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

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ABSTRACT

In this study, it is aimed to establish and refine a translational protocol and compare the osteogenic potential of DPSC and DFSC's on nano mesh containing (nmPCL) and plain (PCL) polycaprolactone scaffolds in vitro and contribute the translational medicine protocols in bone regeneration. DPSCs and DFSCs were osteogenically differentiated on PCL and nmPCL 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 four groups. Among all groups, mean cell counts on scaffolds ranged from 30.8 to 82.6 cells/0.0915 mm2, 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. DFSC/nmPCL 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.

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INTRODUCTION

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 the main objects for craniofacial tissue engineering. Although autologous bone grafts are accepted as the gold standard for reconstruction of bone defects, 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 has gained importance in recent years (2, 3).

Polycaprolactone (PCL) is a biodegradable and biocompatible syntethic material which 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). But 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 has been identified as clonogenic cells which has the ability of 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 lack

of donor site morbidity and 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.

METHODS

Isolation and Expansion of DPSCs and DFSCs

This 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. 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 minched with 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 37oC 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.

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 were done with regard to described 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). At the fourth week of culture, samples were stained with Alizarin red for examining 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.

Scaffolds, Cell Seeding and Culture

Mesenchymal stem cells were seeded on sterile polycaprolactone scaffolds (3D Biotek, USA). Before 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 2.0x105 cells in 25 µl of suspension were transferred into each well. For higher seeding efficiency, careful manipulation was applied in order to avoiding the contact of the pipette tip with the walls of the wells. After three hours incubation in 5% CO2 and 37º 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% CO2 and 37º C. On 14th day of culture, scaffolds were fixed for immunofluorescence staining.

Type I Collagen Formation and Cell Count Analysis

Scaffolds were fixed in 0.05% PFA + 4º 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 + 4º 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 maintained at 4º 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.

Statistical Analysis

Datasets were analysed 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

Characterization of DPSCs and DFSCs

Characterization of DPSCs were 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.

Cell Counts

At the end of the culture period, no deformation was observed in scaffold structures. Immediately after the seeding, cell morphologies were observed to be rounded-shape, whereas on the following days of culture, cell morphologies was observed to be spindle-like as the adhesion and tissue bridge organizations on the PCL fibers begin.

Mean cell count values on the group DPSCm, DFSCm, DPSCnm, and DFSCnm were 30.8 cells/ 0.0915 mm2, 61.6 cells/ 0.0915 mm2, 55.2 cells/ 0.0915 mm2, and 82.6 cells/ 0.0915 mm2, respectively (Figure. 1-3).

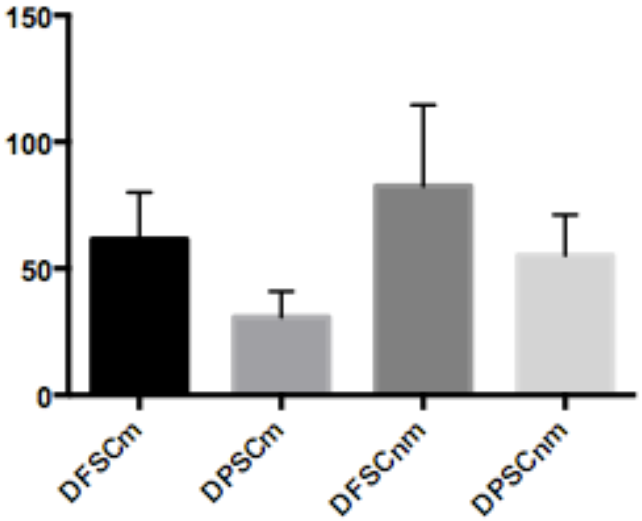

Figure. 1. Cell counts were significantly higher in nmDFSC group.

