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Comperative Investigation of Interaction between Adipose Derived Mesenchymal Stem Cells and Egg White and Polycaprolactone (E-Pcl)

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Abstract

One of the main components of tissue engineering applications for regeneration of damaged tissue or organs are natural scaffolds as well as synthetic ones. ε-Polycaprolactone (ε-PCL) is a preferred soluble polymer scaffold for its low melting temperature (59-64°C) and hydrophilic surface that facilitates the attachment of cells. In recent years in vivo and *in vitro* studies showed that natural scaffold made up of egg white has been shown to mimic the extracellular matrix (ECM) and support and increase the proliferation of somatic cells. The aim of this study is to develop a three-dimensional (3D) cell culture model by comparing the interaction between adipose tissue derived mesenchymal stem cells (AD-MSCs) and ε-PCL and egg white scaffold polymerized by heat. In our experiments; CM-DiI labeled AD-MSCs were cultured for 12 days on polymerized egg white scaffold prepared at 60°C. Microscopic examinations were performed using reverse phase contrast microscope under UV light. MTT analyses were carried out on days 3, 6, 9 and 12. Results showed that the number of AD-MSCs on egg white scaffold increased periodically and cell connections were clearly observed. Although the number of AD-MSCs on ε-PCL scaffold increased until the 6th day of culture, the cell number and cell connections were decreased between day 6 and day 12. The results were statistically evaluated by ANOVA and Student's t-test ($p<0.05$). These results showed that egg white scaffold increases and protects cell adhesion and cell viability for longer than ε-PCL scaffold, thus it can be used as a scaffold in tissue engineering studies using stem cells.

Keywords: Stem cells; ε-PCL; Egg white

Introduction

One of the main components of the tissue engineering applications for damaged or nonfunctional tissues and organs is the structural scaffolds [1]. Up to day, synthetic scaffolds such as Food and Drug Administration (FDA)-approved polycaprolactone (PCL), poly-lactic-co-glycolic acid (PLGA) and Polyethylene glycol (PEG), natural scaffolds such as silk and chitosan [2,3], resorbable and non-resorbable polymers such as polylactic acid (PLA), polyglycolic acid (PGA), nylon, vapor-sensitive polymers, physiological materials such as collagen and hyaluronic acid are all used commonly in tissue engineering studies [4]. Any preferred scaffold construct should be identical to the intended clinical scaffold in composition. The scaffold must be characterized for composition, degradation profile, biomechanical properties and biocompatibility to host and the cell component of the product [5,6]. It is increasingly getting important to control cell growth within artificial scaffolds. Tissues such as skin, blood vessels and cartilage have multi-layer structures with different cells in each layer. With the help of micro-fabrication technology, a novel scaffolding method was developed for biodegradable polymers such as PLA, polyglycolic acid PGA and PLGA, in order to construct three-dimensional, multi-layered, micro-fluidic tissue scaffolds [7]. Synthetic scaffolds are biodegradable and non-immunogenic, however they have serious disadvantages such as hydrolysis reactions which lead to cell death [8]. A commonly used scaffold, ε-polycaprolactone (ε-PCL), is soluble, has low melting temperature (59-64°C) and a hydrophilic surface helping cell adhesion and proliferation; however it also has disadvantages such as local acid production when degraded [9,10].

One great advantage of natural scaffolds over synthetic ones is that they provide the maturation of the natural structure and remodeling of the matrix between the cells [11]. Since the preparation of both natural and synthetic scaffolds require special conditions and expensive setup, obtaining scaffolds are not always possible. For this reason, studies in tissue engineering keep searching for available natural or synthetic scaffolds that do not have the disadvantages of existing scaffolds. Thus, it is very important to develop cheap, easy-to-access, three-dimensional natural scaffolds to be used in tissue engineering studies that do not require special conditions and expensive setup.

In an *in vitro* study where MCF-10 cells and egg white which comprises about 60% of winged-animal eggs were used, it was shown that the scaffold that is prepared by polymerizing egg white via heat increases cell adhesion and supports extracellular matrix (ECM) [12]. Another *in vivo* study reported that transplanted egg white mixed with spleen cells to mice increased cell viability and proliferation [13]. These studies showed that heat-polymerized egg white can be used as a natural scaffold, however up to day, no study was conducted to examine this scaffold in relation to the stem cells which are very commonly used in tissue engineering studies. Hereunder, in this study we aimed to examine the relationship of adipose-derived-mesenchymal stem cells (AD-MSC) and egg white scaffold in comparison to synthetic PCL scaffold and to develop a three dimensional (3D) cell culture model using egg white and AD-MSCs to be used in different areas of tissue engineering.

