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Two Layered Scaffolds (Loofah/PLLA/Cellulose/Chitin) for Repair of Osteochondral Defect

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Abstract

Research of tissue engineering and regenerative medicine continues to develop advanced materials that can better mimic the significant architecture and functional properties of native tissues. Treatment of osteochondral injuries by using scaffolds contains the problem of fixation and integration of the engineered tissue to the surrounding one. Therefore, tissue engineered osteochondral graft design must be directed not only to the injured cartilage but also to the subchondral bone for a sufficient osteochondral repair and integration of the neo-cartilage into the osseous surrounding. In this study, we produced a bilayer scaffold and investigated the ability of co-cultures of chondrocytes and osteoblasts to repair articular cartilage in osteochondral defects. For this purpose, fibrin glued loofah+PLLA+cellulose scaffold with MG-63 cells and loofah+PLLA+chitin scaffold with SW-1353 cells were used to promote bone and cartilage regeneration, respectively. Viability tests and morphology images indicated that this bilayer scaffold had good affinity for osteoblast and chondrocytes cells, encouraging their growth, proliferation and attachment. Histological and immune-histochemical staining analyses confirmed that loofah bilayer scaffolds provided a good support for the cells. Based on the preliminary results *in vitro*, we suggest that the integrated bilayer scaffold consisting of loofah+PLLA+cellulose and loofah+PLLA+chitin, has potential use to repair osteochondral defects, either upon cellular implantation and/or in acellular form.

Keywords: Scaffold; Loofah; PLLA; Cellulose; Chitin; Osteochondral

Introduction

The osteochondral defects containing cartilage and subchondral bone has meagre regeneration capacity because of the different mechanical properties, composition and biological structures of each tissue. The mechanical strength of newly formed articular cartilage in comparison to the natural tissue may create adverse effects, leading to further degeneration of both repaired and adjacent native tissues with a decline toward osteoarthritic conditions.

Recent developments in tissue engineering in the field of orthopedic research have been the design of the relationship between the autologous cells and protein that promotes cell adhering with osteoconductive material in order to generate osteoinductive materials [1].

Critical parameters for the tissue engineering scaffolds are; bio-compatibility, biodegradability, optimum mechanical resistance and arrangement of the appropriate cellular activities [2,3]. The 3D porous membranes provide a microenvironment within the scaffold that contains pores large enough (10 μm) for living cells to move throughout the membrane. The porosity allows the nutrient uptake and cellular waste product removal [4].

Several materials, together with cells, have been suggested for bone and cartilage regeneration including ceramics, hydroxyapatite-based materials or ECM derivatives as well as natural and synthetic polymeric materials [5,6]. Cellulose fibers with linear homopolymer of glucose $(C_6H_{10}O_5)n$, with n ranging from 500 to 5000, are one these potential reinforcing materials [7,8]. They display good mechanical properties and have low weight, and natural advantages such as biodegradability, low cost and renewability. In addition, there is plenty of them in nature.

PLLA is a commonly used non-toxic biodegradable material used in tissue scaffold building. It has a dense and smooth surface morphology suitable for osteoblast cultures [9]. However, PLLA has a few obvious weaknesses as a scaffold material; rapid biodegradation, acidic decomposition by-products and hydrophobicity [10].

Chitin, occurring mainly in crustacea, mollusks and insects as an important constituent of the exoskeleton, is another most abundant form of organic materials [11,12]. It is a biodegradable polymer because the β-1, 4-glycosidic linkage is susceptible to degradation by lysozymes [12-14]. In addition, it can easily be processed into membrane [13-16] nanofiber, gel [17-19] and scaffold [20,21] forms.

In this study, we aimed to examine the characteristics of these two natural materials in combination with loofah as bi-layered scaffolds, and to determine the morphology, adhesion and proliferation capacities of chondrocytes and osteoblasts seeded on these scaffolds.

Materials and Methods

MG-63 Osteosarcoma Human Cells, SW-1353 Chondrosarcoma Human Cells, (ATCC; Manassas, VA, USA), MEM-Eagle, L-Glutamine (200 mM, G7513, Sigma), Pen-Strep-Ampho (03-033-1B Biological Industries), resazurin assay (Cell signaling, 11884), ALP kit (Enzyline PAL Optimise; Biomerieux, France), BCA, Paraformaldehyde (PFA).