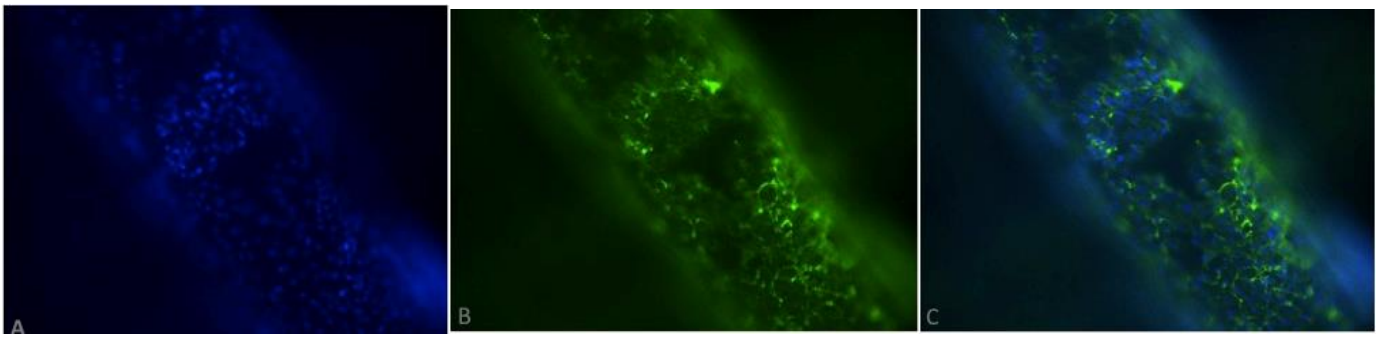

Figure. 2. DAPI (A), Type I Collagen (B) and merged (C) images of DFSCs on PCL fiber structure (X20).

Statistical analysis revealed that, DFSCnm group had statistically significantly ($P < .001$) higher cell counts when compared with every other group. Additionally, observation of DAPI stained samples showed the most frequent and uniform cell organization throughout the DFSCnm group. In comparison of cell counts in DPSCnm/DFSCnm, DPSCm/DPSCnm, DPSCm/DFSCm groups, statistically significantly higher cell counts were observed in the latter groups ($p<0,05$). There was no statistical difference detected in comparison of DFSCm and DFSCnm groups.

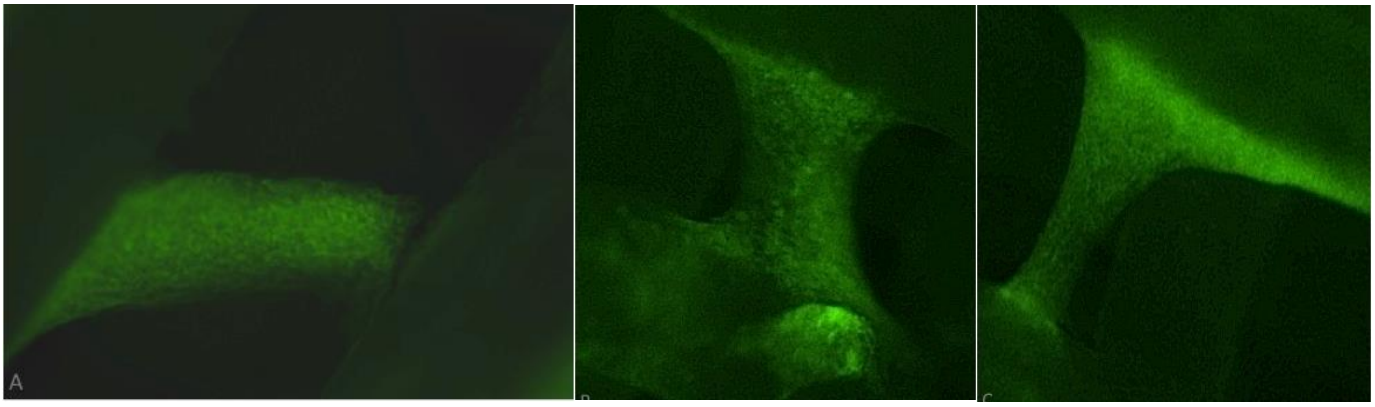

Figure. 3. A-C: Tissue bridging formations of DFSCs on nanomesh scaffold structures.

