Original Article

DOI: 10.14235/bas.galenos.2020.3782
Bezmialem Science 2021;9(3):294-300

Generation of Bone Tissue Using Adipose Tissue-derived Stem Cells

Adipoz Kökenli Kök Hücre Kullanımıyla Kemik Doku Üretimi

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ABSTRACT

Objective: Bone grafts and even bone substitutes do not meet all of the requirements of bony reconstructions. The aim of this study was to generate bone tissue from autologous adipose tissue-derived mesenchymal stem cells (ATDMSCs) and decellularised bone allografts.

Methods: A 1.5 cm bone defect developed in the middle third of the rabbit's ulna. Reconstructions were carried out using miniplate and screws and interpositional autogenous bone grafts according to the designs of the groups: (1) No touch, (2) cryopreserved, (3) decellularised and (4) ATDMSCs-implanted decellularised bones. Before implantation, ATDMSCs in the last group were labelled with Q-dot and identified microscopically.

Results: Graft recovery and irregular callus formation were observed in the first, second and forth groups. In the first group, the organisation of Haversian systems, the structure of the lacunae and the presence of canaliculi ossiums were observed; in the second group, approximately 40% of the Haversian canals contained blood vessels, and canaliculi ossiums in the form of thin filaments were found in 90% of the microscopically examined areas; in the third group, most Haversian canals were empty, most osteocyte canals were devoid of cells, and canaliculi ossiums were absent; in the fourth

ÖZ

Amaç: Kemik doku onarımda altın standart olan otojen kemik greftleri her zaman tüm gereksinimleri karşılayamamaktadır. Bu çalışmanın amacı, otojen yağ doku kaynaklı mezenkimal kök hücrelerin (ATDMSC) ve hücresizleştirilmiş kemik allo greftlerinin kombinasyonunu kullanarak kemik dokusu üretmektir.

Yöntemler: Tavşan ulnasının 1/3 orta segmentinde oluşturulan 1,5 cm'lik bir kemik defektine, grup tasarımlarına göre, gruplar; 1: kontrol; 2: krio ile muamele edilmiş kemik grefti; 3: hücresizleştirilmiş kemik grefti; 4: ATDMSC implante edilmiş hücresizleştirilmiş kemik grefti olacak şekilde mini plak ve vida ile osteosentez yapıldı.

Bulgular: Birinci, ikinci ve dördüncü grupta greft iyileşmesi ve düzensiz kallus oluşumu gözlendi. Birinci grupta, haversian sistemlerinin organizasyonu, lakunların yapısı ve canaliculi ossiumların varlığı gözlendi; İkinci grupta, havers kanallarının yaklaşık %40'ı kan damarları içeriyordu ve mikroskobik olarak incelenen alanların %90'ında ince filamentler şeklinde kanalikül ossiumları vardı; Üçüncü grupta, havers kanallarının çoğu boştu, osteosid kanallarının çoğu hücre içermiyordu ve orada kanaliküller ossiumu yoktu; dördüncü grupta, havers kanallarının bir kısmı kan damarları içermekteydi ve hücresizleştirmeye bağlı olarak

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Cite this article as: Güneren E, Baygöl EG, Karaaltın MV, Canter Hİ, Öztürk K, Ovalı E, Özpur MA, Yıldız K, Eyüboğlu F. Generation of Bone Tissue Using Adipose Tissue-derived Stem Cells. Bezmialem Science 2021;9(3):294-300.

Received: 11.10.2019
Accepted: 24.04.2020

group, some of the Haversian canals contained blood vessels, and there were partly lacunae containing cells due to decellularisation, whereas in approximately 50% of the examined microscope areas, the presence of canaliculi ossiums with evidence of mesenchymal stem cells differentiated into osteocytes was demonstrated by Q-dot traced cells.

Conclusion: In this study, the establishment of a proper niche environment for adipose-derived mesenchymal cells promotes their development into osteogenic cells.

Keywords: Tissue, stem cell, adipose, mesenchyme, regeneration

hücreleri içeren kısmen lakazlar mevcutken, incelenen mikroskop alanlarının yaklaşık %50'sinde, osteositlere farklılaşan mezenkimal kök hücrelerin varlığı ile canaliculi ossium varlığı moleküler testler ile kanıtlandı.