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Scaffold preparation and characterization

Initially, loofah was soaked to swell and washed with water, then dried with NaOH (2 M). The reason for the wetting and swelling process of the loofah sponges is that the loofah has its own unique yellowish juice. This process was done to replace juice of loofah with water at first stage.

Loofah+PLLA+cellulose: Loofah was first dipped in 4% (w/v) PLLA solution in chloroform, and dried. PLLA coated loofah was then soaked in 4% cellulose (in 4% PLLA mixture) and dried at 50°C as previously described [6].

Loofah+PLLA+chitin: Loofah was coated with 4% PLLA solution in chloroform and dried prior to soaking in 4% chitin, instead of cellulose. The liquid is separated and dried at 50°C. All scaffolds were sterilized at 90°C with ethylene oxide prior to use in tissue cultures. Afterward the scaffolds were sticked with fibrin glue (Figure 1).

Cell culture

SW-1353 chondrosarcoma human cells and MG-63 osteosarcoma human cells purchased from the ATCC (Manassas, VA 20108 USA) were cultured in DMEM medium containing 10% FCS, L-Glutamine (200 mM, G7513, Sigma), Pen-Strep-Ampho (03-033-1B Biological Industries) at 37°C in a humidified 5% CO_2 atmosphere. SW-1353 and MG-63 cells were seeded on previously fibrin glued loofah+PLLA+chitin and loofah+PLLA+cellulose scaffolds (Size: 4 × 4 × 3 mm (L × W × H); n=3), respectively to form double layers (Figure 1). Firstly, chondrocytes were seeded on loofah+PLLA+chitin layer and incubated for 30 min at 37°C in a humi dified 5% CO_2 atmosphere for adhesion of cells. Then, osteoblasts were seeded on loofah+PLLA+cellulose turning the scaffolds upside down. In order to avoid cell loss, the cells were added and cultured carefully on either side of the scaffold, allowing them to adhere. After that, culture medium was increased to cover the scaffold for optimum culture conditions. 1×10^6 cells/mL cells were seeded on each layer scaffold. Cells on scaffolds were analyzed at culture days of 3rd, 5th and 8th.

Scanning electron microscopy (SEM)

The scaffolds were washed with PBS three times before the analysis after the culturing period. Then, they were fixed in sodium cacodylate buffer (0.1 M) containing 5% glutaraldehyde (pH 7.2), 7% sucrose and 2% osmium tetraoxide. After being dried in graded ethanol series, samples were covered with gold under 10 kV vacuum (EMITECH K550X) and examined using FE-SEM (FEI Quanta 250 FEG).

Energy dispersive X-ray spectrometry (EDS)

Scaffolds were evaluated by elemental analysis using SEM (Oxford model INCA 300) Energy Distribution Spectrum (EDS) attachment at the end of the culture period. Elemental composition of the scaffold materials was determined along with the cell formation. Ca and P concentrations were calculated.

Cellular viability assay (XTT) of cells

Cellular viability of the cells attached on scaffolds was measured by a commercially available XTT assay kit using a spectrophotometer (Cary 50 UV-Vis).

Lactate dehydrogenase (LDH) activity of cells

Cytotoxicity of the scaffolds was evaluated measuring LDH activity in culture medium by using a commercially available colorimetric assay using a spectrophotometer (Cary 50 UV-Vis).

Analysis of ALP activity of cells

Osteoblastic activity of bone cells was analyzed by a commercially available alkaline phosphatase activity kit. After a 3 min incubation at 37°C ALP activity in conditioned medium was measured according to manufacturer's recommendations at 405 nm spectrophotometrically (Cary 50 UV-Vis).

