandescent (C1) and fluorescent light (A2), CM-DiI labeled AD-MSCs in the egg-white scaffold in incandescent (D1) and fluorescent light (B2), CM-DiI labeled AD-MSCs in the ε-PCL scaffold in incandescent (E1) and fluorescent light (C2) (10X) on the 6th day



Figure 5. Inverted microscope images. (A1) Egg white scaffold, (B1) ε-PCL scaffold, CM-DiI labeled AD-MSCs in incandescent (C1) and fluorescent light (A2), CM-DiI labeled AD-MSCs in the egg white scaffold in incandescent (D1) and fluorescent light (B2), CM-DiI labeled AD-MSCs in the ε-PCL scaffold in incandescent (E1) and fluorescent light (C2) (10X) on the 12th day

3.5. Investigation of Cell Viability of 2D and 3D Cultures of AD-MSCs with MTT Assay

Cell viability of CM-DiI-labeled AD-MSCs in 2D and 3D cultures were analyzed microscopically and by MTT method on the 3rd, 6th, 9th, and 12th days. Studies have shown that there is more surface area in a 3D structure than in a 2D culture, and cell proliferation, differentiation, mechanical responses, and cell survival can be enhanced. Based on this, our data convincingly showed that the number of cells increased in all groups, but the cell number and viability were higher in 3D culture than in 2D culture. According to microscopic examination, purple-colored formazan crystals showed viable cells in cell cultures (Figure 6). In addition, deformation of the ε -PCL scaffold was observed (indicated by arrows in Figure 6-B4. Regarding MTT analysis, AD-MSCs in the egg white scaffold reached maximum survival on day 12, while AD-MSCs in the ε -PCL scaffold reached max survival on day 6. Thus, we observed that AD-MSCs in the egg-white scaffold gradually increased viability over 12 days, whereas the number of cells in the ε -PCL scaffold decreased after day 6 (Figure 7).



Figure 6. Microscopic examination of AD-MSCs on the MTT plate (Day 3: A1, B1, C1, D1, E1, Day 6: A2, B2, C2, D2, E2, Day 9: A3, B3, C3, D3, E3, Day 12: A4, B4, C4, D4, E4)

Blue arrows indicate the degradation sites of the ε -PCL scaffold (**B4**)



Figure 7. MTT analyses of 2D and 3D cultures of AD-MSCs. The bar chart represents the absorbance value that indicates the average cell viability. Data expressed as mean \pm SD. Statistical difference between groups is indicated (*p < 0.1***, p < 0.001)

3.6. Measurement of NO Levels

According to the results, NO production of AD-MSCs in 2D culture was found to be similar throughout 12 days of culture. In addition, despite the increase in the 3rd and 6th days, the NO level in the 3D culture of AD-MSCs and Egg White scaffold decreased on the 9th and 12th days. Interestingly, the NO level of the AD-MSC culture on the ε -PCL scaffold was observed to be the lowest on day 6. However, the NO level increased on day 9, and the highest NO level in all groups was found on day 12 in the ε -PCL scaffold. The NO level of AD-MSCs in the ε -PCL scaffold was found to be lower on days 3 and 6 than in the control AD-MSCs 2D group (P < 0.05). However, the NO level on days 9 and 12 of AD-MSCs on ε -PCL was higher than the control AD-MSCs 2D group (P < 0.05). In addition, the NO level at day 12 of AD-MSCs in the Egg White scaffold was lower than the control AD-MSCs 2D group (P < 0.05) (Figures 8 and 9).



Figure 8. NO production of AD-MSCs in 2D and 3D cultures at days 3, 6, 9, and 12 of culture. Significant differences in NO production are marked with asterisks ($p < 0.1^{**}$, $p < 0.01^{***}$, p < 0.001)



Figure 9. Comparison of NO production of AD-MSCs in 3D cultures at days 3, 6, 9, and 12. Significant differences in NO production are marked with asterisks (*p < 0.1**, p < 0.01***, p < 0.001)

4. DISCUSSION

Newly developed scaffolds with nano and macro architectures are very popular in tissue engineering studies and enable cell attachment, proliferation, and differentiation within a 3D structure. Among them, ε -PCL, an FDA-approved biocompatible synthetic scaffold, is a highly soluble polymer with a hydrophilic surface and low melting point that enhances cell adhesion and proliferation rate [35-38]. In addition, the natural biomaterial egg white is a cheap and easy-to-use protein-based natural biomaterial whose viscosity can be modulated by heat. As a scaffold, egg white also allows the monitoring of cell proliferation with its translucent structure. Besides its biochemical properties, the bioactive molecules of egg white also possess antibacterial properties for wound healing and cell proliferation [39, 40].

In this study, egg white and ϵ -PCL were used comparatively for the first time in a 3D cell culture model with AD-MSCs. The cells were initially characterized by Flow Cytometry and differentiation assay. The results obtained showed that the cells were in line with validated MSC properties.

The ε -PCL scaffold used in this study was characterized by FTIR, DSC, and H1-NMR methods. The results obtained were found to be consistent with the properties shown in similar studies in the literature. Structural analysis of the membranes was investigated by SEM. SEM images show that the membranes have randomly distributed fibers without beads. The average fiber diameters ranged from 517±198 nm to 1076±494 μ m.

3D constructs provide expanded surface area compared to conventional 2D cultures. This feature allows for a high cell proliferation rate [36]. In our study, CM-DiI-labeled AD-MSCs were observed under inverted phase-contrast microscopy and red-light emission showed that the cells proliferated horizontally and vertically. Furthermore, by the literature [41, 42], the degradation of the ε -PCL scaffold was also examined microscopically.

MTT analyses showed that the proliferation rate of AD-MSCs on ε -PCL scaffolds increased until day six and gradually decreased until day 12. These findings are consistent with the knowledge that the ε -PCL scaffold starts to degrade, resulting in the toxic effect of lactic acid [37] and 6-hydroxycaproic acid on cells [43]. Although it is known that the toxic effect is neutralized by metabolizing 6-hydroxycaproic in the citric acid cycle, the acidic environment formed in this process negatively affects cell viability and induces an inflammatory response [44, 45]. Consistent with the literature, in our study, it was observed that the decreasing amount of NO expressed an increased cell proliferation rate according to NO analysis [46]. In contrast, microscopic examination and MTT results of AD-MSCs in egg-white scaffolds showed that the cell number increased linearly for 12 days. The low amount of NO obtained in the experimental results also supports that the egg white scaffold shows a lower toxic effect over time during catabolism compared to the ε -PCL scaffold.

According to cell number and viability analysis, compared to the ε -PCL scaffold, the egg white scaffold promoted the growth and development of ADSCs to a higher extent. These results suggest that egg white has promising potential for use in medical applications. However, the rapid degradation of egg white alone due to its low stability and mechanical resistance is a disadvantage compared to the PCL scaffold.

In conclusion, egg white is a unique biomaterial that can be prepared using tissue engineering approaches. Compared to ε -PCL, egg white is a cheap, available, biocompatible, and easy-to-prepare biomaterial without ethical limitations. These properties make egg white a suitable candidate for 3D cell culture studies. The findings of this study have shown that egg white-based biomaterials can be used in various ways and with different methods in tissue engineering and can be a source of inspiration for tissue engineering studies to be applied in regenerative medicine and other medical fields.

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CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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