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RESEARCH ARTICLE / ARAŞTIRMA MAKALESİ

In Vitro and in Vivo Compliance of 3D Printed PLA Scaffolds with Differentiated Mesenchymal Stem Cells

3D Baskılı PLA-İskelelerin Üzerinde Farklılaştırılmış Mezenkimal Kök Hücrelerin in Vitro ve in Vivo Uyumları

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ABSTRACT

Aim: 3D printing made a rapid entry as a means of production of biocompatible and biodegradable materials for tissue engineering applications. 3D printing made it possible to create custom biodegradable implants and polylactic acid is one of the most promising polymers. In this study, we aimed to form both bone and cartilage differentiated from bone marrow stromal mesenchymal stem cells on 3D printed polylactic acid polymers.

Materials and Methods: The polylactic acid scaffolds were designed, 3D printed and sterilized in dedicated university facilities. Mesenchymal stem cells were collected from rat bone marrow and were then differentiated to either osteoblasts or chondroblasts. The characterization of cells was analyzed using Alizarin Red, Alcian blue staining and osteonectin and collagen II by indirect immunocytochemistry. Differentiated cells were seeded on the 3D scaffold, cultured for 2 weeks. For in vivo tests, 3D scaffolds with or without differentiated bone marrow stromal mesenchymal stem cells were implanted into subcutaneous connective tissue. After the four-month implantation, the rats were sacrificed, and all samples were histochemically and immunohistochemically analyzed.

Results: Osteogenic and chondrogenic differentiation from bone marrow stromal mesenchymal stem cells were performed after 2 weeks culture condition. They were positively stained both histochemically and immunohistochemically. After transfer of the cells onto 3D polylactic acid scaffold, their differentiation continued and both bone and cartilage formation were observed after histochemical and immunohistochemical analyses both under in vitro and in vivo conditions.

Conclusion: 3D printed polylactic acid scaffolds supported both bone and cartilage formation, therefore, it may be conveniently used for experimental cell in vivo studies.

Keywords: Bone, Cartilage, 3D Printing, Mesenchymal Stem Cells,

ÖZ

Amaç: Üç boyutlu baskı, doku mühendisliği uygulamaları için biyolojik olarak uyumlu ve biyolojik olarak parçalanabilen malzemelerin üretim aracı olarak hızlı bir giriş yaptı. Üç boyutlu baskı, özel biyobozunur implantlar oluşturmayı mümkün kıldı ve polilaktik asit (PLA) en umut verici polimerlerden biridir. Biz bu çalışmada, 3D baskı PLA polimerleri üzerinde kemik iliği stromal mezenkimal kök hücrelerinden (BMSC) hem kemik hem de kıkırdağa farklılaştırılan hücreler oluşturmayı amaçladık.

Gereç ve Yöntem: PLA iskeleleri tasarlanmış, Solidworks Yazılımı kullanılarak 3D olarak basılmış ve Yakın Doğu Üniversitesi'nin özel tesislerinde sterilize edilmiştir. Mezenkimal kök hücreler sıçan kemik iliğinden toplandı ve daha sonra osteoblast veya kondroblastta farklılaştırıldı. Hücrelerin karakterizasyonu Alizarin kırmızısı, Alcian mavisi boyama, osteonektin ve kollajen II kullanılarak indirekt immünositokimya ile analiz edildi. Farklılaştırılmış hücreler, 3D yapı iskelesine ekilerek 2 hafta boyunca kültüre edildi. İn vivo test için, farklılaştırılmış BMSC'leri olan veya olmayan 3D yapı iskelesi, subkutan alanın bağ dokusuna implante edildi. Dört aylık implantasyondan sonra sıçanlar sakrifiye edildi ve tüm numuneler histokimyasal ve immünohistokimyasal olarak incelendi.

Bulgular: BMSC'den osteojenik ve kondrojenik farklılaşma 2 haftalık kültür koşulundan sonra gözlendi. Hücreler hem histokimyasal hem de immünohistokimyasal olarak pozitif boyandılar. Hücreler 3D PLA iskelesine aktarıldıktan sonra farklılaşmaları devam etmiş ve hem in vitro hem de in vivo koşullarda histokimyasal ve immünohistokimyasal analizlerden sonra hem kemik hem de kıkırdak oluşumu gözlemlenmiştir.

Sonuç: 3D baskılı PLA iskeleleri hem kemik hem de kıkırdak oluşumunu desteklediği ve bu nedenle bu hücrelerin in vivo çalışmalar için rahatlıkla kullanılabileceği gözlenmiştir.