Histologic staining

Histological examinations were performed on cell seeded

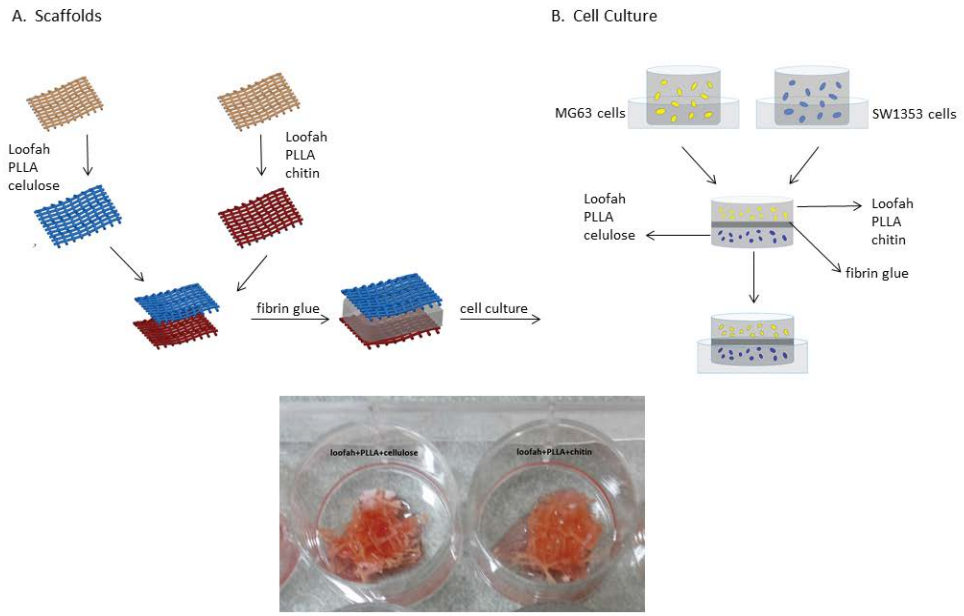

Figure 1: Scaffold preparation with fibrin glue and cell culture. **A.** Loofah, PLLA and cellulose scaffolds and loofah, PLLA and chitin scaffolds were sticked with fibrin glue. **B.** SW-1353 cells were seeded on loofah+PLLA+cellulose scaffold layer; MG-63 cells were seeded on loofah+PLLA+chitin scaffold layer.

scaffolds to determine extracellular matrix (ECM) accumulation and morphological changes in cells. Hematoxylin and eosin (Hand E; for total cellularity), Masson's trichrome (for collagen organization), alizarin red (for calcium depositions), alcian blue and toluidine blue (for proteoglycans) stainings and immunohistochemistry (for type I and II collagens and for chondrocyte and osteoblast phenotype identification) were performed.

The samples were kept in 10% formalin for 48-72 h and then buried in paraffin blocks. 5 μm thick sections were taken from these blocks. Slides were stained with Hand E (01562E, Surgipath, Bretton, Peter Borough, Cambridgeshire) while others stained with Masson's trichrome (2049 GBL, Istanbul, Turkey). De-paraffinized slides were stained with alizarin red solution (ECM815, Chemicon, Germany) after rehydration for 2 min. Excess dye was washed away with acetone-xylene (1:1) solution. Slides were cleared in xylene and mounted in a synthetic mounting medium. Calcium deposits stained orange-red.

Similarly, after de-paraffinization and rehydration some slides were stained with Alcian blue (8GX, Merck, Germany) for 30 min. Slides were washed with tap water for 2 min and dehydrated through 95% alcohol, 2 changes of absolute alcohol, 3 min each. They are cleared in xylol and mounted. Slides were also stained with toluidine blue (C152040, Merck, Germany) for 2 min. After being washed with distilled water slides were kept in 96% alcohol, cleared in xylene and mounted.

Immunohistochemistry staining

Immunohistochemistry analyses were performed for collagen Type I (Bioss bs0578-R) and collagen type II (Abcam Collagen II, ab34712) antibodies. Sections were deparaffinized at 60°C in an incubator, washed in xylol three times for transparency process and digested with 0.25% (w/v) trypsin for 15 min at 37°C. The sections were washed with PBS, treated with blocked solution (TA-125-UB, Invitrogen-, Fremont, CA) for 30 min. After incubated with the primary antibody for overnight at 4°C, sections were washed again with PBS and incubated for 20 min with anti-mouse biotin-streptavidin hydrogen peroxide secondary antibodies (Invitrogen-Plus Broad Spectrum 85-9043). The signal was developed using DAB (Roche, Germany). Finally, the sections were stained with Mayer's Hematoxylin and examined with light microscopy.

Statistical Analysis

The data of the XTT, ALP and LDH experiments were analyzed by using parametric repeated measurement ANOVA and non-parametric Kruskal-Wallis tests. $p<0.05$ was considered statistically significant. Statistical analyses were carried out by using Social Sciences Statistical Package (SPSS), Version 20.

Results

Morphology of Co-Culture cells seeded on scaffolds

Morphology of the loofah+PLLA+cellulose and loofah+PLLA+chitin scaffolds, cultivated with SW1353 and MG-63 cells respectively were characterized by SEM analysis (Figure 2). Cells were observed to adhere to their bi-layered scaffolds and to maintaine their global and the spindle-shaped forms. Furthermore, newly produced extracellular matrix was visible on the scaffolds.