Sonuç: ATDMSC ile uygun bir niş ortamının oluşturulması halinde kemik greftinin yaşayabilirliğinde artış sağlandı ve bunun ATDMSC'lerin osteojenik hücrelere dönüşüm yoluyla olduğu gösterildi.

Anahtar Sözcükler: Doku, kök hücre, yağ, mezenkimal, rejenerasyon

Introduction

Autogenous bone grafting is the gold standard in the repair of bony defects. Infection, nonunion, the need for prolonged immobilisation and recovery, donor area shortage and morbidity and the difficulty of three-dimensional shaping, in addition to suboptimal results, continue to be challenging problems (1-3).

Allografts have advantages such as the lack of donor area morbidity, successful long-term results than autogenous bone grafts and a shorter operation period. However, allografts have disadvantages such as potential rejection, the risk of infectious disease transmission, the need for a high-tech infrastructure and the associated high costs (4,5).

Tissue engineering studies and the use of stem cells to enhance viability, osseointegration and ossification via cell migration into artificial and/or alloplastic demineralised bone matrices or decellularised tissues are promising (6-10).

The aim of our study was to regenerate bone tissue using cultured adipose tissue-derived mesenchymal stem cells (ATDMSCs) integrated in a decellularised bone matrix.

Methods

The present study was approved by the Local Institutional Animal Research Ethics Committee. Moreover, 24 rabbits weighing 3,500-4,000 g were used. The procedures were carried out under general anaesthesia. Fat tissue samples were taken from the inguinal region of an animal that was not used in the study due to the animals being from an inbred animal pool. Furthermore, Q-tracker cell labelling kits were used to culture, characterise and label ATDMSCs. Bone grafts were decellularised and then were incubated with ATDMSCs.

ATDMSCs' Culture

The fat tissue was washed in foetal bovine serum (FBS). The washing procedure was repeated until all blood and connective tissue particles were removed and minced into small pieces. By centrifuging the resultant digested material at 800 g, adipocytes and free oil were separated from the stromovascular components. The cells were suspended and plated into 150-cm^2 culture flasks in Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM-LG) (SH3002101; HyClone) containing 10% FBS (SV3016003; HyClone) and 1% penicillin. The culture medium was changed every 3 days, and the cells were grown to 70% confluence.

Passage 1: When the cells reached 70% confluence, the medium was removed from the flasks. The flasks were then filled with 10 mL of trypsin/ethylenediaminetetraacetic acid solution and incubated in an incubator for 5 min. After trypsin reaction was neutralised with 1 mL of FBS, the cells were collected in a tube and centrifuged with FBS at 400 g. The cells were resuspended in fresh complete medium before being seeded in flasks.

Passage 2: When the cells reached 70% confluence, the medium was removed from the flasks. The cells were then trypsinised and resuspended in fresh complete medium. Following that, a 1 mL sample was taken for analysis.

Characterisation of Mesenchymal Stem Cells

After passage 2, the cells were incubated for 45 min at room temperature with 1 μg of R-phycoerythrin (PE)-, fluorescein isothiocyanate and allophycocyanin -conjugated antibodies and isotype-matched control immunoglobulin Gs. After incubation and washing, the samples were analysed for mesenchymal stem cells with CD45, HLA-DR, CD34, CD90, CD105 and CD73 antibodies using a flow cytometer (FACSCalibur®).

Cell Labelling

Stem cells were labelled using Q-tracker cell labelling kits (Invitrogen®), which are designed for loading cells grown in culture with highly fluorescent Q-dot nanocrystals. Moreover, Q-dot nanocrystals target the cytoplasm of living cells. Components A and B were pre-mixed in a tube and incubated at room temperature for 5 min. The tube was filled with fresh complete medium and centrifuged for 30 s. After that, the cells were added and incubated at 37 °C for 45-60 min before washing twice with complete growth medium. Moreover, flow cytometry was used to visualise labelled live cells using appropriate filters.

Flow Cytometry Analysis of Labelled ATDMSCs

Flow cytometry was used to analyse labelled and unlabelled cells using the appropriate filter (FL2) for red emission. Furthermore, 98.7% of the ATDMSCs were labelled, while 0% were not.