Anahtar Kelimeler: Kemik, Kıkırdak, 3D baskı, Mezenkimal Kök Hücre

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INTRODUCTION

In tissue engineering, 3D printing became an alternative method for in vivo delivery of custom sized and shaped biomaterials seeded with in vitro produced cells. In recent years, research on stem cell interaction with the biocompatible and biodegradable materials obtained by 3D printing and their potential for tissue regeneration has gained evident speed (1).

Stem cells derived from different sources are being continuously investigated as possible "drugs" for cell therapy applications. Mesenchymal stem cells (MSCs) can be isolated easily from bone marrow, adipose tissue, umbilical cord, placenta and dental pulp tissues; therefore, they are preferred for use in tissue engineering with high in vitro exchange capacity (2-6). The compliance of biomaterials with cells is an important issue to provide crosslinking between the biomaterials and the cells in the microenvironment and maintain the integrity for the possible tissue formation and remodeling (7). Biomaterials play an important role in the particular fields by providing matrices for cellular growth, proliferation, and new tissue formation.

For this purpose, polycaprolactone (PCL), poly(l-lactide-co-glycolide) (PLGA), collagen and laminin have been investigated in a variety of studies to determine their supporting capacity of cell differentiation (8-9).

Polylactic acid (PLA) has been variously used in biomedical applications due to its biocompatibility, processability and good mechanical properties (10-15). PLA seeded with different cells has been studied regarding muscle tissue, neural tissue, cartilage, and bone regeneration in the fields of cardiovascular surgery, orthopedics and neurology (11).

3D printing technologies help to create structures using biomaterials with or without cells. The technology itself can control the shape and size of the printed materials. It is therefore advantageous to be able to tweak the properties of 3D printed scaffolds to fully mimic the structure which is aimed to be replaced or augmented. Furthermore, this technique requires a good design, manufacture and viable cells in the model providing the possibility to merge in an appropriate environment (16-19).

In this study, we aimed to investigate to compatibility of 3D-printed PLA bioscaffolds with bone marrow mesenchymal stem cells, and their differentiation potential to osteoblasts and chondroblasts in vivo.

MATERIALS AND METHODS

All procedures were approved by the Ethics Committee on Animal Research, Manisa Celal Bayar University, Manisa, Turkey (Approval #77.637.435-72).

***PLA Scaffold Design and Printing**:* Design and manufacturing of the scaffolds were performed at NEU3D Laboratories (Near East University, 3D Printing Facilities) (Figure 1a). Scaffolds were designed using Solidworks 3D Design Software (Dassault Systemes S.A., MA, USA). Layers consisted of 0.4 mm wide connected parallel bars. Each layer was positioned in perpendicular manner to form a cage like structure in order to capture maximum number of cells in suspension. The spacing between the bars was arranged as 0.4 mm (Figure 1b). The designs were exported as ".stl" files and printed at 215 °C using 1.75 mm polylactic acid filament (Colorfabb B.V, Belfeld, NE) in MakerBot Replicator 5th Generation 3D printer (MakerBot Industries, New York, USA) (Figure 1c). Accoridng to the data provided by manufacturer the density of the polylactic acid filament was 1.2-1.3 g/cm3, tensile strength was 45 MPa and tensile modulus was 3400 Mpa. Vaporized hydrogen peroxide sterilization (low temperature plasma sterilization) of scaffolds was performed in Near East University Hospital, Sterilization Unit. Scaffolds with a solution of hydrogen peroxide, were kept for 30 min in a solution of peroxide at room temperature, then the peroxide was removed, and scaffolds were washed 3 times with a phosphate buffer solution (pH 7.4) (20).

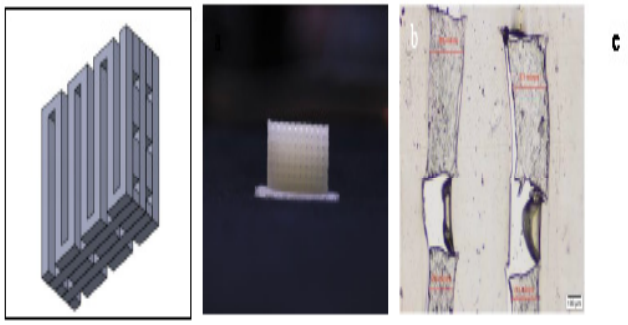

Figure 1: 3D design (a), 3D printed version (b) and sectioned view (c) of PLA scaffold.

