Original Article

Morphological characteristics on a Scanning Electron Microscope of generated hyaline cartilage tissue from adipose mesenchymal stem cells, on Polycaprolactone scaffolds

Pappi Eleni[2], Theodoridis Konstantinos[1,2], Aggelidou Eleni[1], Manthou Maria Eleni[2], Kritis Aristeidis[1]

[1] *Department of Physiology and Pharmacology, School of Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki (A.U.Th), Thessaloniki, Greece.*
[2] *Laboratory of Histology, Embryology and Anthropology, School of Medicine, Faculty of Health Sciences, Aristotle University of Thessaloniki (A.U.Th), Thessaloniki, Greece*

Abstract

Cartilage regeneration is of great interest to the medical community, given the prevalence of osteocartilage defects in the population coupled with the tissue's low intrinsic self-repair potential. A recently new FDA approved biomaterial and 3D-printing technology provided us the opportunity to fabricate tailor made scaffolds. We used collagen coated and uncoated scaffolds to develop hyaline cartilage from adipose mesenchymal stem cells (ADMSCs). The aim of this study is to present the scaffolds' morphological characteristics, as observed on a Scanning Electron Microscope (SEM) of generated cartilage tissue and to compare the two types of scaffolds. Cylindrical shaped PCL scaffolds, 10mm in diameter, were fabricated. ADMSCs were harvested and were cultivated on PCL scaffolds. Half of the scaffolds were treated with collagen I by coating. After 26 days in culture, the scaffolds were examined by SEM. Visualization was succeeded on the top and bottom surfaces and on the cross sections of each scaffold. At day 26, scaffolds revealed extensive colonization and viability of ADMSCs, with concurrent depositions of extracellular matrix. SEM images show that surfaces were covered with a significant amount of material with a glossy, transparent appearance, indicating the development of regenerated cartilage, more apparent on the coated scaffolds. Cultured cells demonstrated aligned direction on the scaffolds' fibers and the ECM that was produced connected the pores of the scaffolds by building apparent bridges between them. The penetration of cells was limited in the coated scaffold. We used 3D printing technology for PCL scaffold production, towards a cartilaginous implant development. SEM images provide us visualization of the scaffolds with the newly developed cartilage tissue and demonstrate that the scaffolds' purpose for chondrogenesis was served successfully in all cases and PCL displayed good biocompatibility. Collagenation of scaffolds led to a higher density of cells on the surfaces but also to a limited penetration within, not fully serving the purpose of a 3D culture.

Keywords: cartilage, regeneration, 3d printing technology, polycaprolactone, scaffolds, scanning electron microscope

Corresponding author:
Maria Eleni Manthou, Assistant Professor, Laboratory of Histology – Embryology, Aristotle University of Thessaloniki, Greece, mmanthou@auth.gr

Introduction

Cartilage is a type of resistant, flexible connective tissue with high content of glycosaminoglycans and proteoglycans in its extracellular matrix. It is found in many areas in the body including the rib cage, ear, intervertebral discs and articular surfaces in the joints. It protects underlying structures, absorbs vibrations and facilitates movements. There are three types of cartilage- hyaline, elastic and fibrocartilage- that differ in their composition of extracellular matrix, adapting to local bio-mechanical needs (Mescher, 2013). Cartilage defects usually result from aging, joint injury and developmental disorders, which often cause joint pain and loss of mobility (Mescher, 2013). However, the regeneration potentiality of damaged cartilage is extremely slow and ineffective, because the tissue is avascular, aneural, alymphatic, with a low metabolic rate (Moutos and Guilak, 2010, Temenoff and Mikos, 2000).

