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RESEARCH ARTICLE

Comparison of Dental Follicle Stem Cells and Dental Pulp Stem Cells in a Translational Bone Tissue Engineering Protocol

Translasyonel Kemik Doku Mühendisliği Protokolünde Diş Folikül Kök Hücreleri ve Diş Pulpa Kök Hücrelerinin Karşılaştırılması

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ABSTRACT

Purpose: This study aims to establish and refine a translational protocol and compare the osteogenic potential of dental pulp stem cells (DPSC) and dental follicle stem cells (DFSC) on nano mesh containing polycaprolactone (nmPCL) and plain polycaprolactone (m) scaffolds in vitro and contribute the translational medicine protocols in bone regeneration.

Materials and Methods: DPSCs and DFSCs were osteogenically differentiated on plain polycaprolactone (m) and nano mesh containing polycaprolactone (nm) scaffolds and four groups were examined for cell proliferation and type I collagen formation rates after two weeks of culture. Following immunofluorescence labeling, nonparametric (Kruskal Wallis) and multiple comparison tests were used to compare the groups.

Results: Among all groups, mean cell counts on scaffolds ranged from 30.8 to 82.6 cells/0.0915 mm^2, and total collagen formation ranged from 2.79% to 17,9%. DFSC and nmPCL complex showed significantly higher cell counts ($p<0.01$) and collagen formation rates ($p<0.01$) in comparison to other groups.

Conclusion: The DFSCnm group is found to show superior properties on cell proliferation and bone matrix formation. This complex is a promising tool for maxillofacial tissue engineering applications.

Keywords: Dental Follicle Stem Cell, Dental Pulp Stem Cell, Polycaprolactone, Tissue Engineering, Bone, Translational Medicine

ÖZET

Amaç: Bu çalışmada, DPSC ve DFSC'lerin nanomesh içeren (nmPCL) ve düz (PCL) polikaprolakton iskeleler üzerindeki osteojenik potansiyellerini in vitro olarak karşılaştırmak ve kemik rejenerasyonunda translasyonel tıp protokollerine katkıda bulunmak amaçlanmıştır.

Gereç ve Yöntemler: DPSC'ler ve DFSC'ler PCL ve nmPCL iskeleleri üzerinde osteojenik olarak farklılaştırılmış ve iki haftalık kültür sonrasında dört grup hücre proliferasyonu ve tip I kollajen oluşum oranları açısından incelenmiştir. İmmünofloresan etiketlemenin ardından, dört grubu karşılaştırmak için parametrik olmayan (Kruskal Wallis) ve çoklu karşılaştırma testleri kullanılmıştır.

Sonuçlar: Tüm gruplar arasında, iskelelerdeki ortalama hücre sayıları 30.8 ila 82.6 hücre/0.0915 mm^2 arasında ve toplam kolajen oluşumu %2.79 ila %17,9 arasında değişmektedir. DFSC ve nmPCL kompleksi diğer gruplara kıyasla anlamlı olarak daha yüksek hücre sayısı ($p<0,01$) ve kolajen oluşum oranları ($p< 0,01$) göstermiştir.

Sonuç: DFSC/nmPCL grubunun hücre proliferasyonu ve kemik matriksi oluşumu üzerinde üstün özellikler gösterdiği bulunmuştur. Bu kompleks maksillofasiyal doku mühendisliği uygulamaları için umut verici bir araçtır.

Anahtar Kelimeler: Diş Folikülü Kök Hücresi, Diş Pulpası Kök Hücresi, Polikaprolakton, Doku Mühendisliği, Kemik, Translasyonel Tıp

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Repair and regeneration of bone defects is one of the main research areas of maxillofacial surgery. Bone tissue diseases, injuries and congenital malformations often require treatment with grafting approaches. Allogenic, xenogenic, alloplastic and synthetic materials, as well as autogenous bone grafts, are currently used for reconstruction of maxillofacial complex. Providing the most efficient vascularization and regeneration of bone tissue within the defect area is one of the main objectives for craniofacial tissue engineering. Although autologous bone grafts are accepted as the gold standard for reconstruction of bone defects, a limited amount can be considered as an important problem. Since autologous bone graft harvesting also has the disadvantage of donor site morbidity and all the other materials lack osteogenic potential, tissue engineering methods need to be studied as an alternative to conventional grafting.[1] With the aim of efficient reconstruction of large defects, studies on tissue scaffolds have gained importance in recent years.[2,3]