Type I Collagen Formation

Mean values of collagen formation ratio of DPSCm, DFSCm, DPSCnm, and DFSC nm 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 were found to be significantly higher than the other groups ($P < .001$). On the confocal microscopic examination, DFSCnm group showed the most profound and uniform distribution of collagen organization (Figure. 4-6). In comparison of type I collagen formation rates in DPSCm/DPSCnm and DFSCm/DFSCnm groups, statistically significantly higher type I collagen formation rates were observed in the latter groups ($p<0,05$). There was no statistical difference detected in comparison of DFSCnm/DPSCnm and DFSCm/DPSCm groups.

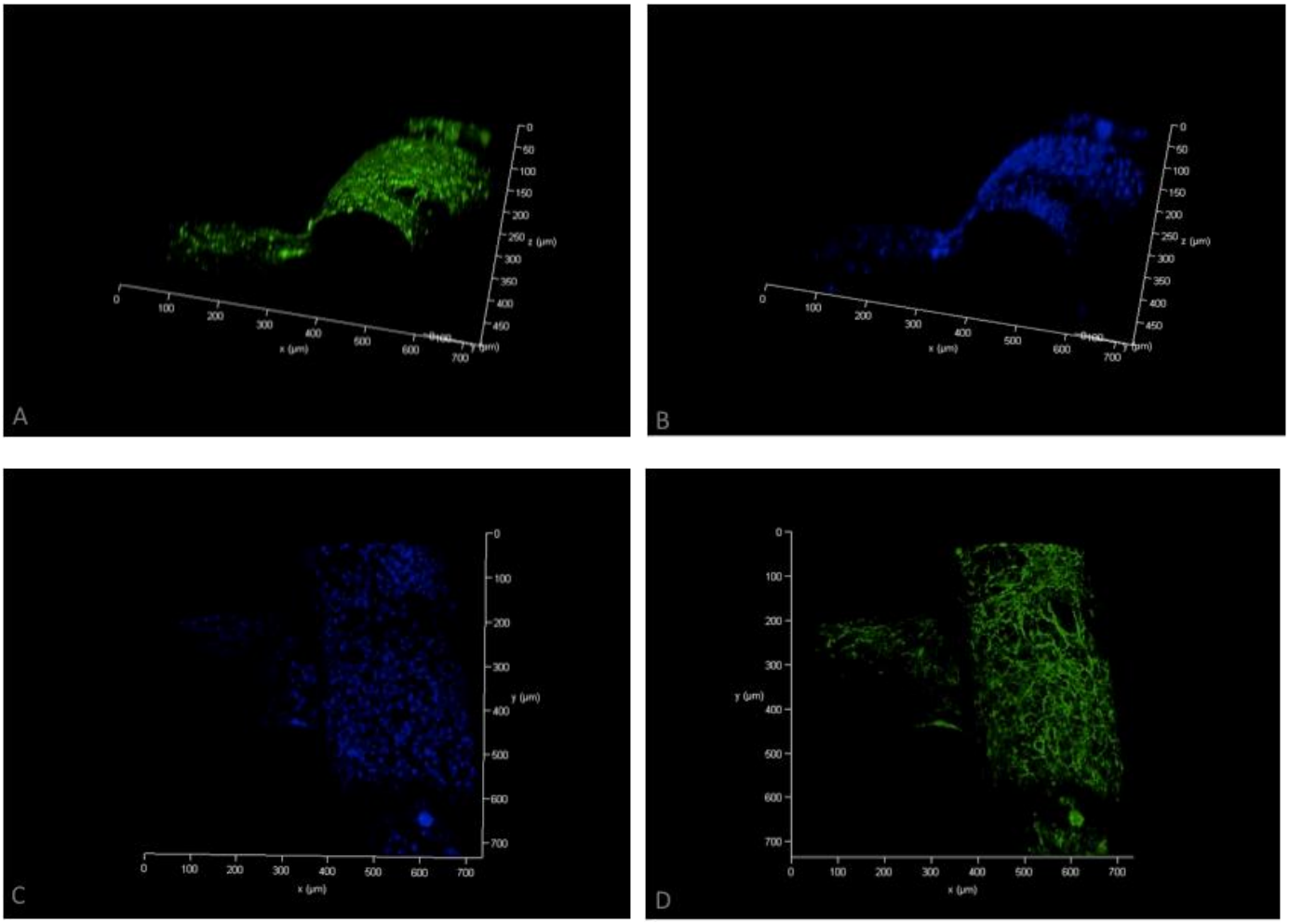

Figure. 4. DAPI (B,C) and Type I Collagen (A,D) confocal microscopy images of tissue bridge forming DFSC lines on nanomesh PCL scaffold fibers. A,B: Cross-sectional vertical bridge formation on and through the space between the fibers. C,D: Axial image of scaffold.