EDS of loofah based scaffolds

Loofah based scaffolds were characterized by EDS to show presence of Carbon (C), Oxygen (O), Nitrogen (N) elements. Due to the main structure of the loofah sponge and its cellulose coating, mainly C and O elements were observed. C and O elements were among the key elements of PLLA. N element, a key element of chitin, was also detected (Figure 2).

Viability of cells in 3D cultures (XTT Assay)

The viability of cells was not influenced by scaffold structure. Cell numbers continued to increase in a time-dependent manner during culture period (Figure 3A). XTT assay showed that on the 8th day, cell numbers were significantly higher compared to the 3rd and 5th days ($p<0.05$). A statistically significant difference was determined in cell viability between culture days 3, 5 and 8 (Figure 3A, $p=0.030$, $p=0.002$, $p=0.010$).

Cytotoxicity in 3D cell cultures (LDH activity)

LDH levels released from cells slightly increased during culture period. Differences in LDH activity were statistically significant between groups ($p<0.05$). Increased LDH activity at 5th day might be

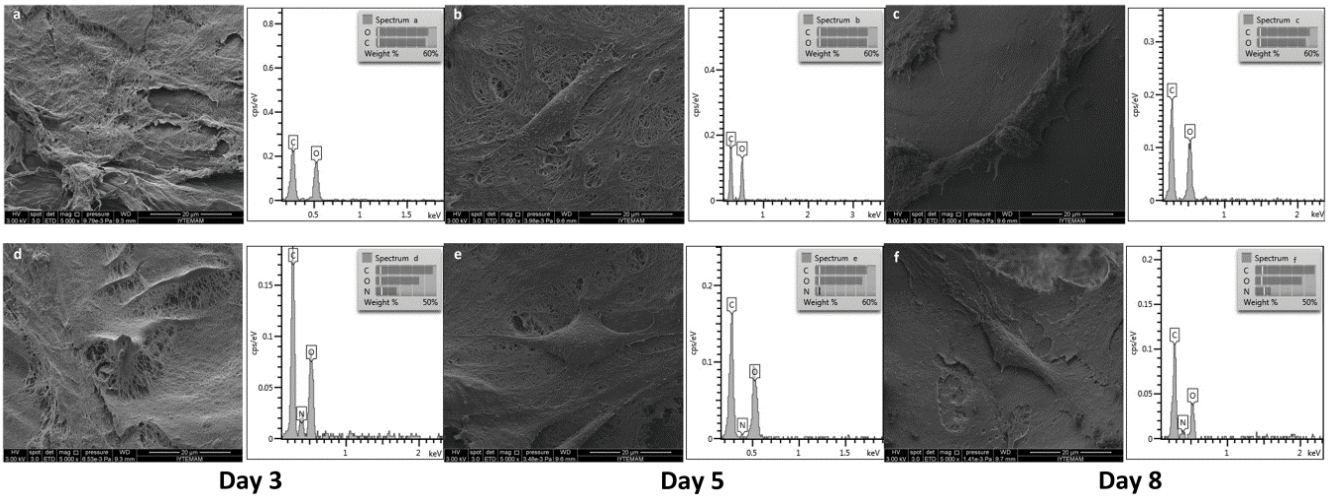

Figure 2: SEM images of bilayer scaffold morphology obtained in different experimental days on 3, 5 and 8. Osteoblast morphology of the (a-c) loofah+PLLA+cellulose part of the bilayer scaffold. The images (d-f) illustrate the chondrocyte cells on loofah+PLLA+chitin part of the bilayer scaffold.