Many surgical techniques and cell-based therapies have been introduced over the last decades in order to enhance cartilage repair and regeneration. Cell-based regenerative therapies appear to be more efficient than classical surgical methods, rapidly providing the patient with better quality of life (Dinescu et al., 2015). Tissue engineering is an interdisciplinary field that combines the knowledge on cells, the use of engineering materials and the application of suitable biochemical factors, in order to create artificial organs and tissues or to regenerate damaged tissues. Regenerative medicine and cartilage tissue engineering have undergone several stages of evolution and nowadays different biomaterials are available to be used as base constructs, called scaffolds, on which cells are encouraged to colonize, proliferate and finally differentiate to chondrocytes (Dinescu et al., 2015). In this study a 3D printing technique was used to fabricate scaffolds for cartilage tissue regeneration, with polycaprolactone (PCL) as a base biomaterial.

Materials and Methods

Human adipose tissue (~ 70 ml) was harvested, from a lipoaspiration procedure, under Papageorgiou Hospital Review Board approved protocols, 263-7/12/2016 and patient informed consent. Cells were isolated and characterized as Mesenchymal Stem Cells.

An additive manufacturing method was used to create cylindrical polycaprolactone (PCL) scaffolds, 10mm in diameter and 3mm in height. The infill fiber, which was 400μm in diameter, followed a rectilinear pathway, thus resulting to a pore network of 200μm rectangular pores. After fabrication, PCL scaffolds were immersed in a 4M NaOH bath, for approximately 20h, to increase their surface hydrophilicity and clean their fibers (Moutos and Guilak, 2010, Moutos et al., 2016, Tsuji et al., 2003). Half of the scaffolds (designated as Col-group) were further treated with Collagen I (rat tail, BD), by gelation method. All scaffolds were seeded with 2×105 cells/scaffold. At the 7th day chondrogenic medium (TGF-β2) was added on both types of scaffolds, for a period of 26 days.

Morphology of seeded adipose derived stem cells (ADMSCs) of gelated and non-gelated scaffolds was observed visualizing sample surfaces and cross sections by means of SEM. Scaffolds were fixed with 3% v/v glutaraldehyde, rinsed, post-fixed with osmium tetroxide, rinsed again and then were dehydrated in increasing concentrations (30%-100% v/v) of ethanol in water. The samples were dried, sputter-coated with carbon and

observed under a SEM (JEOL JSM-6390 LV) at an accelerating voltage of 20 kV.

Results

At day 26 of chondrogenic differentiation scanning electron microscopy (SEM) images were taken from top and bottom surfaces, from the first layers beneath these surfaces and from a cross section of each scaffold. Images show an overall extensive colonization and viability of ADMSCs, with concurrent depositions of extracellular matrix. Top and bottom surfaces were covered with a significant amount of a glossy material, indicating the development of new regenerated cartilage (Fig.1A).

The extracellular matrix (ECM) produced in all scaffolds connected their pores by building apparent bridges between the scaffolds' fibers (Fig.1.B). The ECM appeared either spongioid or fibroid and covered the whole surface of each polymer (Fig.2, Fig.3A). In the Col-PCL scaffolds the extracellular matrix was denser compared to the control scaffolds (Fig. 2). They seemed to be covered by a thick membrane which often appeared crystallized (Fig.2A).

Cells were found mostly on the top surfaces of all scaffolds with the highest density appearing in the Col-PCL scaffold, but they were not so distinct because they were covered by ECM. This was probably also the reason their outer surfaces appeared irregular (Fig.2B). They often demonstrated an aligned direction on the scaffolds' fibers. They were relatively sparse within the scaffold (Fig.3B), but often formed bigger, tighter groups (Fig.2B). They usually appeared large and round, but sometimes they were oval or spindle shaped (Fig.3). In areas with less ECM we were able to observe cells that were attached to the scaffold by small anchors (Fig.3B).

Inside the scaffolds, as shown by cross sections of both types, there were fewer cells and less ECM, compared to the surfaces. In addition, the cells in Col-PCL scaffolds did not succeed to penetrate to the extent that was noticed in the controls.

Discussion

The effectiveness of a scaffold is related to many parameters, such as the material used, its mechanical and physico-chemical properties, its biocompatibility and biodegradability (Khan and Tanaka, 2018). Research aims to create the most appropriate scaffold, for the ideal recreation of the in vivo microenvironment (Vinatier et al., 2009). For this reason a group of different materials have been tested, each of them presenting its own advantages and drawbacks (Doulabi et al., 2014). In this study 3D printed scaffolds were fabricated by polycaprolactone (PCL).