Polycaprolactone (PCL) is a biodegradable and biocompatible synthetic material that is reported to have suitable chemical and physical properties in osteogenic differentiation and bone tissue engineering research.[4] With the goal of autologous bone regeneration, allogeneic and xenogeneic stem cell transplantation, have been studied and obtained successful results.[5-7] However the success of treatment also varies according to the characteristics of the tissue scaffolds and type and source of transplanted stem cells[8,9]. Evaluation of the osteogenic differentiation and regeneration capacity of stem cell colony types is an important criterion in the specific reconstruction of bone defects.[10]

Stem cells have been identified as clonogenic cells which have the ability to self-renewal, differentiating into various cell types and forming new cell lines.[11] In the area of bone tissue engineering, bone-marrow derived stem cells are one of the most reported sources in the literature.[3,12,13] Due to the lack of donor site morbidity and the presence of strong osteogenic potential, investigations on dental pulp (DPSC) and dental follicle (DFSC) derived stem cells are recently increased and satisfactory results on bone regeneration are reported.[14-19]

In order to apply successful clinical translation of the tissue engineering techniques, tissue specific differentiation potential of stem cell types is needed to be carefully evaluated. The studies that directly compare the results of osteogenic potential of different mesenchymal stem cell colony types are rare in the literature.

Instead of bone marrow-derived cell lines, mesenchymal stem cell (MSC) colonies that are cultured in this study can be obtained from tissues that will be discarded as medical waste at the end of an oral surgical operation. Hence, clinical translation of DPSC and DFSC studies will result in improved patient comfort by eliminating donor site morbidity and complications.

The purpose of our study is to compare the osteogenic potential of DPSC and DFSC colonies which are cultured on polycaprolactone mesh and polycaprolactone nanomesh scaffolds.

MATERIALS AND METHODS

1. Study Groups

For 10 scaffolds for each of four different groups (DPSCm, DPSCnm, DFSCm, DFSCnm), a total of 40 scaffolds were examined. For each group, two additional scaffolds were cultured as negative controls.

2. Isolation and Expansion of DPSCs and DFSCs

This comparative experimental study was approved by Medipol University Research Ethics Committee (number of approval: 10840098-153). The authors have read the Helsinki Declaration and have followed the guidelines in this investigation. A fully impacted wisdom tooth with its follicle was extracted in aseptic conditions and cracked in sterile conditions with an osteotome. Removed pulp tissue and follicle tissues were finely minced with a scalpel and transported into a 15 ml falcon tube. 2 ml of collagenase type I was added (1:500, ab 34710; Abcam, Cambridge, UK) to each sample and mixture was incubated at 37°C for 1 hour. Samples were then filtered through a 70 µm cell strainer and washed two times with equal volumes of phosphate buffered saline (PBS). After washing, supernatant was removed and 1 ml of culture medium was added to the pellet. Then, cells were seeded into the cell culture flasks and incubated. Culture media was changed every three days and cells were passaged when 80% of confluency was achieved.

3. Characterization of DPSCs and DFSCs

Analyses were performed in every sub-culture from passage 1 to passage 5 using flow cytometry. Characterization of the DPSCs and DFSCs was done with regard to described

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characteristic MSC markers including CD45, CD14, CD34, CD25, CD28, CD105, CD146, CD90, CD73 and CD29.

For osteogenic differentiation, culture medium was supplemented by 50 µg/ml ascorbic acid (Sigma, USA), 10 mm β-glycerophosphate (Sigma, USA) 100 nmol/L dexamethasone (Sigma, USA). In the fourth week of culture, samples were stained with Alizarin red to examine the formation of mineralized nodules within the culture. For differentiation into adipocytes, cells were cultured in adipogenic differentiation media. Adipocytes were identified by inverted microscopy examination and Oil red O (Sigma, USA) staining. For differentiation into chondrocytes, culture medium was supplemented with chondrogenic differentiation media. Chondrocytes were characterized by Alican blue staining.