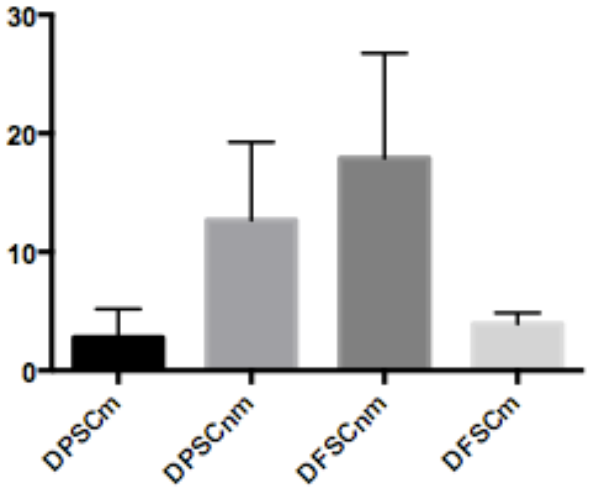

Figure. 5. Type I collagen formation rates were significantly higher in nmDFSC group.

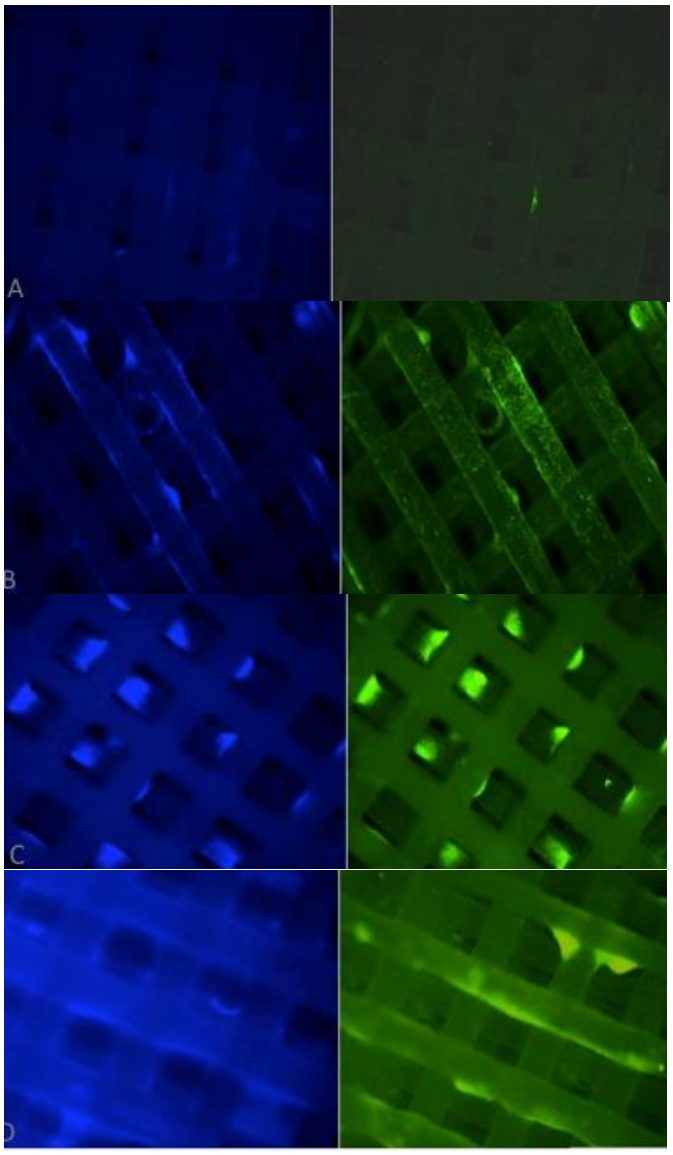

Figure. 6. Immunofluoroscence microscopy images of (A) DPSC, (B) DFSC, (C) DPSCnm and (D) DFSCnm groups (X4). DAPI (left) and Type I Collagen (right).