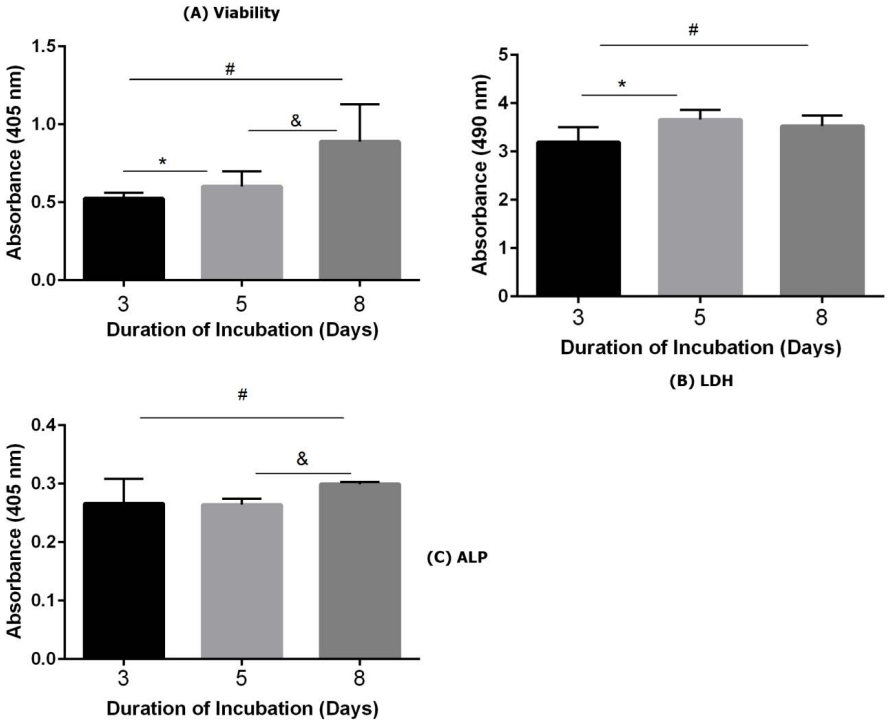

Figure 3: (A) The viability of the cells on bi-layer scaffolds over 8 days period. Error bars represent ± SD of the mean values (n=3). *3rd vs. 5th day (p=0.03); #3rd vs. 8th day (p=0.01); and 5rd vs. 8th day (p=0.01). (B) LDH activity of the seeded cells after 8 days culture on bi-layer scaffolds. Error bars indicate ± SD of the mean values (n=3). Statistically significant represents that cytotoxicity has significantly different on different days. Compared with the *3rd vs. 5th day (p=0.016); # 3rd vs. 8th day (p=0.035). (C) The ALP activity of cells seeded on bilayer scaffolds at different period of culture. Error bars represent ± SD of mean values (n=3). #3rd vs. 8th days (p=0.006); and 5th vs. 8th days (p=0.004).

the result of release of PLLA polymer which can be toxic during its degradation (Figure 3B).

ALP activity in 3D cells cultures

ALP activity increased significantly during 8-day assay period ($p<0.05$). It was the highest in the conditioned media at the 8th day. Slight decrease in ALP levels on the 5th day was followed by an increase on the 8th day (Figure 3C). This finding might be the result of the temporary decrease of osteoblastic activity of MG-63 cells due to the degrading polymer toxicity.

Histological analysis

Hand E-staining revealed heterogeneous cell morphologies with extracellular matrix formation within bilayer scaffolds (Figures 4A-4C) Masson's trichrome staining confirmed the existence of fibrin glue, scaffolding and the cells in cultures (Figures 4D-4F). Histological analyses using toluidine blue (Figure 4) and alcian blue (Figure 5) staining confirmed well-formed chondrocytes 8 days after seeding. Toluidine blue staining showed the production of aggrecan and sulfated glycosaminoglycans in ECM on bi-layer scaffolds. Scaffolds showed highly stained proteoglycan by toluidine and alcian blue. To detect individual chondrocyte-like cells, sections were stained with Alcian blue (pink colour). Osteoblast lineage on scaffolds was determined by alizarin red staining which is commonly used in osteoblast cultures. Dark cell staining demonstrated osteoblastic function in bi-layer scaffold structures (Figure 5). Similar to the results of Masson's trichrome staining, immunohistochemical analyses revealed the presence of both collagen types I and type II expression in cultures (Figures 6A-6F).