4. Scaffolds, Cell Seeding and Culture

Mesenchymal stem cells were seeded on sterile polycaprolactone scaffolds (3D Biotek, USA). Before the cell seeding procedure, cell colonies were washed with PBS (phosphate buffer saline), and incubated with alpha-MEM with 10% fetal calf serum. Then, DPSC and DFSC suspensions were seeded into the 5x1.5 mm PCL mesh (m) and 5x1 mm PCL nanomesh (nm) scaffolds in 96 well plates. For 10 scaffolds for each of four different groups (DPSCm, DPSCnm, DFSCm, DFSCnm), total of 40 scaffolds were examined. For each group, two additional scaffolds were cultured as negative controls. A number of $2.0x10^5$ cells in 25 µl of suspension were transferred into each well. For higher seeding efficiency, careful manipulation was applied in order to avoid the contact of the pipette tip with the walls of the wells. After three hours of incubation in 5% CO_2 and 37° C, 175 µl of medium containing 10% FCS and 1% Penstrep was added to the wells. After examining the cell morphology by microscopic examination, cells were taken into the incubator. After adhesion of the cells to the tissue scaffolds for two days, cell-scaffold complexes were supplemented with alpha-MEM, gentamycin (50 µg/ml) and 15% FCS containing 50 µg/ml ascorbic acid (Sigma, USA), 10 mm β-glycerophosphate (Sigma, USA) 100 nmol/L dexamethasone (Sigma, USA) for osteogenic differentiation. Culture medium was changed in every 2 or 3 day of intervals. Cell-scaffold complexes were incubated in 5% CO_2 and 37° C. On 14^{th} day of culture, scaffolds were fixed for immunofluorescence staining.

5. Type I Collagen Formation and Cell Count Analysis

Scaffolds were fixed in 0.05% PFA + 4° C for overnight, then washed with PBS for 2 minutes. After incubation with 70% cold ethanol for a period of 15 minutes, permeabilization was performed for 15 minutes with 0.1% PBS Tween. Cells were blocked with 10% goat serum for 1 hour. Scaffolds were incubated with primary antibody (1: 500 by rabbit anti-collagen I, EU 34710; Abcam, Cambridge, UK) for overnight at + 4° C. A conjugated goat anti-rabbit IgH antibody (DyLight488, Abcam, Cambridge, UK) was used as the secondary antibody. Nuclear staining was performed with 4',6-diamidino-2-phenylindole (DAPI, Sigma) for 5 minutes. Examples were maintained at 4° C until examination. For total cell count, seven representative images were captured using a phase contrast fluorescent microscope at 20x and 40x magnification (Leica, Germany). For each scaffold, seven random representative sections were obtained by confocal laser scanning microscopy (CLSM) (Leica, Germany). The total area of the collagen formation was quantified and total cell counts were calculated for each slide with ImageJ software (National Institutes of Health, Bethesda, MD) and also confirmed manually by the same observer.

6. Statistical Analysis

Datasets were analyzed by using GraphPad Prism 5 software (GraphPad Software, Inc., CA, USA). Comparisons of the multiple groups were performed with nonparametric Kruskal-Wallis test and Bonferroni correction was used when comparing the groups. For all analyses, a P value less than 0.05 was considered statistically significant.

RESULTS

1. Characterization of DPSCs and DFSCs

Characterization of DPSCs was performed using flow cytometry analysis and differentiation potentials of the cell colonies. Flow cytometric analysis demonstrated that DPSCs express stem cell markers CD73, CD90, CD105, CD146 and CD29 and do not express hematopoietic cell markers CD45, CD34, CD25, CD28 and CD14 on their cell surface. With supplementation of the culture by the according differentiation media, isolated DPSC colonies were shown to be capable of in vitro adipogenic, chondrogenic and osteogenic differentiation.

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2. Cell Counts

At the conclusion of the culture period, no deformation was observed in the scaffold structures. Immediately following the seeding process, the cells exhibited a round morphology.