Culture and Differentiation of Bone Marrow Stromal Cells: Male Wistar Albino rat bone marrow stromal cells (BMSC) which were stocked in Department of Histology and Embryology, Manisa Celal Bayar University, were cultured in standart culture medium containing 10% fetal bovine serum (F4135, Sigma-Aldrich, MO, USA), 1 % L-glutamine (G7513, Sigma-Aldrich, MO, USA), 1 % penicillin-streptomycin (P0781, Sigma-Aldrich, MO, USA), 1 % gentamycin (G1397, Sigma-Aldrich, MO, USA), and 0,1 % amphotericin B (A2942, Sigma-Aldrich, MO, USA) in α-MEM (M4526, Sigma-Aldrich, MO, USA) until 80 % confluency. The osteoblastic differentiation of stem cells was performed using osteogenic stimulatory mesencult ™ Kit (05504, StemCell Technologies, MA, USA) chondrogenic differentiation was performed using Stempro® Chondrogenesis Differentiation Kit (A10071-01, Gibco/Life Technologies, MD, USA). Cells were incubated in a humidified atmosphere of 95% air, 5% CO2 at 37°C (Hera Cell, Kendro Laboratory, Germany). The medium was changed every other day. Cells were microscopically observed under inverted microscope with a phase-contrast attachment and photomicrographs were obtained (21).

Characterization of Osteoblasts and Chondroblasts

Alizarin Red Staining: Alizarin red staining was done for identification of osteoblast cells. The culture medium from osteoblast differentiated cell layers was removed, and the cells were rinsed with phosphate buffered saline (PBS) for 3 times before fixation in 4% paraformaldehyde for 1 hour at 4°C. Then, they were washed with deionized water and allowed to dry. The fixed cells were stained with 0.1% of Alizarin Red's (A5533, Sigma-Aldrich, MO, USA) solution in distilled water (pH 7.2) for 1 hour at 37°C. They were then washed with deionized water and observed under inverted phase-contrast (IX-71, Olympus, Tokyo, Japan) microscope (22).

Alcian Blue Staining: Alcian blue staining was done to identification of chondroblast cells. The culture medium from chondroblast differentiated cell layers was removed, and the cells were rinsed with PBS for 3 times before fixation in 4% paraformaldehyde (P6148, Sigma-Aldrich, MO, USA) (pH 7.4) for 30 minutes at room temperature. They were then washed with PBS and stained with 1% of Alcian blue solution (1263395-3, Carlo Erba, Val-de-Reuil, France) in 0.1 N HCl for 30 min in the dark at room temperature. They were washed 3 times with 0.1 N HCl and distilled water for neutralization (23).

Immunocytochemical Methods

Differentiated cells were washed with PBS and they were fixed with 4% paraformaldehyde (P6148, Sigma-Aldrich, MO, USA) for 30 min at room temperature. They were then washed with PBS for 3 times, 3% of hydrogen peroxide solution (H2O2 solution, 3%, H1009, Sigma-Aldrich, MO, USA) was added for 10 minutes to inhibit of the endogen peroxidase activity. After washing with PBS for 3 times, the cells were incubated with blocking solution (85-9043, Invitrogen, ThermoFisher Scientific, MA, USA) for 1 hour and primary antibodies; anti-collagen II (sc-52658, Santa-Cruz, CA, USA), anti – osteonectin (33-5500, Invitrogen, ThermoFisher Scientific, MA, USA) were added and incubated overnight at 4 °C. After washing with PBS, they were incubated with biotinylated secondary antibodies for 30 min and streptavidin hydrogen peroxidase for 30 min (85-9043, Invitrogen, ThermoFisher Scientific, MA, USA). They were washed again with PBS and stained with diaminobenzidine (DAB, 85-9043, Invitrogen, ThermoFisher Scientific, MA, USA) for 5 min. They were mounted with mounting medium (107961, Merck Millipore, Darmstadt, Germany) and examined under light microscope (BX-40, Olympus, Tokyo, Japan (22).

Culture, Characterization and in vivo Transplantation of Differentiated Cells in PLA Scaffolds

Sterile 3D PLA scaffolds were conditioned for 12 hours in medium before seeding with the cells. Differentiated cells ($1x10^6$ cells / cm^3 density of either osteoblast or chondroblast)

were seeded on PLA and cultured for 14 days. After the culture time they were then either fixed in 10 % formalin solution for histochemical and immunohistochemical analyses at 24-48 hours or transferred animals for in vivo studies.