Bone Decellularisation

The bone graft, kept at 2 °C-8 °C, was washed in PBS containing 1% povidone iodine. In order to remove the povidone iodine, it was washed twice with PBS. The washed graft was decellularised for 24 hours in 1% SDS and 1% Triton X-100. The graft was cleared of SDS and Triton X-100 after being washed in a physiological saline solution containing antibiotics and DNase for 24 hours. Further, the graft was kept at 2 °C-8 °C in a container fluid with 1% antibiotics until it was surgically adapted or loaded with cells.

Preparation of Biobone

ATDMSCs were cultured on the outside and inside of the bone graft and were grown in a low glucose DMEM containing 10% PBS and 1% antibiotics. After medium and cell replenishment on the third day of culture and after its addition to the bone graft containing ATDMSCs and bone meal on the fifth day, it was cultured for another day before implantation.

Groups

Groups were formed as (n=6) (1) no touch, (2) cryopreserved, (3) decellularised and (4) ATDMSCs-implanted decellularised bones used as grafts.

Surgical Procedures

After cleaning, the rabbit was placed in a supine position, and both forearms were shaved up to the proximal of the elbow and covered with a sterile polyvinylpyrrolidone iodine (polyvidon-iodine) 10% (Batticon®, Adeka İlaç Sanayi ve Ticaret A.Ş., TR) solution. Skin flaps were elevated after a 5 cm incision was performed on the dorsal side of the right forearm. By dissecting the forearm muscles, the ulna was exposed through the subperiosteal plane. Then, at the middle, one-third of the ulna was resected to form a full-thickness defect.

The first group consisted of autografts (Figure 1). In the second group, the resected fragments were stored at -80 °C for 24 hours before being preserved as a bone graft. In the third group, the defect was reconstructed using decellularised bone grafts prepared from the other leg of the rabbit. In the fourth group, the defect was again reconstructed with a decellularised graft and implanted with previously cultured adipose tissue-derived mesenchymal stem cells prepared from the other leg. In all groups, the prepared bone grafts were secured with six-hole plate and four screws. After wound was closed, a splint was placed on the forearm.

Before the rabbits were sacrificed at the end of the sixth week of study, all groups were subjected to a perfusion test with India ink.

Evaluation

Following decalcification, specimens were stained with hematoxylin and eosin (H&E) for a general morphological evaluation. The second series of sections taken for histomorphological evaluations, on the other hand, were stained with Schmorl's picro-thionin stain. The stained sections were examined with an Olympus BX51 photomicroscope and photographed with an Olympus DP72 camera. For histopathological scoring, bone tissues in each section were examined with x400 zoom in at least three similar areas. A semiquantitative scoring was carried out: (1) the number of empty osteocyte lacunae, (2) the presence of stained canaliculi ossium in each area by dividing the microscope area in four, and (3) the number of empty Haversian canals were evaluated. Furthermore, two experienced histologists performed the histopathological scoring blindly.

Statistical Analysis

An ANOVA test was used to compare more than two groups, taking into account the number of samples and the distribution of data within the groups. In addition, the post hoc Tukey test was used to compare the groups to one another. In the measurements that were taken, there is a 95% confidence interval. For the analysis, the MedCalc statistical software (MedCalc Software, Acacialaan 22, B-8400 Ostend, Belgium) was used, and $p<0.05$ was considered statistically significant.

Results

During the macroscopic examination, graft recovery and irregular callus formation on the plate-screws were observed in all groups. In the third group, graft recovery is lower than in others.

The Results of Bone Staining with India Ink Injection

According to the flow cytometry analysis of labelled ATDMSCs for red emission, 98.7% were labelled.

All grafts from the first group were stained. Moreover, four of the six grafts from the second and fourth groups, as well as three of the six grafts from the third group, were stained.