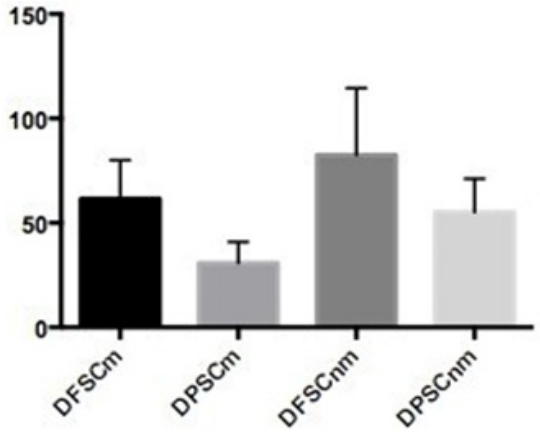

Fig. 1. Cell counts were significantly higher in DFSCnm group.

However, as the culture progressed, a transition to a spindle-like morphology was observed. This change occurred in response to the initiation of adhesion and tissue bridge organization on the PCL fibers.

The mean cell count values for the group DPSCm, DFSCm, DPSCnm, and DFSCnm were 30.8 cells/ 0.0915 mm^2, 61.6 cells/ 0.0915 mm^2, 55.2 cells/ 0.0915 mm^2, and 82.6 cells/ 0.0915 mm^2, respectively (Fig. 1-3).

The results of the statistical analysis indicated that the DFSCnm group exhibited a statistically significant increase ($p<.001$) in cell count compared to the other groups. Furthermore, observations of DAPI-stained samples revealed the most frequent and uniform cell organization throughout the DFSCnm group. A comparative analysis of the cell counts in the DPSCnm/DFSCnm, DPSCm/DPSCnm, and DPSCm/DFSCm groups was performed. The analysis revealed statistically significantly higher cell counts in the latter groups ($p<0.05$). An analysis of the data yielded no statistically significant differences between the DFSCm and DFSCnm groups.

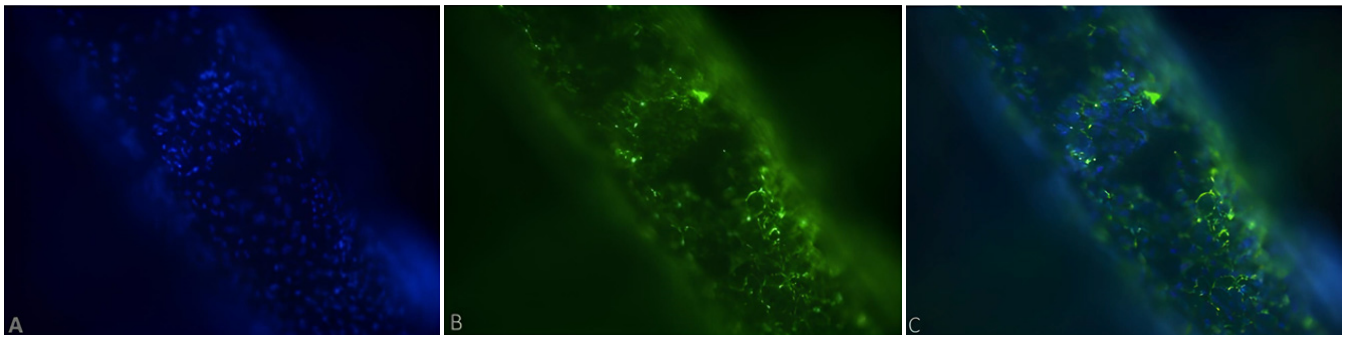

Fig. 2. DAPI (A), Type I Collagen (B) and merged (C) images of DFSCs on PCL fiber structure (X20).