DISCUSSION

In bone tissue engineering applications, cells and expressed extracellular matrix have to be supported by scaffold materials in order to obtain successful organization of bone tissue in qualitative and quantitative aspects. 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). Subsequently, in another study by Abukawa et al, porcine bone marrow stem cells are cultured on a channeled PLGA scaffold for 10 days of duration, and then implanted in a porcine mandibular critical-sized defect. On 2nd, 4th and 6th weeks of healing, histologic sections are obtained and more intensive, uniform and highly vascularized bone formation on channeled-PLGA scaffolds was observed. On non-channeled scaffolds, bone tissue formation was observed only on the surface area. Addition of channels and micropores to the scaffold structure was concluded to enhance the permeability and transport of the culture medium, cell number and cell distribution, thus the formation of the bone tissue. Also, the highest cell count was reported to be on the second week of the culture, and significant decrease on the cell counts after this timepoint was indicated (25).

Scaglione et al. indicated the importance of total porosity, fully connected interior structure and chemical composition of a scaffold and suggested a new “open-pore” tissue scaffold architecture. In vitro an in vivo tissue formation and vascular infiltration was found to be satisfactory on mesh-formed, calcium phosphate coated hydoxyapatite 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. By culturing rat bone marrow derived mesenchymal stem cells on these structures, they reported enhanced cell adhesion, viability, and elevated bone tissue biochemical markers on 1st, 2nd, and 3rd weeks on nano surfaces compared to control groups. In this study, three-dimensionally printed, solvent-free PCL scaffolds are concluded to have enhancing effects on biological performance of mesenchymal stem cells and can be used as a successful form of tissue scaffolds on 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 to 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 beginning of the fourth week of culture. In the bone tissue engineering studies that reported in literature, cell-scaffold complex is mostly indicated to be implanted after the mineralization of tissues was observed. In our study, the interconnected multilayer mesh scaffold design and culture duration was selected due to fulfill the maximum nutrient and oxygen diffusion, vascular penetration and uniform bone formation for clinical translation of the technique.

Jensen et al. compared three scaffold models for osteogenic differentiation of DPSCs on 1st, 7th, 14th and 21st days. Cell proliferation, migration, osteoblastic activity and calcium deposition was 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 comparison the osteogenic potential of DPSCs and DFSCs are rare in the literature. Shoi et al. evaluated the cell proliferation, colony forming capacity, gene expression, cell surface markers and differentiation capacity of DPSCs and DFSCs which are isolated from supernumerary incisors. Due to greater obtainable tissue amount, DFSCs are indicated to be easier stem cell sources 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 were shown. Despite 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 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, highest cell count and type I collagen formation rate were observed in DFSCnm group. The effect of electro-spun nanomesh basement membrane structure was evaluated to have enhancing effects on cell spreading, adhesion and proliferation. The differences observed in morphology, cell counts and type I collagen expression rates may have resulted by specific osteogenic differentiation potentials of the cell types, as well as asynchronous differentiation of cell lines.

In conclusion, using nanomesh PCL scaffold and DFSC complexes are 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 understand the effect of the technique on in situ osteogenesis.

Funding

No funding was obtained for this study.

Conflict of Interest

There is no conflict of interest.

Ethical Approval

This study was approved by Medipol University Research Ethics Committee (number of approval: 10840098-153).

Consent to Participate

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

Authors Contributions

Elif M Ozcan was responsible for all experimental design and process, cell culture experiments, data collection & analyses and manuscript preparation; Yucel Erbilgin was responsible for all experimental process, data collection, confocal microscopy and review of manuscript; Selin Yıldırım. and Noushin Zibandeh were responsible for isolation and expansion of related stem cell lines, experimental process and review of the manuscript; Tunç Akkoç and Kamil Goker were responsible for the project supervision and coordination. All authors gave their final approval of the work.

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