PCL is an aliphatic polyester that has been widely investigated for use in biomedical applications and has received FDA approval and CE Mark registration. PCL is a kind of polymer with a glass transition temperature of about −60 °C and a melting point of 55–60 °C, depending on the degree of crystallinity, which in turn is dictated by the molecular weight and the scaffold fabrication process. It demonstrates a slow degradation rate 1-2 years (Hutmacher et al., 2001). Due to its semi-crystalline and hydrophobic nature, the mechanical properties of PCLs are suitable for a variety of applications (Xue et al., 2017).

PCL represents a biomaterial that recreates the physical and biomechanical properties of the native tissue and encourages cell infiltration, growth, and differentiation. It displays
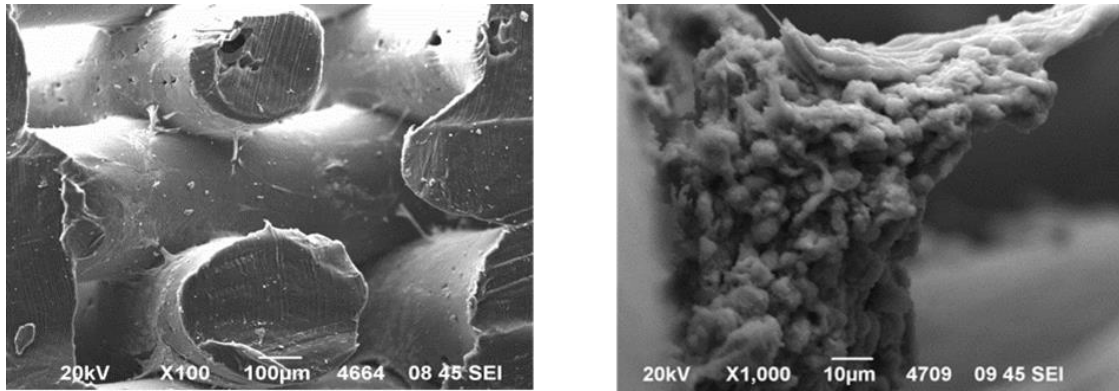
Figure 1: SEM of the adipose derived stem cells (ADMSCs) seeded in PCL scaffolds. A. X100. Surfaces of the scaffold covered with a significant amount of a glossy, whitish material, indicating the development of new regenerated cartilage. B. X1000. The ECM connects the scaffold's pores by building apparent bridges between the scaffolds' fibers. Cells appear round shaped.
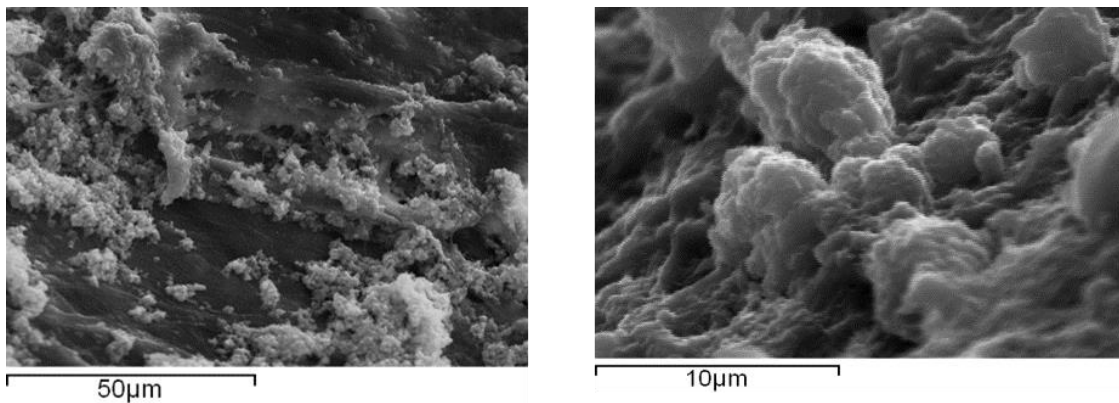