A total of eighteen male Wistar rats were used for the in vivo studies. Implantation area was shaved and sterilized with iodine solution at the dorsal side of each rat after anesthesia with ketamine (60 mg/kg, Ketalar, 002 038, Eczacıbasi) and xylazine (5 mg/kg, Alfazyne, 0804125-11, Alfasan) (Figure 2). Animals were sacrificed 4 months after the in vivo implantation of PLA scaffolds with or without cells. For light microscopic analyses, biomaterials with or without differentiated cells were incubated for 14 days in borax-sodium carbonate buffer (0.01 M Na2CO3 and 0.3 mm Na2B4O7, pH:11) to achieve softening of PLA (20). All samples were embedded in paraffin and 5µ sections were taken. For histochemical analyses, sections were stained with alcian blue and alizarin red (see above). For immunohistochemical analysis, distributions of osteonectin and collagen II were evaluated using standart protocol of indirect immunoperoxidase staining (see above).

Figure 2: PLA scaffolds with or without cells were implanted subcutaneously in the rat's neck.

RESULTS

Culture of Bone Marrow Stromal Cells and Differentiation of Bone Marrow Stromal Cells to Osteogenic or Chondrogenic Lineage

BMSCs had characteristic fibroblast-like morphology under standart culture medium condition (Figure 3a). Culture of the cells was continued until 80 % confluency was reached after 14 days in culture (Figure 3b).

The BMSCs were cultured in either osteogenic or chondrogenic medium for 14 days. After the differentiation, cuboidal osteogenic-like cells (Figure 3c-d) and epitheloidal chondrogenic-like cells (Figure 3e-f) were identified. While BMSCs maintained fibroblast morphology in standart culture condition at 14 days of culture, the morphology of the cells differentiated into osteogenic or chondrogenic lineage were different. After 14 days in culture, osteogenic and chondrogenic cells were positively stained with Alizarin Red (Figure 4a). and Alcian blue (Figure 4c), respectively. They also expressed osteonectin (Figure 4b) or collagen II (Figure 4d) after differentiation of the cells.

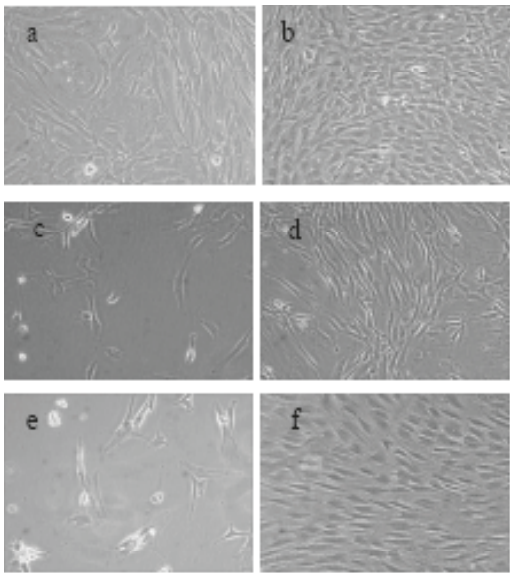

Figure 3. Photographs of BMSCs at 7 (a) and 14 (b) days of culture time. Differentiation of BMSC to osteogenic (c, d) or chondrogenic (e, f) lineage after 3 (c, e) and 14 (d, f) days of culture. Original Magnification X400.

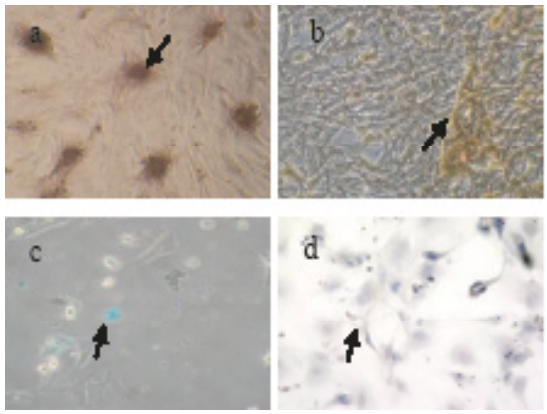

Figure 4: Osteogenic (a, b) or chondrogenic (c, d) differentiated cells were identified after 14 days in culture. a: Alizarin Red (arrow), b: Osteonectin (arrow), c: Alcian blue (arrow), d: Collagen II (arrow). Original Magnification X400.

