Osteogenic differentiation of canine adipose derived mesenchymal stem cells on B-TCP and B-TCP/Collagen biomaterials

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ABSTRACT

Mesenchymal stem cells are adult stem cells that can differentiate into osteogenic, chondrogenic, adipogenic and myogenic lineages. In orthopedics and traumatology, mesenchymal stem cells, combined with biomaterials, are used mainly for treating bone fractures and diseases in humans and animals. This study aims to promote the growth, proliferation, and osteogenic differentiation of mesenchymal stem cells isolated from the adipose tissue of canines on B-TCP (Beta-tricalcium phosphate) and B-TCP/Collagen biomaterials. MTT analysis was performed to test the cell adhesion and proliferation on B-TCP and B-TCP/Collagen biomaterials used to mimic the extracellular matrix of three-dimensional bone tissue. Scanning electron microscope analysis was performed to show general surface characters of B-TCP and B-TCP /Collagen biomaterials. The osteoinductive capacities of the B-TCP and B-TCP/Collagen biomaterials were determined by alkaline phosphatase and Von Kossa stainings, and RT-PCR analysis. The ALP activity of the B-TCP/Col containing material was significantly higher than the B-TCP in the early days. In terms of gene expression, there were no significant differences except 14th-day SPARC gene expression. The results of Von Kossa staining indicated that B-TCP/Col has above the desired level degradation capacity. As a result of this research, although it is advantageous in terms of alkaline phosphatase activity and osteogenic gene expression compared to B-TCP material, it is thought that B-TCP/Collagen biomaterial should be developed for use in bone tissue engineering due to its high degradation property.

Introduction

Mesenchymal stem cells (MSCs) are adult stem cells that were first isolated from bone marrow (17, 18). After this isolation, they were separated from many tissues, especially tissues with abundant vascular connective tissue (6).

In 2001, Zuk et al. (53) showed that MSCs can also be obtained from adipose tissue. Adipose tissue derived mesenchymal stem cells (Ad-MSCs) are obtained by explant culture method or using collagenase enzyme (21, 33). Ad-MSCs have advantages over other sources of MSCs. It has been reported that Ad-MSCs have more osteogenic differentiation potential than mesenchymal stem cells obtained from bone marrow (9, 12, 28). On the other hand, unlike embryonic stem cells, there are neither ethical nor political concerns regarding Ad-MSC isolation since they can be isolated from the patient's adipose tissue (10, 13, 40). All these advantages make Ad-MSCs one of the most preferred sources of mesenchymal stem cells.

One of the most common uses of mesenchymal stem cells today is bone tissue engineering studies in orthopedics and traumatology, especially for treating bone, cartilage and tendon diseases (8). Various biomaterials can be used to provide structural and mechanical support during the healing process of large defects and multi-component fractures with insufficient bone regeneration capacity (13, 19, 20, 30).

One of the important developments in the field of biomaterials has been the use of bioceramics for bone replacements. Calcium phosphate ceramics have been

used as synthetic bone graft substitutes for over 30 years, as they have similarities with the inorganic composition of bones (46).

B-TCP is the most studied bioceramic in the treating bone fractures and diseases (46). According to the numerous in vivo and in vitro evaluations in the literature, B-TCP has excellent biocompatibility and osteoconductivity. It has also been shown to support the differentiation and proliferation of mesenchymal cells (7, 16, 44, 52). Using porous ceramic implants in bone tissue engineering can provide an environment for cells to grow and differentiate (3).

Collagen is a natural polysaccharide widely used in bone tissue engineering, because of its structural resemblance to natural bone tissue. It is a biocompatible material that the body can absorb. Moreover, collagen is proven to be minimally immunogenic and nontoxic material. Despite these advantages, collagen shows poor mechanical properties. Today, collagen is used in prosthetic implants and tissue engineering of many organs, including bone (1, 26, 36).

