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3D Printed Polylactic Acid Scaffold For Dermal Tissue Engineering Application: The Fibroblast Proliferation in Vitro

Dermal Doku Mühendisliği Uygulaması için 3B Baskılı Doku İskelesi: In vitro Fibroblast Proliferasyonu

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Abstract: Dermal fibroblasts are mesenchymal cells that produce extracellular matrix. Fibroblasts play an important role in the skin wound healing process and skin bioengineering. The aim of this study is to evaluate the behaviour of 3D printed polylactic acid (PLA) scaffolds in terms of biocompatibility and toxicity on human dermal fibroblasts (HDFs). Scaffolds were prepared with the PLA filament using a custom made fused deposition modeling (FDM) printer. We fabricated scaffolds with two different pore sizes (35% and 40%). HDFs were seeded at different densities on PLA scaffolds. The cell growth was measured by WST-1 colorimetric assay after 12 and 18 days of seeding HDFs on 3D PLA scaffolds. The morphology and the adhesion property of HDFs were visualized by scanning electron microscopy (SEM). HDFs showed a significant cell proliferation in 3D printd PLA scaffolds. The cell proliferation was highest at a density of 4 x 10^4 cells per well. SEM images showed that HDFs attached the surfaces of the scaffolds and filled the inter-fiber gaps. Our results showed that PLA scaffolds fabricated by 3D bioprinting is a promising candidate for HDF seeding and could have a potential application wound healing or personalized drug trials.
Keywords: PLA Scaffolds, Skin tissue engineering, Dermal Fibroblast, 3D printing

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Özet: Fibroblastlar ekstrasellüler matriks üreten mezenkimal hücrelerdir. Fibroblastlar deride yara iyileşme sürecinde ve deri biyomühendisliğinde önemli bir role sahiptir. Bu çalışmanın amacı 3B baskılı Polilaktik asit (PLA) iskelelerinin insan dermal fibroblastlar (HDF) üzerindeki biyouyumluluk ve toksisite etkilerinin değerlendirilmesidir. İskeleler PLA malzemesi ile özel tasarım birleştirmeli yığma modellemesi (BYM) 3B yazıcı kullanılarak hazırlanmıştır. Çalışma için iki farklı por boyutunda (%35 ve %40) PLA iskeleler üretilmiştir. HDF'ler PLA iskelelere farklı sayılarda ekilmiştir. 3B PLA iskelelere ekilen HDF'lerin hücre çoğalması çalışmanın 12. ve 18. gününde WST-1 kolorimetrik yöntemi ile ölçülmüştür. HDF morfolojisi ve adhezyon özellikleri taramalı elektron mikroskopisi (SEM) ile görselleştirilmiştir. En yüksek hücre yoğunluğu 4 x 10^4 hücre ekilen grupta ölçülmüştür. SEM görüntüleri HDF'lerin iskele yüzeylerine tutunduklarını ve fiber-arası boşlukları doldurduklarını göstermiştir. Sonuçlarımız 3B baskılı üretilen PLA iskelelerin HDF ekiminde kullanılabileceğini ve yara iyileşmesi ve kişiselleştirilmiş ilaç denemelerinde potansiyel bir uygulamaya aday teşkil ettiklerini göstermiştir.
Anahtar Kelimeler: PLA Doku İskelesi, Deri Doku Mühendisliği, Dermal Fibroblast, 3B baskı

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1. Introduction

Dermal fibroblasts are mesenchymal cells located in the dermal skin layer. They produce and organize extracellular matrix (ECM) components to strengthen the dermis (1). Dermal fibroblasts are characterized by an elongated, spindle morphology on planar surfaces (2). In contrast, the *in vivo* cells are stellate-shaped cells forming dendritic networks with extended branches (3). Dermal fibroblasts play an essential role in skin wound healing. Fibroblasts move into the wound site via chemoattractants and fill the wound area with the ECM proteins that they produce (4). These proteins constitute the newly formed granulation tissue providing structural integrity. Dermal fibroblasts also play an important role in skin tissue engineering (5).