Materials and Methods

Conditions for Isolation and Tissue Culture of Adipose-Derived Stem Cells

Discarded liposuction material was used to isolate human adipose-derived stem cells. Since the study was not classified as human subject research according to Yildiz Technical University guidelines, it did not require ethics approval. Patient informed consent is not needed for the use of discarded tissue for research as the hospital's genera informed consent for surgery/procedures specifies that the removed tissue may be collected and used anonymously for research. Liposuction material was washed briefly twice with 10 ml DPBS (01-042-1A, Biological Industries, Beit Heamek, Israel). The tissue was digested using collagenase (Sigma Aldrich, St. Louis, MO, Cat Number C2674) solution (0.1 g/L type 1 collagenase). After 1 hr of digestion, the digested tissue was applied to a 70 µm nylon cell strainer (Corning, NY, USA). The suspension was centrifuged at 400g, for 10min and the pellet was resuspended in 1 ml of DMEM Anti- Anti and GlutaMax™ plus 10% fetal bovine serum (FBS) (Gibco). Cells were plated in DMEM Anti- Anti and GlutaMax™ plus 10% FBS (Invitrogen, Grand Island, NY, USA) and grown to confluence at 37°C in a humidified 5% CO_2-95% air chamber. Cells covering the surface of the flask were detached using 10x Trypsin. The cells were centrifuged at 400g for 5 minutes, the supernatant was discarded and the pellet was resuspended in 1 ml medium containing 10% FBS. Live cells with a concentration of 100.000 cells/ml were cultivated in T25 flasks in 5 ml fresh medium after counting the cell number using Thoma slide.

Fluorescence-Activated Cell Sorting Analysis

ADSCs that were passaged for the second time were grown until confluent, trypsinized and pelleted by centrifugation at 200g for five minutes. For analysis of surface markers, each sample was incubated for 30 minutes at 4°C with fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated antibodies against the following surface markers: Anti-human CD34-PE, CD73-PE, CD90-FITC, and CD105-PE (Becton Dickinson, San Jose, CA). Samples were incubated at room temperature for 30 minutes in dark to allow the antibodies bind to the antigens. At the end of this period, 500µl isodiluent was added onto the cells and the cells were centrifuged at 1000rpm for 5 minutes. The supernatant was discarded. 500µl isodiluent was added onto the pellet for re-suspension and Cell Lab Quanta Flow Cytometer (Beckman Coulter, USA) was used for observation.

Differentiation Assays

ADSC cultures were grown to confluence after which the standard adipose stem cell medium was replaced with the inductive media.

Cells were cultured in adipogenic induction medium (43.2 mL DMEM, 5 mL FBS, 0.5 mL dexamethasone (Sigma-Aldich, St. Louis, MO), 0.05 mL insulin (Sigma), 2 mL indomethacin (Sigma), 0.05 mL isobutylmethylxanthine (IBMX) (Sigma-Aldich), 0.5 mL penicillin/streptomycin (Sigma) and 0.5 mL l-glutamine (Sigma) for two weeks with three medium changes per week in order to induce adipocytic differentiation. For histochemical examination, adipogenic cultures were stained for Oil Red-O as an indicator of intracellular lipid accumulation. Briefly, cells were washed three times with sterile PBS and then fixed in 10% formaldehyde for 1 hour. Later, they were stained using Oil red O solution including 60% isopropanol for 10 minutes, washed using water, incubated in 100% isopropanol for 15 minutes, washed using water once gain and finally, observed under microscope.

Marking Adipose-Derived Stem Cells using CM-DiI

Adipose-derived stem cells reaching surface coverage of 90% were deprived of culture medium. Cells were washed twice with PBS. CM-DiI (CellTrackerTM CM-DiI; C7000; Invitrogen Life Technologies) in 5 ml PBS was added onto the cells. Cells were incubated first at 37°C for 4 minutes, then at 4°C for 15 minutes. Upon completion of staining process, cells were washed with PBS; culture medium was refreshed and the cells were incubated at 37°C for 4 hours until the stains become visible.

Preparation of Scaffold using Egg White

Organic chicken eggs were used in the study. Under sterile conditions, the egg shell was wiped with 70% ethanol and a hole was opened onto the shell using a sterile pincer. The white of the egg was transferred to a 50 ml falcon tube passing through this hole, not mixing with the yellow of the egg. A 1000ml pipette tip cut with sterile scissors on the tip was used to transfer 100µl of egg white to the middle of a four-well plate. In order to help egg white polymerize, the culture plate was covered and incubated for 45 minutes on a 60°C heat block. The water vapor accumulating on the cover was discarded in laminar flow cabin using micropipettes. It was observed that the egg white becomes half-solid and sticks to the bottom of the well after 45 minutes. Egg parts that did not stick to the plate were removed by pipette-wash using 500µl DMEM medium.