This study aims to compare the osteogenic differentiation capacities of canine Ad-MSCs biomaterials containing B-TCP and B-TCP/Collagen (B-TCP/Col). These biomaterials provide structural and mechanical support and create the microenvironment for stem cells during the healing process of large bone defects and multi-part fractures of the bone. It is thought that the biomaterial prepared as a combination of B-TCP and collagen will be advantageous in terms of adhesion, proliferation, compatibility and osteogenic differentiation. Comparing these biomaterials as microenvironments for stem cells may increase the treatment success of bone fractures and accelerate the post-operative healing process.

Materials and Metods

Isolation of Ad-MSCs: For the isolation of MSCs, inguinal adipose tissues were harvested from n= 5 dogs during ovariohysterectomy operation at Ankara University Faculty of Veterinary Medicine. This study was approved by Ankara University Animal Experiments Local Ethics Committee (2017-5-37). The explant culture method was preferred for mesenchymal stem cell isolation from adipose tissue. Adipose tissue ($1cm^3$) was divided into small pieces in a sterile petri dish under laminar flow and kept at 37 °C in an incubator with 5% CO_2 for 20 minutes. Then Modified Eagle Medium (Lonza) which contain 20% Fetal bovine serum (Biowest), 2% L-Glutamine, 1% Penicillin, Streptomycin and 77% Dulbecco's medium was added (53).

Characterization of Ad-MSCs: At the end of the third passage, adipogenic, osteogenic, and chondrogenic differentiation and flow cytometry analyzes were performed for the characterization of canine Ad-MSCs.

For adipogenic differentiation, adipocyte differentiation basal medium and supplements (standard medium high-glucose DMEM (10% FBS), 0.5 mM 3-isobutyl-1-methylxanthine, 1 µM dexamethasone, 10 µg/mL insulin, 0.5 mM indomethacin (Sigma-Aldrich, Switzerland); for osteogenic differentiation, osteocyte differentiation basal medium (DMEM-LG, 0.05 mM ascorbate-2-phosphate, 100 nM dexamethasone, and 10 mM sodium β-glycerophosphate (Sigma-Aldrich); and for chondrogenic differentiation, chondrocyte differentiation basal medium (high-glucose DMEM containing 6.25 µg/mL insulin-transferrin-selenious acid, 0.1 mM ascorbate2-phosphate, 10–7 M dexamethasone, 1.25 mg/mL bovine serum albumin, 5000 IU/mL penicillin, 50 µg/mL ascorbate 2-phosphate, and 100 nM dexamethasone and 10 ng/mL human transforming growth factor) were used. Adipogenic, osteogenic and chondrogenic differentiation were evaluated using Oil Red O, Von Kossa and Alcian Blue staining methods. The development of cells was observed with an inverted microscope (Olympus Cx45).

For flow cytometry analyzes, $1x10^6$ cells were placed in flow cytometry tubes and these cells were washed 3 times each in 3% Bovine serum albumin/PBS (BSA/PBS) solution. Stem cells were incubated with 0.1-10 µg/ml primary antibodies [CD 29 (P4611, Chemicon), CD 34 (ICO115, Novusbio, USA), CD 44 (G44-26, BD biosciences, USA), CD73 (P21589, Bioss, USA), CD 81 (J5-81, BD biosciences, USA), CD90 (OX-7, BD biosciences, USA) and CD 271 (C40-1457, BD biosciences, USA)]for 30 minutes at room temperature. Cells were washed 3 times in 3% BSA/PBS solution and then precipitated by centrifugation. For unconjugated primary antibodies, cells were incubated with secondary antibody for 30 minutes at +4 °C and washed 3 times in 3% BSA/PBS solution. After the cell sediments were dissolved in 1 ml of 3% BSA/PBS solution, the cell was analyzed in flow cytometry device (BD Accuri Plus flow cytometer) (48).