Skin tissue engineering is an innovative approach for the treatment of skin conditions and large wounds originating from traumas, burns, or other skin diseases. It uses artificial supporting structures called "scaffolds". Scaffolds are three-dimensional (3D) templates that can mimic special structures for cell attachment, proliferation and differentiation. They serve as a replacement to the natural ECM until host cells could synthesize a new natural matrix. They are also used as carriers for the controlled delivery of drugs or growth factors (6).

An ideal scaffold for tissue engineering should possess considerable mechanical properties, suitable biodegradability and good biocompatibility (7-8). Scaffolds are prepared using biodegradable materials, allowing the material gradually degrades after the formation of a new tissue. Scaffolds are seeded with suitable cells (depending on the tissue type) *in vitro* and a cell proliferation occurs and then implemented *in vivo* into the injury area. The synthetic materials that scaffolds are usually made of are polymers such as polylactic acid (PLA), polycaprolactone, polyglycolic acid. Polylactic acid is a widely used material in biomedical applications because of its excellent biodegradability, mechanical properties, and biocompatibility. The principal raw material of PLA is lactic acid, which is nonirritating and safe for human use. The degradation products of PLA are CO_2 and H_2O, which are nontoxic (8-9).

Many techniques have been used and improved to fabricate PLA scaffolds. Compared to conventional techniques used to produce scaffolds for tissue engineered constructs (10), 3D printing has the advantages of more precise pore size and distribution, cell proliferation, and high mechanical strength (11). 3D printing enables the production of high-precision structures of a wide variety of materials with complex architecture, shape or size (12).

In this study, we aimed to analyze the behaviour of 3D printed PLA scaffolds in terms of biocompatibility and toxicity on human dermal fibroblasts (HDFs). We used PLA scaffolds with two different pore sizes to test the optimum structure for the adhesion and the proliferation of HDFs *in vitro*.

2. Materials and Methods

Preparation of HDFs

HDFs were purchased from Lonza (USA). They were maintained in fibroblast growth medium (FGM) at 37°C in a humidifed atmosphere containing 5% CO_2.

3D Printing PLA Scaffold

Scaffolds were prepared with the PLA filament using a custom made fused deposition modeling (FDM) printer. Scaffolds were fabricated according to the FDM method in a single extruder 3D printer, which uses hot end extruder for printing PLA filament. This printer has a mechanical precision of 100-100-200 µm in the X-Y-Z axis. 0.3 mm nozzle and 1.75 mm filaments were used for the production of tissue scaffolds. Tissue scaffolds are produced with two different filling rates (20% and 40%). The nozzle temperature for PLA was set to 230°C.

The design and production parameters of the scaffolds are defined and optimized by SolidWorks and Slic3r software to set the correct printing procedure. Scaffolds template (Ø = 4 mm, thickness = 2.5 mm) were designed using SolidWorks 2017 software and subsequently filled and sliced using and Slic3r 1.2.9 software to obtain cylindrical STL models. Briefly, clump generator software was used to create squared pores into a 3D object in a "stl"file format. The printing head was computer-controlled in three axes (X, Y, Z)

while extruding the PLA filament using the Slic3r software.

The melted PLA was then extruded through a 0.3 mm diameter stainless-steel nozzle on to a printing plate heated at 40°C. Porous scaffolds were printed layer-by-layer in the form of squares surrounded by a dense PLA perimeter. We determined the optimal speed of 15 mm/s for the filling speed and 25 mm/s for the gap speed. The travel speed of extruder was set to be 100 mm/s. We fabricated scaffolds with two different pore sizes (35% and 40%).

After printing, the scaffolds were carefully removed from the printing bed. Prior to biological evaluations, printed scaffolds were sterilized by ethylene oxide (EtO).