Poly -Ɛ-Caprolactone (PCL) Scaffold Design and Sterilization

FDA (Food and Drug Administration) approved, bio-degradable, low molecular mass (10 kDa) and high molecular mass (85 kDa) poly-ε-caprolactone (PCL) were synthesized by Dr. Alper Isoglu and colleagues (Abdullah Gul University, Department of Chemical Engineering) from ε-caprolactone monomer under catalysis of tin octoate. Synthesized PCLs were prepared by mixing 80% low molecular mass and 20% high molecular mass polymer. Webless, nanofiber-structured membranes containing micro-pores were prodıced using the polymer by electro-spinning. The molecular mass of the produced membrane was examined using GPC (Gel permeability chromatography), chemical and structural analyses were made using FTIR (Fourier Transform Infrared Spectrophotometry) and H-NMR (Proton Nuclear Magnetic Resonance), thermal behavior was observed using DSC (Differential Scan Calorimetry), morphological structure was shown using SEM (Scan Electron Microscobe). Electro-spinned, micro-pore containing membranes were designed to fit 8 mm in diameter and 1 mm in thickness after characterization, sterilized using ethylene oxide gas and thus, became ready for 3D culture studies.

Culture of CM-DiI Marked Adipose-Derived Stem Cells on Scaffolds

CM-DiI marked adipose-derived stem cells were detached from flask surface using Trypsin, washed with culture medium containing 10% FBS and centrifuged at 400g for 5 minutes. Supernatant was removed, cells were suspended and 2µl of the suspension was diluted in 1/50 ratio, added trypan-blue and cells were counted on Thoma slide in order to determine the cell number in 1 ml. Later, ADSCs were planted on scaffolds in 4-well plates for microscopic follow-up (n: 3). 500.000 cells per 50 µl for each well were added onto the egg white and the cells were incubated for 5 minutes in a chamber at 37°C with 5% CO_2 in order for them to attach. At the end of this 5 minutes, 1ml DMEM medium containing 10% FBS was added to each well. In parallel, 500.000 CM-DiI marked ADSCs per 50µl were planted on PCL scaffold that is 8 mm in diameter and 1 mm in thickness and the cells were incubated at 37°C in 5% CO_2 containing chamber for 2-6 hours for the PCL scaffold to absorb the cells. At the end of this period, 1 ml culture medium was used to cover the 3D culture and cells were left for incubation. At the end of 24-hour incubation, CM-DiI marked ADSCs were examined on egg white and PCL scaffold using inverted microscope.

Survival Analysis using MTT in Cell Culture

At this point in our study, ADSCs not marked with CM-DiI were cultured on 4 of 96-well plates on scaffolds. MTT study was performed for survival analyses of ADSCs on egg white and PCL scaffold on 96-well plates at third, sixth, ninth and twelfth days of culture that was continued for a total of 12 days. Each plate was used for follow-up of one period and the process was repeated three times (n: 3). For this reason, 10.000-15.000 cells were cultured on 1 cm^2 egg white or PCL. MTT salt (Sigma-Aldrich M2003-1G) was prepared with PBS to get a final concentration of 10mg/ml. 10µl of MTT suspension was added onto the sample in each well. The plates were incubated for 4 hours at 37°C and incubated for another 30 minutes at room temperature in dark after adding 100µl DMSO. The results were examined using ELISA reader (Thermo Scientific Multiskan EX Microplate Photometer - Multiskan EX Photometer, Model 51118177).

Results

Isolation and Characterization of Human Adipose-Derived Stem Cells

The ADSC appeared initially as adherent, spindle-shaped cells during the first three to four days of culture; then, the cells started growing rapidly and become easily expandable. Immuno-phenotypic analysis with fluorescently labeled antibodies against a panel of cell surface markers was performed in order to determine the antigen expression profile of the established ADSC. The analysis revealed that the cells did not express endothelial markers (CD31-PECAM1), hematopoietic markers (CD34, CD45 and CD117-c-kit) or the marker for macrophages (CD11b). However, the cells expressed high levels of CD44, CD105 (SH2), adhesion markers (CD29-integrin β1 and CD90-Thy-1) and mesenchymal stem cell marker CD73, consistent with the published profile from adult adipose tissues. Also, the ADSC were negative for HLA-class II (HLA-DR), but positive for HLA-class I (HLA-ABC). Adipogenic potential of the cells was analyzed *in vitro* for the verification of the ability of established ADSC cultures to differentiate into adipocytes. Cells were maintained in adipogenic induction medium for five weeks to induce adipocytic differentiation. For histochemical examination, the adipogenic culture was stained for the presence of intracellular lipid droplets as an indicator of intracellular lipid accumulation. Oil Red-O staining of five-week cultures for intracellular lipid vacuoles did not reveal any cells resembling adipocytes in control cultures, whereas upon treatment with adipogenic inductive medium, cells with a typical adipocyte phenotype containing intracellular lipid droplets appeared.