Figure 2: SEM of coated PCL scaffolds. A. X1000. Spongioid and crystallized ECM on a scaffold fiber. B. X5000. Spongioid ECM completely covers the cells.
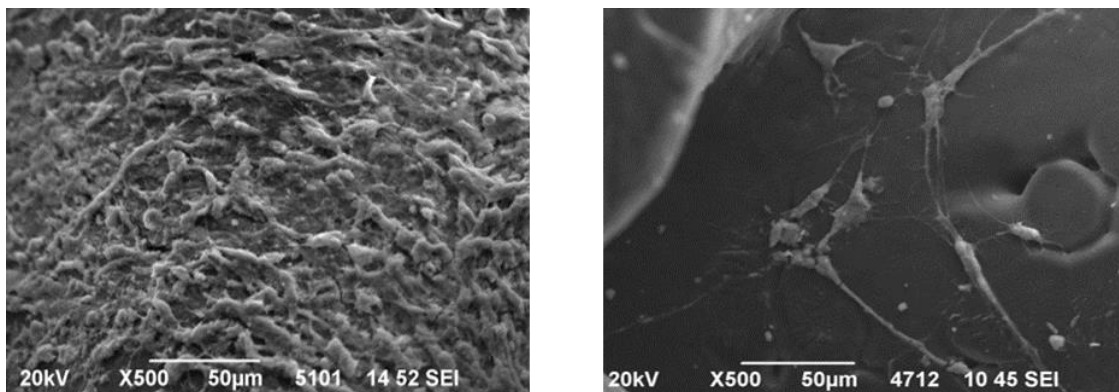

Figure 3: SEM of non-coated PCL scaffolds. A. X500. ECM appears fibroid. B. X500. Cells are attached to the scaffold by small anchors. A.B. Cells appear spindle shaped.

excellent dimensional stability, so the scaffolds are able to resist the cell-mediated contractile forces generated by the developing tissue. The PCL scaffolds provide initial flexibility, coupled with the strength and stiffness of the thermoplastic PCL yarn (Moutos and Guilak, 2010). It has however a hydrophobic surface that does not allow cell interaction, therefore there is an increasing interest in the development of PCL-composite/blend scaffolds with collagen, as a means to enhance hydrophilicity (Doulabi et al., 2014).

Collagen is a structural protein with undisputed beneficial characteristics that is found in excess throughout the human body. It is extensively used as a natural biomaterial in tissue engineering, presenting outstanding biocompatibility, biodegradability and non-toxic characteristics (Neves et al., 2011). Among natural biomaterials, collagen has attracted many interests because it is the most abundant protein constituting the natural ECM of articular cartilage which is responsible for expressing the chondrocytes phenotype, maintaining GAG production and supporting the chondrogenesis (Doulabi et al., 2014). It is usually used as a vehicle carrier of cells because cells can attach to its surface via a2 β1intergins (Sayin et al., 2017). On the other hand collagen presents two crucial limitations, low mechanical stability and rapid biodegradation rate. However, these disadvantages can be overcome by crosslinking collagen with natural or synthetic polymers, like PCL (Doulabi et al., 2014).

The ability of differentiated stem cells to secrete ECM material is crucial to the formation of tissue-engineered cartilage (Theodoridis et al., 2019). All our constructs developed a smooth and glistening gross appearance resulting from newly synthesized ECM, indicating that the scaffolds' purpose for chondrogenesis was served successfully in all cases. The differentiated stem cells appeared well attached to the surfaces, in both types of scaffolds. They proliferated and grew together to form confluent cells, which indicates that PCL displayed good biocompatibility. The cells could infiltrate within the porous scaffolds but penetration was rather limited in the Col-PCL scaffolds. This could be attributed to the thicker membrane formed on the surface of the scaffold, as seen on SEM, which, to some extent, prevented the penetration of cells within.