Cell Seeding on the PLA Scaffolds

PLA scaffolds with two different pore sizes (35% and 40%) were used in the experiments. Prior to cell cultivation, sterile scaffolds were immersed in FGM in the 96-well plate and incubated 1 h in a humidified incubator (5% CO_2, 37°C). Then suspension of HDFs in FGM were seeded on the scaffolds at different cell densities ($1x10^4$, $2x10^4$, $3x10^4$ and $4x10^4$ cells per well) and incubated in a humidified incubator (5% CO_2, 37°C) for 1 h. An additional 150 µL FGM was added into each well. HDFs were also seeded in the wells without scaffolds as control groups. The cell-seeded PLA scaffolds were cultured for 12, and 18 days. The medium was replaced every 2 days.

Measurement of HDFs proliferation activity on PLA scaffolds

Cell proliferation activity on the scaffolds was measured by ready-to-use colorimetric WST assay (Biovision, Milpitas, CA, USA) 12 and 18 days days after the seeding of cells. The assay principle is based upon the cleavage of the tetrazolium salt WST-1 to formazan by cellular mitochondrial dehydrogenases. The generation of the dark yellow coloured formazan is measured at 450 nm and is directly correlated to cell number. The measurement was performed according to the manufacturer's instructions by using a microplate reader (Biotek Synergy HTX, USA).

Cell visualisation on PLA scaffolds

HDFs on the scaffolds cultured 18 days after seeding were fixed to evaluate the adhesion property and the proliferation activity by scanning electron microscopy (SEM). The cell-containing scaffolds were transferred to the 24-well plate, fixed in 4% paraformaldehyde, dehydrated with a graded series of ethanol concentrations of 60%, 70%, 80% and 99% (v/v). Then the scaffolds were wrapped in aluminum foil and dried in desiccator for 2 days (13). The scaffolds placed on the platform were plated with 5 nm gold for 20 minutes by the Q150R S (Quorum) instrument. Copper banding was then applied to the platform to eliminate charging effect. Images were taken with ZEISS Sigma 500 VP FE-SEM.

3. Results

HDFs proliferation on 3D printed PLA scaffolds

Our results showed that HDFs proliferation was highest at a density of 3 x 10^4 cells per well on the 12th day and 4 x 10^4 cells per well on the 18 day. Different pore sizes (35% and 40%) did not affect the proliferation activity of HDFs. The results on the 12th and 18th days are shown in Figures 1 and 2, respectively. The precentages of cell proliferation on days 12 and 18 are presented in Tables 1 and 2, respectively.

HDFs morphology on 3D printed PLA scaffolds

SEM images showed that HDFs attached the surfaces of PLA scaffolds and filled the inter-fiber gaps. SEM also demonstrated that HDFs formed dendritic networks with extended branches SEM images are shown in Figure 3.

Table 1.
Percentages of HDFs proliferation activity on the 12th day

Cell number	Control	%40 PLA	%35 PLA
10^4	100	47,03	74,49
$2x10^4$	100	74,43	64,11
$3x10^4$	100	77,58	78,56
$4x10^4$	100	74,91	77,07

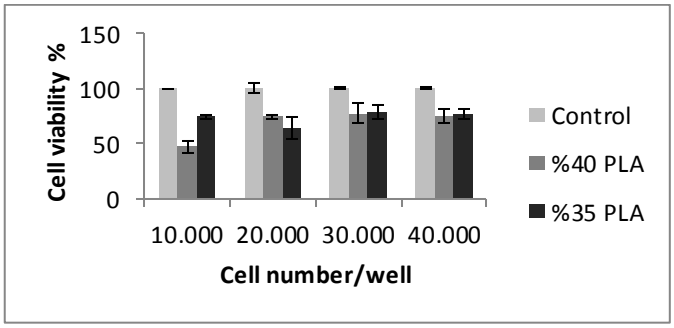

Figure 1.
HDFs proliferation activity on the 12th day.