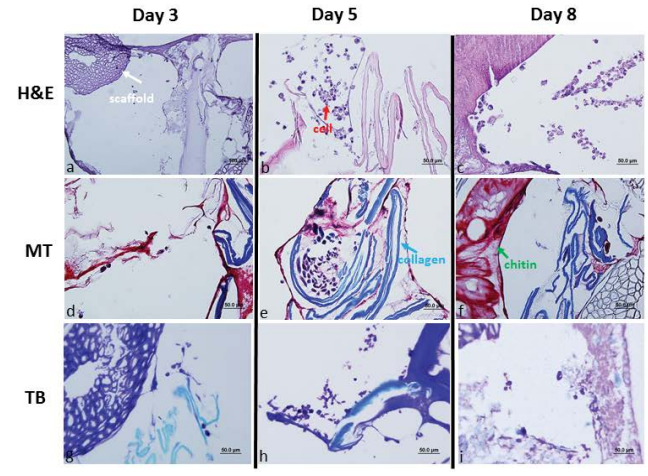

Figure 4: Histological examination of cell-seeded, bilayer scaffold constructs at 3rd, 5th and 8th days of cultures. H&E staining showed extracellular matrix and heterogeneous cell morphologies; round-elongated osteoblast- and chondrocyte-like cells through constructs (a-f). Masson's trichrome staining reveals collagen and keratin inside the chitin. Proteoglycans and glycosaminoglycans were stained in blue with toluidine blue staining (m-s). Red arrows indicate cells, white arrows indicate loofah, green arrows indicate chitin, and blue arrows indicate collagen (g-l). Magnifications: 20x (a-c, g-i), 40x (d-f, j-l).

TEM analysis

TEM were assessed to show the osteoblast and chondrocyte

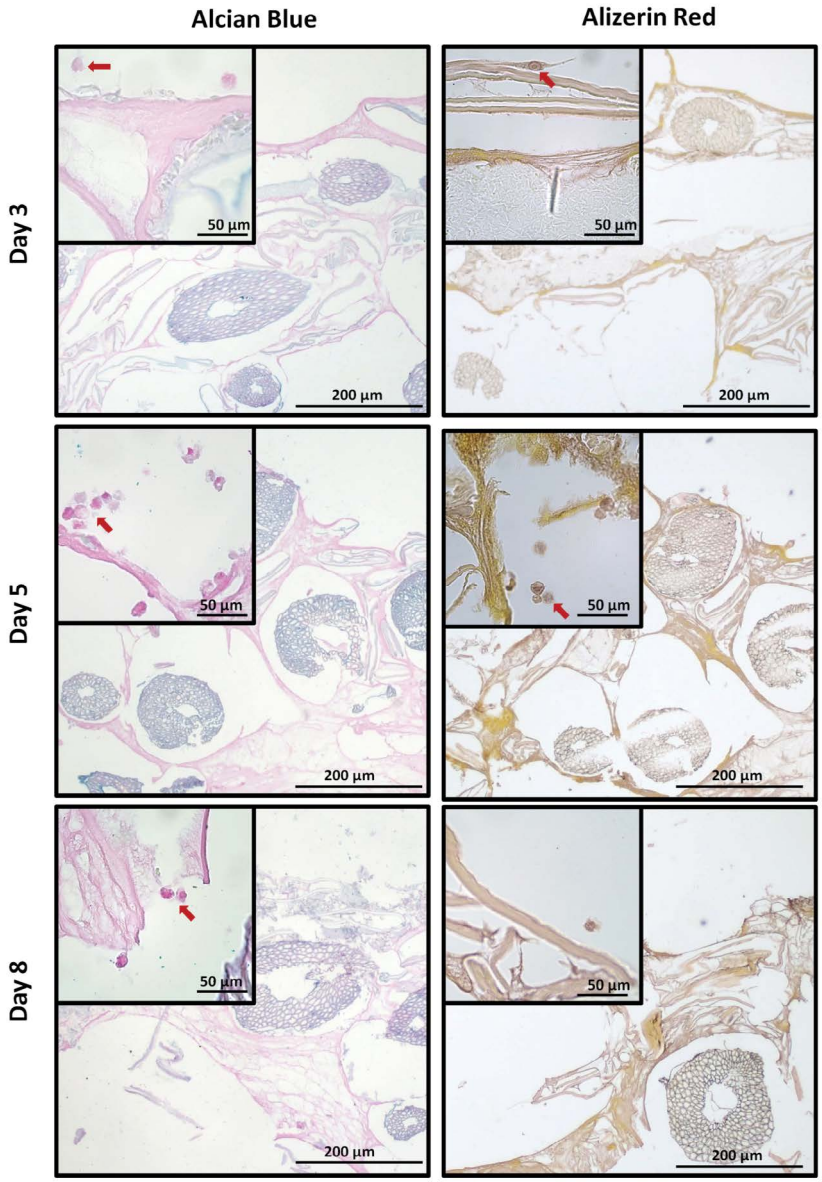

Figure 5: Chondrocytes (a-c) and osteoblasts (d-f) with Alcian blue and Alizarin red staining, on days 3, 5 and 8. Red arrows indicate the cells. Calcified-bony structures were observed stained orange-red (a-c). Nucleuses were bright pink-red, cytoplasms observed as pale pink and acidic structures were observed in blue (d-f). Scale bars: 40x, 10x (a-f).