Examination of ADSC-Egg White Scaffold and ADSC-PCL Interactions

Interactions between CM-DiI labeled ADSCs and egg white scaffold and PCL scaffold were examined using an inverted phase contrast microscope under UV light using a green filter for 12 days. The examination revealed that ADSCs on egg white scaffold proliferated periodically for 12 days as indicated by the increased density of the red florescent labeled cells; and ADSCs on PCL scaffold were most rich in red fluorescent labeled cells on the 6th day (compared to days 1 to 12) (Figure 1). Connections between ADSCs on egg white scaffold were clearly visible through day 1 to 12, whereas connections between cells on PCL scaffold were not definitively determined.

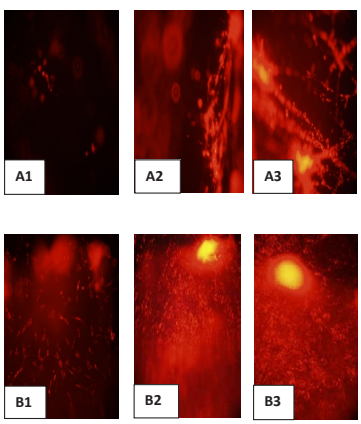
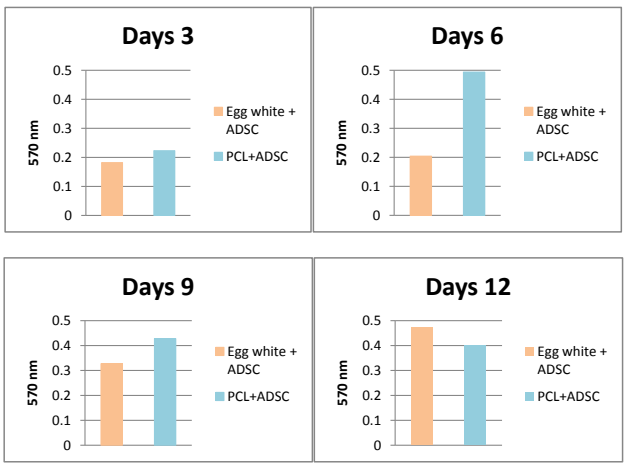


Figure 1: Inverted microscope images of CM-DiI labeled ADSC on egg white scaffold (A1, A2, A3) and PCL scaffold (B1, B2, B3) (10X).

Examination of Survival of ADSC on Egg White and PCL Scaffold 3D Culture

Four time points were determined for MTT assay (days 3, 6, 9 and 12) and ADSCs were cultured on egg white scaffold and PCL scaffold on 96-well culture plates for each time point (n:3). After the incubation, optical densities of ADSCs both on egg white scaffold and PCL scaffold were measured using ELISA Reader at 570 nm at each time point to understand vitality (Graph 1). According to this, ADSCs on egg white scaffold reached maximum survival on day 12, whereas ADSCs on PCL scaffold reached maximum survival on day 6. Furthermore, it was observed that the vitality of the cells on PCL scaffold started decreasing after day 6, however ADSCs on egg white scaffold increased vitality periodically through 12 days. This confirmed that the MTT assay results were consistent with the microscopic follow-up of the 3D cultures of the cells.



Graph 1: MTT Assay Results

Statistical Analysis

Statistical analysis of MTT assay results were determined using ANOVA and *Student's t test*. All data was run through Statistical Packages of Social Sciences (SPSS, version 19.0 for Windows) program and a $p<0,05$ was accepted to be statistically significant.

Consequently, ADSCs were shown to interact with both scaffolds and provided a 3D culture. Besides, it was observed that 3D culture using egg white scaffold had periodically increased vitality through 12 days, whereas PCL scaffold 3D culture had a decrease in vitality after the 6th day.