Microscopic Analysis

In the first group, osteocytes in regular morphology, canaliculi ossium and Haversian canals together with Haversian systems were observed (Figure 2A); in the second group, Haversian systems similar to the first group were observed, but there was

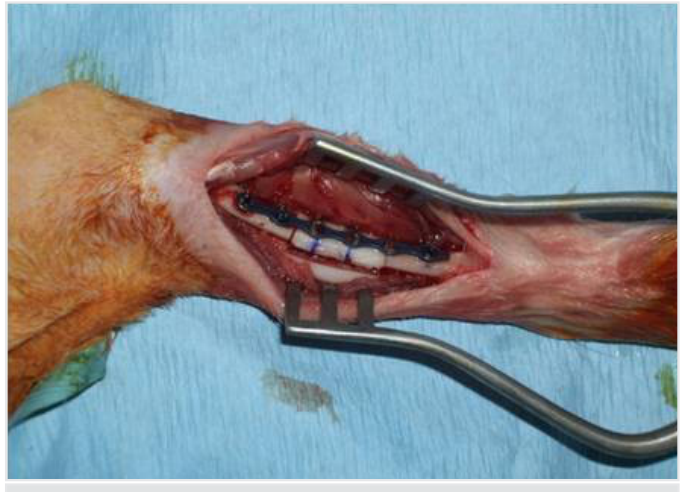


Figure 1. Interpositional bone graft fixation with plate and screws and some polypropylene sutures

an increase in the presence of empty osteocyte lacunae (Figure 2C). In the third group, most of the osteocyte lacunae within the structure of Haversian systems were empty, with only a few lacunae filled with osteocyte (Figure 2E); in the fourth group, the osteocyte lacunae within the structure of Haversian systems were empty, but the number of lacunae filled with cells was increased (Figure 2G).

Schmorl's picro-thionin stained specimens in all groups, lacunae containing osteocyte were observed in fusiform and in brown, canaliculi ossium were observed as thin brown filaments between the lacunae, and the blood vessels within the Haversian canals were observed in various shades of brown.

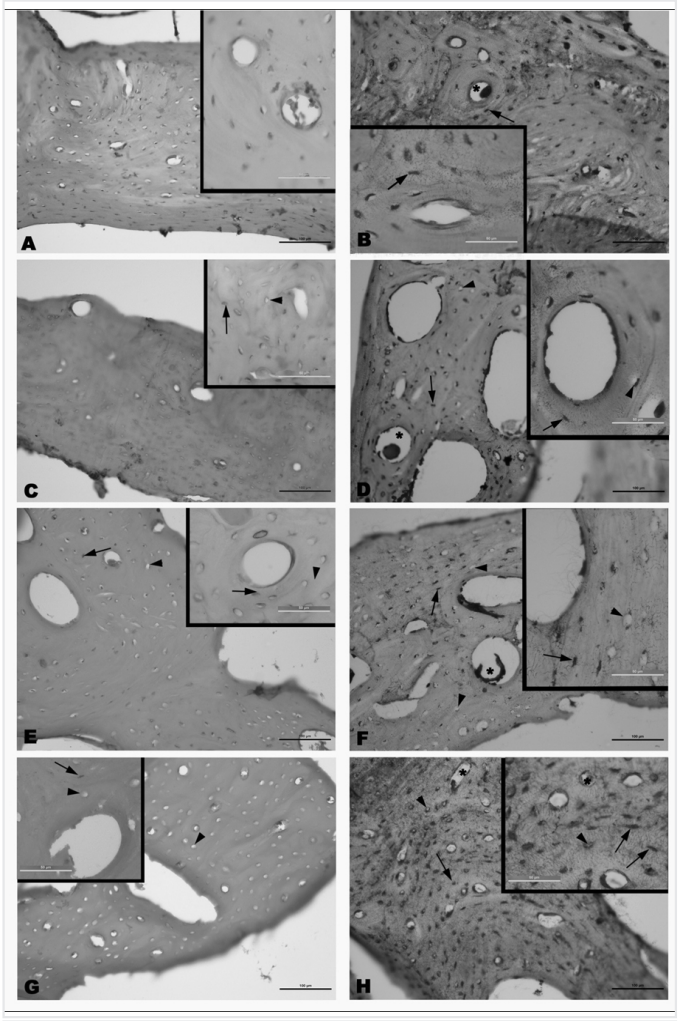


Figure 2. On H&E and Schmorl's picro-thionin stained specimens: 2A, regular osteocytes, canaliculi ossium with Haversian systems; 2C, an increase in the presence of empty osteocyte lacunae; 2E, most of the osteocyte lacunae were empty; 2G, osteocyte lacunae were empty; 2 B, Haversian systems, the lacunae and the presence of canaliculi ossium; 2D, near 40% of the Haversian canals contained blood vessels; 2F, the Haversian canals were empty, the osteocyte canals did not contain any cells and no canaliculi ossium; 2G, the Haversian canals contained blood vessels, empty osteocyte lacunae