Identification of Differentiated Cells in PLA Scaffolds

The differentiated cells were cultured in PLA scaffold for 14 days. During culture periods, the proliferation of both

osteogenic and chondrogenic cells were observed in 3D PLA scaffolds. The differentiation and proliferation were continued until 14 days of cell culture. Osteogenic (Figure 5a, b) and chondrogenic (Figure 5c, d) cells were highly affected by the geometry of individual pores within the scaffold

Indeed, differentiated cells had long projections onto surface of 3D PLA scaffolds. These results showed that differentiated cells grow and proliferate on 3D PLA scaffolds. Alizarin calcium deposits in areas with red staining was observed in reddish brown color (Figure 5e) In alcian blue stained areas, the chondrogenic accumulation was also observed with blue (Figure 5f). The cells expressed osteogenic or chondrogenic markers such as osteonectin or collagen II, respectively (Figure 5g, h).

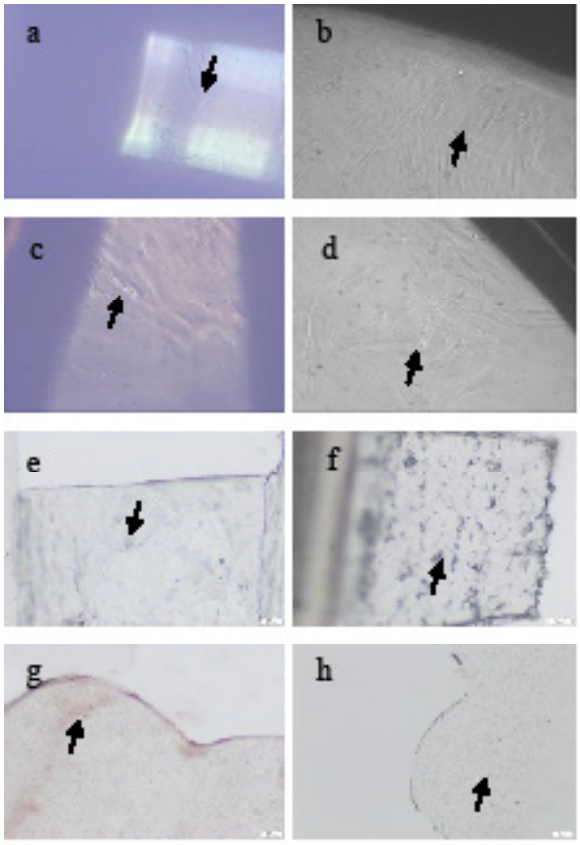

Figure 5: Culture of differentiated cells in 3D PLA Scaffolds at 14 days. a-b: Osteogenic differentiation (arrow), c-d: Chondrogenic differentiation (arrow). Characterization of differentiated cells on 3D PLA scaffolds. e: Alizarin Red staining (arrow), f: Alcian Blue staining (arrow), g: Osteonectin (arrow), h: Collagen II (arrow). Scale Bars: 20 µm.

In vivo Implantation of 3D PLA Scaffold

The 3D PLA scaffolds with or without differentiated cells were implanted in the subcutaneous area of the rat necks and they were examined after 4 months. All of the rats stayed alive until sacrification without any obvious inflammatory response. Upon histochemical observation, the cells or connective tissue which covered the scaffolds with differentiated cells were seen (Figure 6 a, b, d, e). No cells or extracellular matrix were detected in the unseeded scaffolds (Figure 6c, f). During immunohistochemical evaluation, immunoreactivities for osteonectin and collagen II were stained positively in 3D PLA scaffolds with differentiated cells. (Figure 6). No staining was observed in negative controls (Figure 6 g-i).

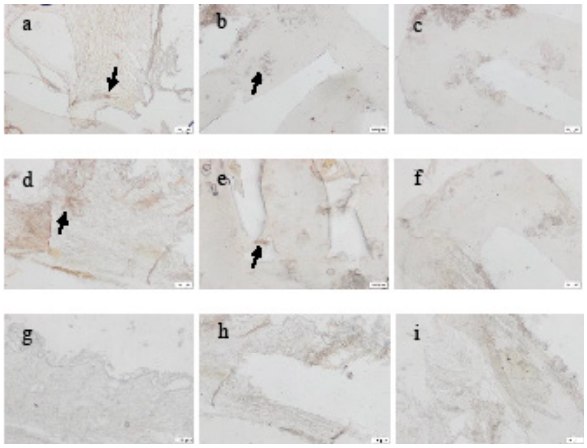

Figure 6: In vivo implantation of osteogenic (a, b) (arrow), chondrogenic (d, e) (arrow) and without cells (c, f) on 3D PLA scaffolds. a, b, c: Osteonectin, d, e, f: Collagen II. Scale Bars: 100 µm. Negative control (g: osteogenic, h: chondrogenic, i: without cells).