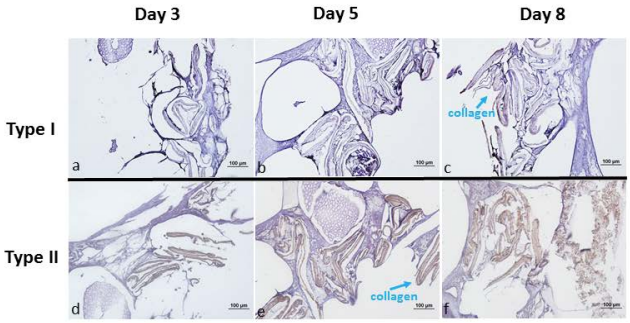

Figure 6: Immunohistochemical staining of bilayer scaffolds sections. As part of bilayer scaffolds; collagen type I (a-c) in loofah+PLLA+cellulose scaffolds and collagen type II (d-f) in loofah+PLLA+chitin scaffolds were present as densely stained fibrils after 8 days. Blue arrows indicate the collagen fibrils. Magnifications: 20x.

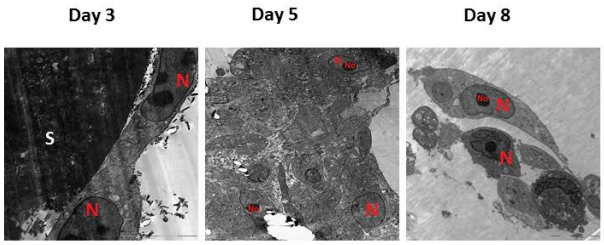

Figure 7: TEM images of cells in the bilayer scaffolds. Adherent cells on the surface of the bilayer scaffolds with normal ultrastructural morphology. Nucleus and nucleolus can clearly be observed. N: Nucleus, No: Nucleolus, S. Scaffold. Scale bar: 10.000 nm.

interactions. Ultrastructural morphology showed that cells developed bud-like extensions and were in contact with each other (Figure 7).

Discussion

Cell therapy and tissue engineering is a promising alternative to artificial permanent implants for repair of injured tissue. As a widely accepted discipline in biological and medical sciences, bone and cartilage tissue engineering achieves successful results in growth of human bone and cartilage tissues. This study presents a loofah based bilayer scaffold for chondrocyte and osteoblast co-cultures to mimic and repair osteochondral defects. Previously, Cecen et al. [6] reported biocompatibility and biomechanical characteristics of loofah based scaffolds and suggested possible use of these constructs in bone and cartilage tissue engineering.

Bio-characteristics of implant materials mostly depend on their chemical compositions, composite surface characteristics and porosities. The first objective in our work to use loofah sponge material was to have a biocompatible, natural entity with porosity. This study was a follow up of the previous publication showing no toxic effects of loofah matrix on chondrocytes in which the cellulosic structure of loofah had been aimed especially for the use of cartilage. The purpose of the cartilage tissue engineering is to design biodegradable scaffolds that can be integrated with a porous texture that ensures nutrients and waste products to spread.

On the other hand, bone graft materials are required to be not only biocompatible but also biodegradable, osteoprotective and osteoinductive. As a matter of principle, osteochondral graft design should be directed to sub-chondral bone not only for injured cartilage, but also for sufficient osteochondral repair and to be integrated into the neo-cartilage around the bone [22].

Biocompatibility is one of the important factors in adherence of the cells on the surface of a biomaterial. Cell adherence is determined by experimental methods relying on morphological and biomechanical approaches. According to our EDS analyses of the scaffolds, the C, O and N elements which are in the main structure of PLLA, cellulose and chitin were present as expected (Figure 2). Morphology of scaffold surface is an important factor. While porosity of scaffold material mimics the microstructure of cancellous bone or cartilage, the surface properties of biomaterials play a major role in cellular interactions, such as cell adhesion, infiltration and proliferation [23,24]. In present study, morphological analyses demonstrated that osteoblasts and chondrocytes adhere on the surface and moved within the pores of the bilayer scaffolds of loofah+PLLA+cellulose and loofah+PLLA+chitin. Loofah sponge structure does not have a porous structure because it is in a fiber nature. In our system, after five days of culture, chondrocytes were observed to spread along the scaffolds. Cells were heterogeneously allocated on and in the bilayer scaffolds, penetrating from the seeded side to the opposite side so that they can pass through to the adjacent scaffold. In bilayer scaffolds cell growth was observed at all-time points within extracellular matrix (Figure 2). Our results show that, this novel loofah construct may provide an opportunity for cells to proliferate and penetrate in fiber oriented structures.