In the bone tissue samples from the first group, the organisation of Haversian systems, the structure of the lacunae and the presence of canaliculi ossium were all observed to be in normal morphology (Figure 2B); in the second group, approximately 40% of the Haversian canals contained blood vessels, the number of osteocyte canals observed to be empty increased in comparison to the control group, and canaliculi ossium in the form of thin filaments were found in 90% of microscope areas (Figure 2D); in the third group, most of the Haversian canals were empty, most of the osteocyte canals did not contain any cells, and there were no canaliculi ossium in most of the microscope areas (Figure 2F); in the fourth group, it was discovered that some of the Haversian canals contained blood vessels, that the number of osteocyte lacunae observed to be empty was high once more, but that there were partly lacunae containing cells. In approximately 50% of the examined microscope areas, canaliculi ossium was observed (Figure 2H).

ATDMSCs labelled with Q-dot were identified microscopically in histologic samples from the fourth group. These microscopic views were compared to histologic samples from the third group that had not been marked with Q-dot and others (Figures 3).

Statistical Results

Tukey test was used to compare each of the groups. Moreover, Tukey test shows that the fullness of lacunae in groups 1 and 3-4 is significantly different ($p<0.05$). The fullness of lacunae also differs significantly ($p<0.05$) between groups 2 and 4. The fullness of canaliculi differed significantly between groups 1 and 3-4. The fullness of canaliculi in group 4 is 47% and in group 3

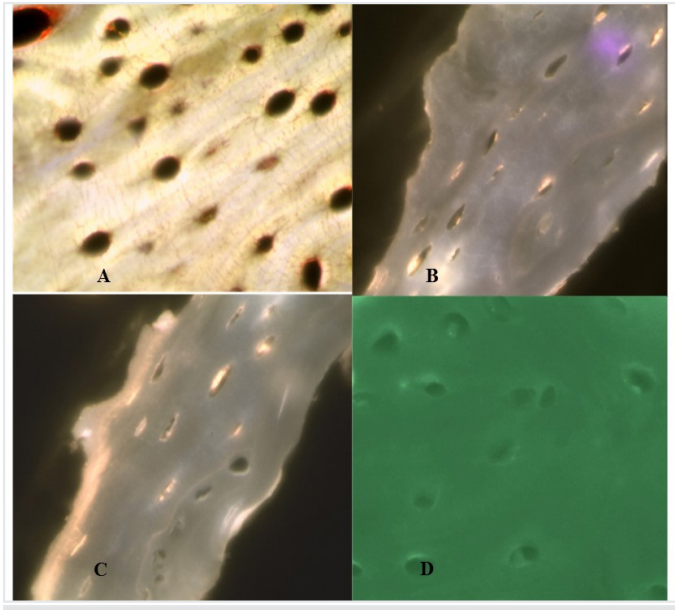


Figure 3. ATDMSCs marked with Q-dot were microscopically identified in histologic samples of fourth group. 3A, Preimplant view of Q-dot nanocrystals; 3B-C, postimplant view of Q-dot nanocrystals, showing the cytoplasm of living cells; 3D, postimplant view of the third group, with no Q-dot nanocrystals

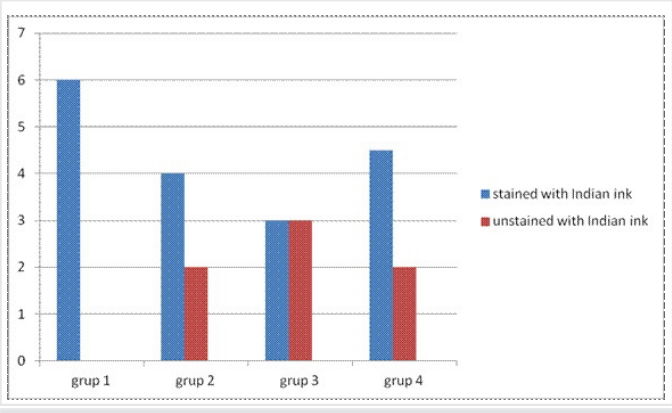
is 20%, but there is no statistically significant difference (p>0.05) (Graphs 1 and 2).