DISCUSSION

3D printed scaffolds may help to solve the problems during in vivo experimental studies with cells as different types of the scaffolds provide a three-dimensional environment for cell attachment and growth by mimicking the in vivo environment. Three-dimensional printing allows production of bioscaffolds of variable sizes and shapes to serve the needs of the patients. Obtaining an optimal high resolution 3D printed bioscaffold is a complex process which needs careful selection of both software and hardware (16). Currently, there are some in vitro culture studies, aiming production of bone graft materials seeded with osteoblasts and osteoclasts (7).

Biocompatibility and mechanical properties of the PLA has attracted great interest to this material, and it is being widely investigated in medicine. Our results demonstrated that 3D printed PLA scaffolds supported the differentiation, proliferation and attachment of BMSCs. In addition, the osteonectin which is a marker of osteogenic differentiation and collagen II which is a marker of chondrogenic differentiation continued their expressions on 3D PLA scaffolds. Therefore, we may state that PLA is not a toxic material for differentiated cells.

The structure and porosity of scaffolds are extremely important for adequate cell attachment during the culture process. Accurate pore size of scaffolds may provide appropriate signaling to encourage the differentiation of stem cells. Currently, three-dimensional printing allows the scientists to determine the size, shape, mechanical strength, and porosity of the intended scaffold. Mechanical performance of a 3D scaffold affects the response of the cells, and it may also affect the nutrient diffusion. Mesenchymal stem cells from differentiated cells in the osteoblastic differentiation and proliferation have been reported to be effective in the geometric structure of the pier (17). However, some biomechanical studies showed that PLA implants are not suitable for use in the weight bearing bones (18). In our study, 3D PLA scaffolds provided in vivo cell survival and it was possible to achieve BMSCs' proliferation and differentiation.

Intercellular communication is related directly to scaffold. For good adhesion and proliferation of cells on synthetic scaffolds, natural polymers extracted from the native extracellular matrix (ECM) have been used to modify the surface of PLA scaffolds (24, 25).

Polymer degradation rate is determined by the water content of the environment, temperature, humidity and lactic acid concentration. Several studies examined endochondral and intramembranous ossification in implanted biodegradable scaffolds seeded with cultivated osteogenic stem in the bone defects (26,27). The possibility to create 3D culture system with PLA was also shown (28). In our study, the implanted PLA scaffolds stayed for 4 months without apparent signs of major degradation. This finding shows that PLA scaffolds are probably more suitable for the implants which need long term mechanical stability instead of rapid degradation. But we should also keep in mind that polylactic acid is not strong enough to be used in weight bearing bones.

Biomaterials with or without cells can be used for implantation. In regenerative medicine, different kind of biomaterials has been tested for clinical use. In the early 1990's, the focal articular cartilage defects were treated with autologous chondrocytes with biomaterials (19). PLA is shown to be non-toxic in bone or cartilage repair applications. PLA was successfully used to accelerate the rat tibia bone healing and bone formation. At the same time absence of infection or inflammation with PLA was shown in the studies regarding articular healing in osteochondral defects in the rabbit (11). There were no differences between osteogenic and chondrogenic differentiation on 3D PLA scaffolds in our study. Therefore, we can also say that 3D printed PLA scaffolds may be safely used both in bone and cartilage studies.

One of the concerns regarding PLA is that it may cause inflammatory reaction in long term (29, 30). Some studies have shown that the implanted PLA was found to be well tolerated with no chronic inflammation for up to 39 weeks in rats (11). In our study, inflammatory response of tissue to PLA was not detected in 4 months' period, however, the long-term effects of PLA with stem cells have to be investigated.

Among the limitations we should note that we did not perform thermal, spectroscopic, and elemental analysis of our 3D printed scaffolds because it was not among the primary focus of our study.

CONCLUSION

3D printed PLA scaffolds were used as three-dimensional environment during differentiation of bone marrow stromal mesenchymal stem cells to osteoblast or chondroblast cells showed excellent support for both bone and cartilage cell formation. In addition, there were no toxic effects of PLA scaffold during differentiation period of mesenchymal stem cells. Therefore, patient-based 3D printed PLA scaffolds with different type of mesenchymal stem cells may be conveniently used for in vivo and further clinical studies.

Ethics

There are no ethical issues after the publication of this manuscript.

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