The viability analyses on 8th day showed that cells were viable and continued to proliferate (Figure 3A). Sung et al. reported that fast degradation of the scaffold might negatively affect the cell viability and migration into the scaffold *in vitro* and *in vivo* [25,26]. Indeed, we examined the LDH activities slightly increased (Figure 3B) at day 5. This simultaneous differentiation might be due to the release of PLLA polymer which can be toxic during its degradation. This result is in agreement with a study by Marques et. al which also reported that starch-based polymers had a higher degree of cytotoxicity [20]. Nonetheless, our results suggest that loofah degradation is slower due to its cellulose content and thus minimized the negative effects of polymer release from degrading scaffold on cell viability. These results fully demonstrate that, there is not only one factor to be considered on its own, from the standpoint of material science, affecting cellular viability and adhesion [24]. Following day 5, there was a decrease in LDH levels suggesting that cells might recover from the toxic effects of degrading polymers if they were kept in cultures longer.

ALP activity by cells indicated osteoblastic phenotype of the cultured cells. ALP levels demonstrated that cell proliferation on the scaffolds significantly improved in eight days' experimental period, suggesting that the loofah+PLLA+cellulose scaffolds are suitable for osteoblast seeding and growth. The slight decrease in ALP activity on day 5 may again be explained by the early degradation of PLLA causing a temporary toxicity.

Heterogeneous round-elongated osteoblast or chondrocyte like cells in construct were observed in various sections of bilayer scaffold structures on days 3rd, 5th and 8th with Hand E staining (Figures 4A-4C). Cells were in close vicinity of the collagen structures. Masson's trichrome staining indicated increased formation of collagen organization in scaffolds (Figures 4D-4F). We believe that this staining was the outcome of newly produced collagen by the cultivated cells. This newly produced collagen fibers might be the triggering effect of absorbed fibrin glue during the culture period. Loofah+PLLA+chitin stained red with masson Trichrome staining at the edges due to the presence of keratin in chitin in the scaffold structures. Dense alizarin red staining in the bi-layer scaffolds also supported the presence of osteoblast to in cultures. Simultaneously, presence of chondrocytes in bilayer scaffolds construct were confirmed by Alcian blue. Accumulation of chondrocytes in scaffolds were observed as pink staining. Additionally, deep blue and pink coloured staining in scaffolds indicated chondrogenesis. Cells tend to aggregate and to be surrounded by cartilaginous ECM. These results indicated GAG production in loofah based bi-layer scaffolds (Figure 4). The histological data indicated that both the cartilage and bone sections of the scaffold exhibit homogeneous cell distribution and matrix formation.

Immunohistochemical procedures distinguished collagen types produced by cells in the bilayer scaffolds. Chondrocytes on the loofah+PLLA+chitin site of the scaffolds were observed to produce more type II collagen compared to type I whereas on the loofah+PLLA+cellulose site type I collagen staining was more prominent. This finding might confirm co-existence of chondrocytes and osteoblasts in bilayer scaffold cultures keeping their individual phenotypic characteristics (Figure 6).

TEM analysis confirmed the existence of cells in the loofah based scaffolds. Histological results were consistent with the TEM investigations. Some cells were found to be more rounded whereas others were more in fusiform shape (Figure 7). We think that fusiform shaped cells to be chondrocyte-like cells while round ones were more likely to be osteoblasts. TEM images implied that cells kept their normal morphologies with clear nuclei and nucleoluses. Moreover, cells also formed aggregates as observed in normal *in vivo* tissues and performed contact with each other via bud-like formations. However, further surface marker analyses are required to determine the exact localization of different cell types in co-cultures.

Loofah can be considered to design double layer osteochondral structures due to its nontoxic, cellulosic structure and the difficulty in dissolving. This is the first study in literature showing that cultivation of chondrocytes and osteoblast on loofah based scaffolds have potential as a novel approach for applications in the field of cartilage tissue engineering to repair articular defects.

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