Discussion

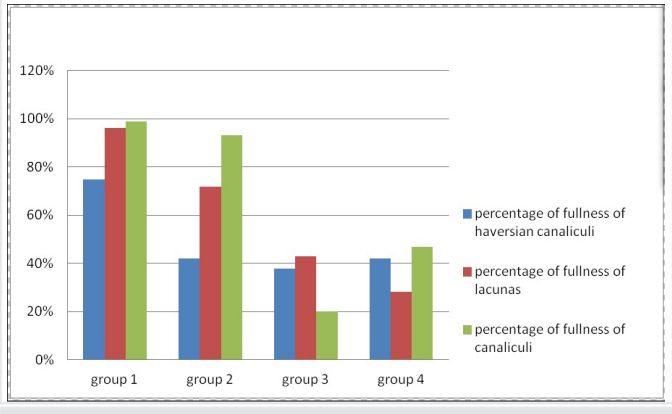
The bone graft serves as a template on the recipient area on for recovery (osteoconduction) and leads to the formation of a new bone tissue by inducing osteoprogenitor cells in the host tissue (osteoinduction). It takes at least 3 weeks to start forming new bone tissue (4-6).

Although autogenous bone grafts are the gold standard, they have some disadvantages, including limited availability, the creation of a new operative field, prolonged operative time, donor area morbidity, additional cost, resorption risk and difficulty in shaping (1-3).

Allografts, which come in cortical, cancellous and demineralised bone matrix forms, have advantages such as not causing donor area morbidity, having successful long-term results when compared to autogenous bone grafts and requiring no additional operative time. However, allografts also have disadvantages such as the risk of rejection, the risk of infectious disease contagion, the risk of resorption and the need for a high-tech infrastructure with correspondingly high costs (4,5).



Graphic 1. The number of the grafts that were stained or unstained with Indian ink



Graphic 2. The percentage of fullness of Haversian canaliculi, fullness of lacunae, and fullness of canaliculi

Alternative bone grafts include synthetic ceramics, silicate-based materials and polymers, which do not cause donor area morbidity and do not pose a risk of disease transmission. Hydroxyapatite made of synthetic ceramics, bioactive glass made of silicate-based materials and polymethyl methacrylate made of polymers are materials that are preferred in cosmetic and reconstructive facial surgery (3-5). However, besides their costs, these substances cannot provide adequate stability for the repair of defects in big and load-bearing bones due to their fragile structure, so their use is limited to the craniofacial zone (4,6).

Adult stem cells, such as ATDMSCs and bone marrow-derived stem cells (BMDSCs), hold tremendous promise in regenerative medicine (10). Traditional treatments for degenerative skeletal defects and wounds include allograft, autograft and synthetic implant applications, but these are inadequate for some defects (11). However, complications of these techniques include donor area morbidity, inadequate tissue provision, the emergence of immunogenicity and implant loss (12-14). The use of autologous ATDMSCs in the treatment of tissue replacements reduces the immune response and provides more sources than BMDSCs. In order to form a tissue engineering structure specific to the tissue, two important components must be present: (1) tissue-specific cells and (2) a biocompatible, mechanically suitable tissue scaffold to which the cells can stick and form an extracellular matrix have been used by researchers in the treatment of tendon, cartilage and bone repair (15-19), which encourages regeneration in the defect area. The cells cultivated scaffolds as a result of tissue engineering. As the use of ATDMSCs and other stem cell types in tissue engineering studies increases, so do studies on improving the tissue scaffold medium. In order to discuss the use of ATDMSCs in the formation of bone tissue-equivalent tissue, it is important to know the differences between BMDSC and ATDMSCs.

Most ATDMSC tissue engineering studies up to this point have been basically derived from BMDSCs studies, so excluding BMDSCs would be a huge mistake. Until now, the majority of stem cell studies have relied on BMDSCs (20). However, because the source of these cells is limited, their extensive commercial use is limited, and the studies focus on ATDMSCs. In contrast to bone marrow, fat tissue is more plentiful and easily accessible. ATDMSCs and BMDSCs have comparable cell yield, cell expansion, growth kinetics and differentiation (21).