Discussion

Scaffolds in tissue engineering are 3D structures made up of natural or synthetic materials that can preserve cell viability, do not create immune rejection in the host organism and are biodegradable. There are many available natural or polymeric scaffolds with defined efficiency that are used in cellular therapies. In the field of tissue engineering and regenerative medicine biodegradable scaffolds based on natural or synthetic polymers have received special attention. These scaffolds can provide porous matrices that temporarily support and guide the cells and their development. An ideal polymeric scaffold requires several structural and chemical properties to control and promote specific events at the cellular and tissue level such as a target tissue adapted structure, a sufficient porosity as well as interconnected pores of a suited size, an appropriate surface chemistry, a defined degradation rate and an easy fabrication in a variety of shapes and sizes. However, designing a suitable scaffold for regenerative medicine applications has become one of the most challenging issues in material sciences [15-20]. Egg white which comprises about 60% of winged-animal eggs works as a shock-absorber to keep the egg yolk fixed, provides an anti-microbial barrier and plays a role in the development of the embryo with proteins and water. Together with having these biological properties and being a rich food source, egg white is used in food and medicine industry for it is cheap and rich in high quality proteins. It is also a very important product in biotechnology because of the antibodies, phospholipids, lysozyme, ovotransferrin and avidine it contains. Egg white contains 6.5% ovoalbumin, 1.5% ovotransferrin, 0.4% lysozyme and 0.2% ovomucine. With these valuable components, it is used in biomedical studies and areas of protein chemistry and also it is shown to have cytotoxic effect on tumor cells and insecticide, antiviral and antimicrobial properties that protect against cerebral hemorrhage [21]. Egg white is also commonly used in cell biology studies since it has effects on maintaining cell survival and differentiation. An in vivo study on mice has shown that transplanted egg white together with spleen cells helped maintaining spleen cell viability and differentiation [13,21,22]. Besides, an *in vitro* study showed that a scaffold prepared with egg white had supported cell growth in 3D [16]. Commercially available 3D cell culture media require special conditions and expensive setup, thus it is not usually possible to reach such sources by many laboratories and researchers. In our study, we used PCL scaffold to compare to egg white scaffold, because it is FDA-approved, easily processed, biodegradable and has high usage potential in many different areas of tissue engineering [23,24]. PCL scaffold dissolves easily, has low melting temperature, and helps cell adhesion and cell proliferation through its hydrophilic surface [23]. Besides, PCL scaffold is easily obtained by any lab with a polymer processing technology as it is a versatile, biodegradable, easily produced polymer with outstanding rheological properties [24].

Thus, with this study we suggested a natural scaffold made from egg white that is easily obtained, does not have ethical limitations, is compatible with host organism and supports cell growth in 3D in comparison to the PCL scaffold that is commonly used in tissue engineering studies.

In this experiment, CM-DiI labeled AD-MSCs were cultured for 12 days on ε-PCL or egg white polymerized at 60°C. The microscopic examinations showed that the AD-MSCs on egg white scaffold were increased in number periodically for 12 days and the connections between the cells were clearly visible on day 12. AD-MSCs cultured on ε-PCL were concentrated at day 6; however the cell number and connections were decreased at day 12. In accordance with the microscopic examinations, MTT analyses made on day 3, 6, 9 and 12 showed that AD-MSCs cultured on egg white scaffold reached maximum viability on day 12 and AD-MSCs cultured on ε-PCL scaffold reached maximum viability on day 6. Furthermore it was shown that the viability of AD-MSCs on ε-PCL scaffold started decreasing by day 6, but the AD-MSCs on egg white scaffold remained viable for 12 days. We think that the decrease in AD-MSC number after day 6 on PCL scaffold is because the PCL creates local acid as it is degraded [8]. Thereby, the 3D cell culture model prepared by AD-MSCs and egg white scaffold is proposed to

be superior to the 3D cell culture model prepared by AD-MSCs and PCL scaffold according to cell viability and proliferation studies. The results of MTT analyses performed were in accordance with the results observed with microscopic examinations. Furthermore, for the first time CM-DiI is shown to be an appropriate marker for microscopic examinations of 3D structures in tissue engineering studies.

With this study, by comparing the interaction between adipose derived mesenchymal stem cells and egg white scaffold and PCL scaffold, it was shown for the first time that mesenchymal stem cells can interact with both scaffolds and form a 3D culture. This study proposes the egg white-AD-MSC 3D cell culture model as an alternative to other 3D culture models used in tissue engineering studies for the first time. Thus, egg white is suggested as an affective candidate for use in tissue engineering studies as scaffold since it is a natural, easy to obtain and prepare, bio-compatible and cheap material. Our findings suggest that egg white based scaffolds can be prepared for use in tissue regeneration studies on damaged solid tissues such as bone and cartilage.

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