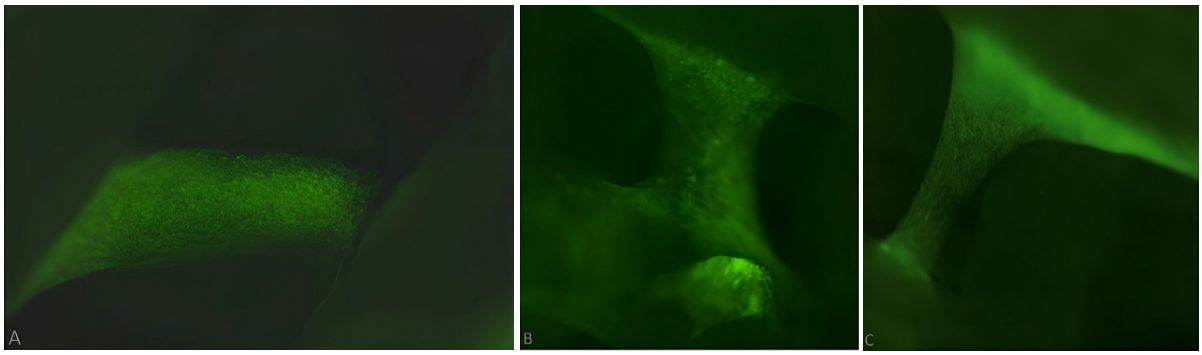

Fig. 3. A-C: Tissue bridging formations of DFSCs on nanomesh scaffold structures.

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3. Type I Collagen Formation

The mean values of collagen formation ratio for the DPSCm, DFSCm, DPSCnm, and DFSCnm groups were 2.79%, 3.93%, 12.7%, and 17.9%, respectively. In the statistical analysis, the rate of type I collagen formation in the DFSCnm group was found to be significantly higher than in the other groups ($p < .001$). Evaluation via confocal microscopic analysis revealed that the DFSCnm group exhibited the most profound and uniform distribution of collagen organization (Fig. 4-6). A comparison of type I collagen formation rates in DPSCm/DPSCnm and DFSCm/DFSCnm groups revealed statistically significantly higher rates in the latter groups ($p<0.05$). The comparison of the groups, DFSCnm/DPSCnm and DFSCm/DPSCm, revealed no significant statistical difference.

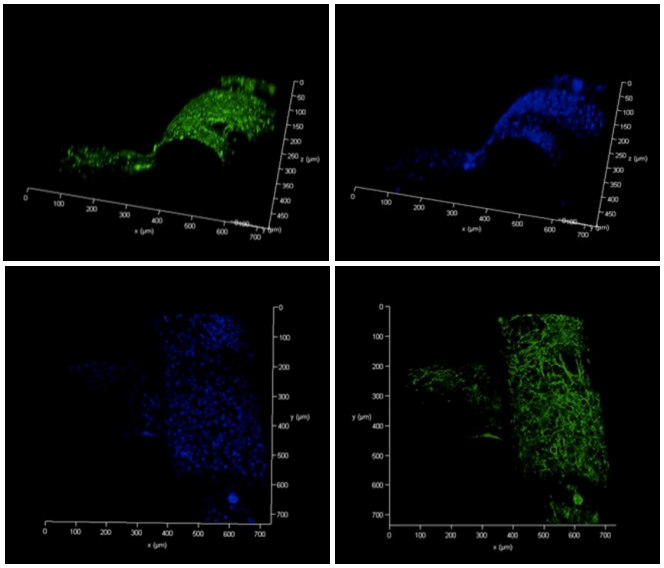

Fig. 4. DAPI (B, C) and Type I Collagen (A, D) confocal microscopy images of tissue bridge forming DFSC lines on nanomesh PCL fibers.

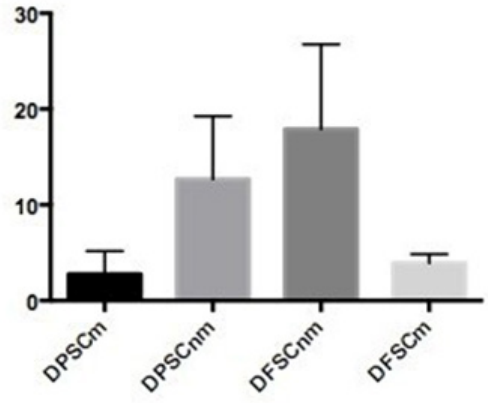

Fig. 5. Type I collagen formation rates were significantly higher in DFSCnm group.