Table 2.
Percentages of HDFs proliferation activity on the 18th day

Cell number	Control	40% PLA	35% PLA
10^4	100	63,94	76,00
$2x10^4$	100	65,89	54,69
$3x10^4$	100	55,98	71,55
$4x10^4$	100	82,53	73,85

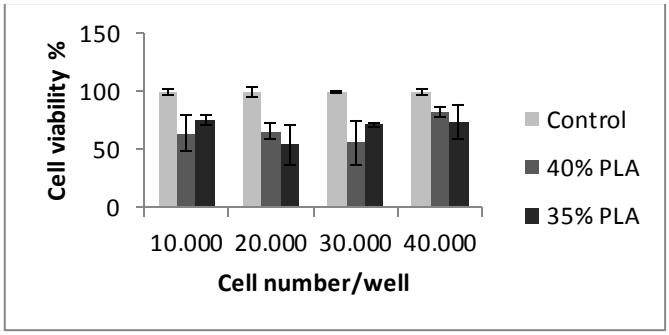

Figure 2.
HDFs proliferation activity on the 18th day

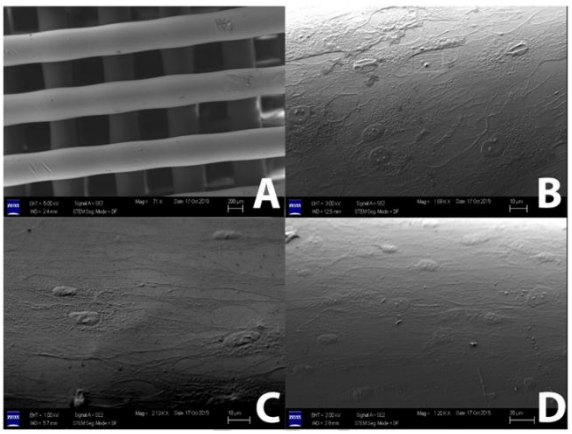

Figure 3.
SEM images of A) PLA scaffold B) HDFs on the scaffold, 40% porosity and $4x10^4$ cell/well C) HDFs on the scaffold, 40% porosity and $3x10^4$ cell/well D) HDFs on the scaffold, 35% porosity and $4x10^4$ cell/well

4. Discussion

In this study, we investigated the behaviour of 3D printed PLA scaffolds in terms of biocompatibility and toxicity on HDFs in vitro. Our results showed that HDFs attached and proliferated on PLA scaffolds fabricated by 3D printer. According to WST-1 assay, the highest proliferation activity was determined at a density of 3 x 10^4 cells per well on the 12th day and 4 x 10^4 cells per well on the 18 day after seeding. These results revealed that the cell density of seeding is important for the proliferation activity of HDFs on scaffolds. We also demonstrated that different pore sizes (35% and 40%) did not affect the proliferation of HDFs. Our findings of cell proliferation in HDFs on PLA scaffolds are consistent with a previous report. Mohiti-Asli et al. measured the proliferation activity of HDFs seeded on PLA scaffolds prepared by the conventional method by Alamar Blue assay. They showed that the cell proliferation was the highest on day 7 after seeding and decreased on the 10th and 14th days (14). However, in our study, the proliferation activity of HDFs on 3D printed scaffolds was still high both on days 12 and 18 after seeding. Since HDFs continue to attach on the scaffolds on the 12th and 18th days, 3D printed PLA scaffolds are still confirmed to be biocompatible and safe in the longer period. Gregor et al. seeded MG-63 osteosarcoma cells on 3D printed PLA scaffolds and stated that these scaffolds could be used for bone tissue regeneration (15). Our in vitro biocompatibility results of PLA scaffolds demonstrated that the microenvironment formed within the scaffolds is suitable and sufficient for the cells to preserve their viability, which is crucial for tissue engineering. Further studies in humans and animals are recommended for further assessment of the mechanical performance of the designed scaffolds containing fibroblast cells. In future studies, to obtain more evidence about the properties of 3D scaffolds to improve tissue regeneration, these 3D scaffolds will be evaluated in a context that mimics a clinical feature using the wound healing model in diabetic rats in vivo.

5. Conclusion

The data presented here indicate that HDFs attach and proliferate in biocompatible 3D printed PLA scaffolds. In this method, existing FDM printers produced scaffolding structures that provide higher resolution and accuracy than the existing direct printing methods for PLA. These results would provide new valuable knowledge and development for further research in the scaffold bio-fabrication.

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