Obtaining a large number of ATDMSCs and a high cell yield is much easier than isolating BMDSCs. While some studies show that ATDMSCs, like BMDSCs, undergo osteogenic, myogenic, adipogenic and chondrogenic transformations, the results of other studies have led to increased discussions about the differentiation potential of ATDMSCs (21-25). While some researchers claim that there is no difference in the differentiation of ATDMSCs and BMDSCs (26), others have reported that ATDMSC differentiation is inadequate in comparison to BMDSC differentiation (27-31). Aust et al.

(21) compared the differentiation potential of BMDSCs to that of ATDMSCs and found no difference in their ability for chemically induced differentiation. Similarly, Hattori et al. (24) found that BMDSCs and ATDMSCs cultured in osteogenic medium produce similar results in terms of calcium phosphate accumulation and osteocalcin release. According to Im et al. (29) and Mehlhorn et al. (27), these two cell types do not have the same ability for osteogenic and chondrogenic differentiation. After 2 and 3 weeks of tracking, Im et al. (29) revealed that BMDSCs are clearly superior to ATDMSCs in terms of mineralisation and alkaline phosphatase activity and have similar results in chondrogenic markers. While drawing attention to the concerns that ATDMSCs have lower stem cell potential than BMDSCs in different studies, it is important to note that there is no agreement on the data. Considering their differences in surface markers that they express, medium requirements for differentiation (31) and genes that they are influenced by during differentiation, ATDMSCs are not less potent than BMDSCs, but they behave differently. For recent specific cell populations, there are obvious differences in gene expression profiles between ATDMSCs and BMDSCs (27). Despite evidence supporting the natural differences between ATDMSCs and BMDSCs, there is a need for further studies on cell behaviours before they can be used effectively in tissue engineering practices.

The reason for the overall superior results in the bone autograft group compared to the allograft ATDMSC group could be due to the decellularisation method, which might have damaged the ultrastructure of the bone scaffold, impairing the optimal environment for osteogenesis (32,33).

Study Limitations

In the examination of the samples from the third group, where reconstructed bones were applied as graft after decellularisation, and from the fourth group, where bones were similarly applied as graft with ATDMSC cultivation after reconstruction and decellularisation, the fact that the fourth group showed a more positive result in terms of fullness of canaliculus can be interpreted as a positive effect on recovery caused by stem cells through regeneration.

The presence of Q-dot stained cells in the lacunae in the specimens of group four strongly suggests that these cells have been developed from the mesenchymal stem cells that were loaded on the decellularised bone grafts. This finding indicates the ability of the ATDMSCs to develop into osteogenic cells that can promote tissue regeneration and perhaps may serve as the foundation for future bone tissue engineering studies.

Conclusion

In an optimal stem cell niche environment, ATDMSCs have a high potential for osteogenic tissue formation. Now that the osteogenic effect of stem cells has been proven, we believe that focusing studies in bone tissue engineering on tissue scaffolds as well as bone decellularisation methods will yield more positive results in clinical practices.

Acknowledgement: This study was funded by the Turkish Ministry of Science and Technology (SanTez Code; 01103. STZ.2011-2) and was carried out in Bezmialem Vakıf University Animal Research Laboratory.

Ethics

Ethics Committee Approval: The present study was approved by the Local Institutional Animal Research Ethics Committee.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: E.G., E.G.B., M.V.K., H.İ.C., K.Ö., E.O., M.A.Ö., K.Y., F.E., Design: E.G., E.G.B., M.V.K., H.İ.C., K.Ö., E.O., M.A.Ö., K.Y., F.E., Data Collection or Processing: E.G., E.G.B., M.V.K., H.İ.C., K.Ö., E.O., M.A.Ö., K.Y., F.E., Analysis or Interpretation: E.G., E.G.B., M.V.K., H.İ.C., K.Ö., E.O., M.A.Ö., K.Y., F.E., Literature Search: E.G., E.G.B., M.V.K., H.İ.C., K.Ö., E.O., M.A.Ö., K.Y., F.E., Writing: E.G., E.G.B., M.V.K., H.İ.C., K.Ö., E.O., M.A.Ö., K.Y., F.E.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

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