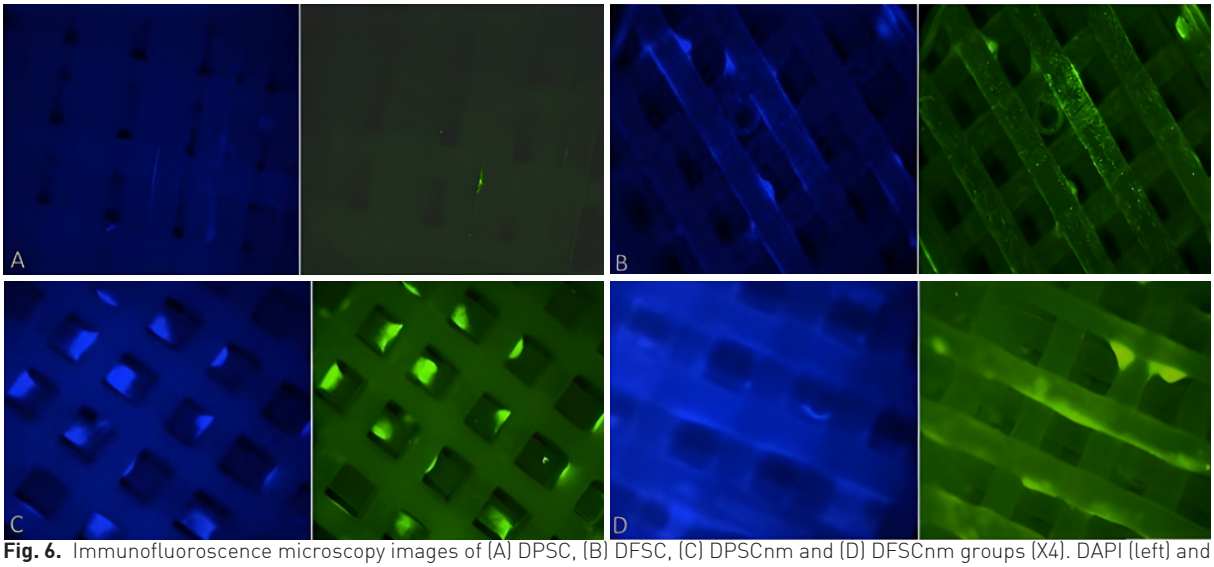

Fig. 6. Immunofluoroscence microscopy images of (A) DPSC, (B) DFSC, (C) DPSCnm and (D) DFSCnm groups (X4). DAPI (left) and Type I Collagen (right).

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DISCUSSION

In the context of bone tissue engineering, the successful organization of bone tissue in qualitative and quantitative aspects is critically dependent on the utilization of scaffold materials to provide support for cells and expressed extracellular matrix. Also, these materials are able to provide guidance to the geometric shape of the tissue growth. The most important task of the material to be used as scaffold is to help fulfill tissue function by providing a temporary support to the cell colonies and an environment that will create the biological orientation of the cells.[4,20-23] Abukawa et al. studied pig mandibular condyle reconstruction by culturing mesenchymal stem cell colonies on PLGA (polylactic-co-glycolic acid) scaffolds, and reported that the bone formation was observed only on the surface of the construct[24]. In a subsequent study, Abukawa et al. cultivated porcine bone marrow stem cells on a channeled PLGA scaffold for a period of 10 days. The cells were then implanted into a porcine mandibular critical-sized defect. In the second, fourth, and sixth weeks of healing, histologic sections were obtained, and there was observation of more intensive, uniform, and highly vascularized bone formation on channeled-PLGA scaffolds. In the case of non-channeled scaffolds, the formation of bone tissue was observed exclusively on the surface area. The addition of channels and micropores to the scaffold structure was found to enhance the permeability and transport of the culture medium, cell number and cell distribution, thereby facilitating the formation of bone tissue. In addition, the maximum cell count was documented to occur during the second week of the culture, and a substantial decline in cell counts after this period was reported.[25]

Scaglione et al. indicated the significance of total porosity, fully connected interior structure, and chemical composition of a scaffold. They further suggested a new "open-pore" tissue scaffold architecture. The formation of tissue and vascular infiltration in both in vitro and in vivo models was found to be satisfactory when using mesh-formed, calcium phosphate-coated hydroxyapatite polymer structures.[26]