Overall, collagenation of scaffolds led to a higher density of cells on the surfaces but also to a limited penetration within. Collagen may induce the formation of a thick membrane on the scaffolds' surfaces which sometimes appears crystallized, however, to some extent, it seems to prevent a large number of cells to infiltrate, thus not fully serving the purpose of a 3D culture.

References

Dinescu, S., Galateanu, B., Radu, E., Hermenean, A., Lungu, A., Stancu, I. C., Jianu, D., Tumbar, T. & Costache, M. (2015). A 3D Porous Gelatin-Alginate-Based-IPN Acts as an Efficient Promoter of Chondrogenesis from Human Adipose-Derived Stem Cells. Stem Cells International, 2015, 17.

Doulabi, A. H., Mequanint, K. & Mohammadi, H. (2014). Blends and Nanocomposite Biomaterials for Articular Cartilage Tissue Engineering. Materials (Basel), 7, 5327-5355.

Hutmacher, D. W., Schantz, T., Zein, I., NG, K. W., Teoh, S. H. & Tan, K. C. (2001). Mechanical properties and cell cultural response of

polycaprolactone scaffolds designed and fabricated via fused deposition modeling. J Biomed Mater Res, 55, 203-16.

Khan, F. & Tanaka, M. (2018). Designing Smart Biomaterials for Tissue Engineering. International Journal of Molecular Sciences, 19, 17. MESCHER, A. L. 2013. Junqueira's basic histology : text and atlas.

Moutos, F. T., Glass, K. A., Compton, S. A., Ross, A. K., Gersbach, C. A., Guilak, F. & Estes, B. T. (2016). Anatomically shaped tissue-engineered cartilage with tunable and inducible anticytokine delivery for biological joint resurfacing. Proc Natl Acad Sci U S A, 113, E4513-22.

Moutos, F. T. & Guilak, F. (2010). Functional properties of cell-seeded three-dimensionally woven poly(epsilon-caprolactone) scaffolds for cartilage tissue engineering. Tissue Eng Part A, 16, 1291-301.

Neves, S. C., Moreira Teixeira, L. S., Moroni, L., Reis, R. L., Van Blitterswijk, C. A., Alves, N. M., Karperien, M. & Mano, J. F. (2011). Chitosan/poly(epsilon-caprolactone) blend scaffolds for cartilage repair. Biomaterials, 32, 1068-79.

Sayin, E., Rashid, R. H., Rodriguez-Cabello, J. C., Elsheikh, A., Baran, E. T. & Hasirci, V. (2017). Human adipose derived stem cells are superior to human osteoblasts (HOB) in bone tissue engineering on a collagen-fibroin-ELR blend. Bioact Mater, 2, 71-81.

Temenoff, J. S. & Mikos, A. G. (2000). Review: tissue engineering for regeneration of articular cartilage. Biomaterials, 21, 431-40.

Theodoridis, K., Aggelidou, E., Vavilis, T., Manthou, M. E., Tsimponis, A., Demiri, E. C., Boukla, A., Salpistis, C., Bakopoulou, A., Mihailidis, A. & Kritis, A. (2019). Hyaline cartilage next generation implants from adipose-tissue-derived mesenchymal stem cells: Comparative study on 3D-printed polycaprolactone scaffold patterns. J Tissue Eng Regen Med, 13, 342-355.

Tsuji, H., Ishida, T. & Fukuda, N. (2003). Surface hydrophilicity and enzymatic hydrolyzability of biodegradable polyesters: 1. effects of alkaline treatment. Polymer International, 52, 843-852.

Vinatier, C., Bouffi, C., Merceron, C., Gordeladze, J., Brondello, J. M., Jorgensen, C., Weiss, P., Guicheux, J. & Noel, D. (2009). Cartilage tissue engineering: towards a biomaterial-assisted mesenchymal stem cell therapy. Curr Stem Cell Res Ther, 4, 318-29.

Xue, R., Qian, Y., LI, L., Yao, G., Yang, L. & Sun, Y. (2017). Polycaprolactone nanofiber scaffold enhances the osteogenic differentiation potency of various human tissue-derived mesenchymal stem cells. Stem Cell Res Ther, 8, 148.