Polycaprolactone structures can be made by three-dimensional fabricating technologies without exposure to chemical solvent materials. Porter et al. evaluated short term biocompatibility and long-term bioactivity assays of PCL nanomesh structures that were produced with three-dimensional printing methods. In this study, the cultivation of rat bone marrow-derived mesenchymal stem cells on PCL structures was conducted. The results indicated that there was an enhancement in cell adhesion, viability, and elevated levels of bone tissue biochemical markers on nano surfaces during the first, second, and third weeks in comparison to the control groups. In this study, the findings suggest that three-dimensionally printed, solvent-free PCL scaffolds have a positive impact on the biological performance of mesenchymal stem cells and can be utilized as an effective form of tissue scaffolds for bone regeneration.[27]

Binulal et al. evaluated the adhesion and proliferation potential of human mesenchymal stem cells on nanofibrous and microfibrous electro-spun PCL scaffolds. Adhesion, organization, proliferation and osteoblastic differentiation features of the stem cells were observed to be superior on nanofibrous structures.[28]

After the implantation of a cultured cell-scaffold complex into a defect area, viability and successful fusion depends on the angiogenesis activity within the first three days. In in-vitro conditions, mineralization of the extracellular matrix and formation of bone nodules is observed from the beginning of the fourth week of culture. In the studies on bone tissue engineering that have been documented, the cell-scaffold complex is predominantly indicated to be implanted subsequent to the observation of mineralization in the tissues. In the present study, the interconnected multilayer mesh scaffold design and culture duration were selected to ensure optimal nutrient and oxygen diffusion, vascular penetration, and uniform bone formation, thereby facilitating the clinical translation of the technique.

Jensen et al. compared three scaffold models for osteogenic differentiation of DPSCs on the 1st, 7th, 14th and 21st days. Cell proliferation, migration, osteoblastic activity and calcium deposition were observed to be increased at day 21 in nano-structure hyaluronic acid / TCP modified scaffold group when compared to control group. According to the results, DPSC/PCL scaffold complexes were stated to be a suitable implementation method for in vivo bone regeneration studies[29].

Studies that directly compare the osteogenic potential of DPSCs and DFSCs are rare in the literature. Shoi et al. evaluated the cell proliferation, colony forming capacity, gene expression,

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cell surface markers and differentiation capacity of DPSCs and DFSCs isolated from supernumerary incisors. Due to the increased obtainable tissue amount, DFSCs are indicated to be a more accessible stem cell source for isolation protocols. The rate of cell proliferation and colony forming capacity of DFSCs were found to be significantly higher in comparison to the DPSCs. In the appropriate culture medium, osteogenic differentiation potential of both cell lines was shown. Despite the similar stem cell characteristics of the DPSCs and DFSCs, due to easier access and higher proliferation rate of DFSC, it is indicated that DFSCs are a more favorable source of stem cells in regenerative applications.[30-33]

Surface topography is one of the main factors in determination of the differentiation of mesenchymal stem cell lines. This process is based on cell-cell, cell-extracellular matrix and cell-biomaterial interactions via signaling mechanisms.[34] Osteogenic differentiation is reported to be more effective on fibrillar nanostructured constructs.[35-37]

In our study, the highest cell count and type I collagen formation rates were observed in DFSCnm group. The effect of the electro-spun nanomesh base membrane structure was found to have a positive impact on cell spreading, adhesion, and proliferation. The observed variations in morphology, cell counts, and type I collagen expression rates may have resulted from the distinct osteogenic differentiation potentials of the cell types, as well as the asynchronous differentiation of cell lines.

In conclusion, using nanomesh PCL scaffold and DFSC complexes is found to be a suitable and promising method for bone tissue engineering applications. In vitro characteristics of stem cell-tissue scaffold complexes are needed to be correlated with in vivo bone regeneration studies. Based on these results, an experimental orthotopic critical size defect model should be studied in order to elucidate the impact of the technique on in situ osteogenesis.

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COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

ETHICAL APPROVAL

This study was performed in line with the principles of the Declaration of Helsinki. This study was approved by Medipol University Research Ethics Committee (number of approval: 10840098-153).

CONSENT TO PARTICIPATE

Informed consent was obtained from individual participants included in the study.

CONSENT TO PUBLISH

Non applicable.

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