Biomaterial fabrication, Cell seeding, and Osteogenic Differentiation Protocol: B-TCP (SupraBone, 0.5–1mm particle size, Figure1) and B-TCP/Col biomaterials were kindly donated by BMT Calsis Health Technologies (41). The pack of B-TCP/Col was split into 0.5 mm x 0.5 mm x 0.5 mm pieces (Figure1). Both biomaterials were placed in 24-well dishes and sterilized under UV light. Following sterilization, 200,000 canine Ad-MSCs reaching passage 3 were seeded onto each scaffold for further analysis. Before adding 2 ml of cell culture medium to each well, they were incubated for 4 hours at 37°C. During the 4-hour incubation, 20 µl of the medium was added to each cell scaffold every 30 minutes to prevent the materials from drying out. After cell cultivation, media were added to the biomaterials and incubated at 37°C in an incubator containing 5% CO_2 (25). To initiate osteogenic

differentiation, osteogenic differentiation basal medium was added to each well after 1 day of incubation with cell culture medium.

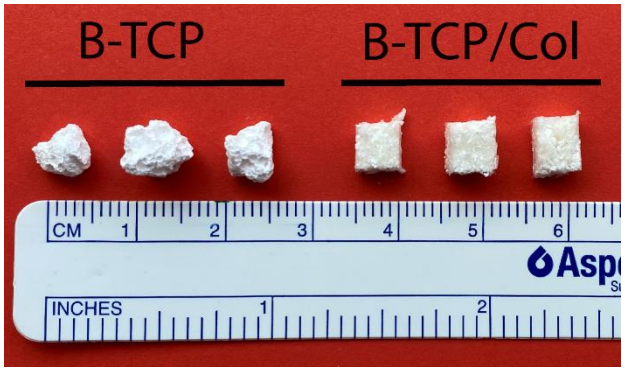

Figure 1. Image of B-TCP (SupraBone, 0.5–1mm particle size) and Image of B-TCP/Col (0.5 mm x 0.5 mm x 0.5 mm).

MTT Assay (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide): Colorimetric MTT test was performed on days 7, 14 and 21 to examine the growth and proliferation of cells on B-TCP and B-TCP/Col structured tissue scaffolds. Each 200,000 cells seeded biomaterials were kept in a solution (5 mg / mL) medium and MTT reagent (Biovision) was mixed at a ratio of 10:1 by volume for 4 hours at 37 °C in an incubator with 5% CO_2. Then, 100 µl of 1% SDS was added to each sample and kept at 37 °C in an incubator with 5% CO_2 and in an environment without light for 24 hours. Finally, 200 µl of the solution was taken and transferred to a 96-well plate. The absorbance was measured at 590 nm with a microplate reader (SpectraMax i3) (5, 32).

Scanning Electron Microscopy (SEM): To analyze Ad-MSCs in B-TCP and B-TCP/Col scaffolds 250,000 cells were seeded in both materials. After 2 days of incubation at 37°C in the incubator, the biomaterials were fixed in 5% glutaraldehyde at 4°C for 24 hours. After washing with distilled water, dehydration was done by holding in 35%, 50%, 75%, 95% and absolute alcohols twice for 15 minutes. B-TCP and B-TCP/Col biomaterials were left to dry in a desiccator at room temperature after they were kept in 2 ml of Hexamethyldisilazane (HMDS). Dried biomaterials were coated with copper and examined under an SEM (EVO50 Zeiss) (31).

Alkaline Phosphatase (ALP) Assay: Cell-seeded biomaterials on days 1, 7, 14 and 21 were fixed for 5 minutes using 10% neutral buffered formalin and washed with PBS. After washing, the biomaterials were dyed with the p-nitrophenyl phosphate (p-NPP) solution for 45 minutes in an incubator containing 5% CO_2 at 37 °C without light. After stopping the reaction with the stop solution, the absorbance values were recorded at 405 nm in a microplate reader (SpectraMax i3) (27, 32).

Von Kossa Staining on Biomaterials: At the end of 7, 14 and 21 days, cell-seeded biomaterials were fixed for 30 minutes with 10% neutral buffered formalin. After washing with distilled water, silver nitrate solution (5%) was added onto the biomaterials and they were subjected to UV light for 60 min. Then they were rewashed, and mineralization was demonstrated by adding 1% sodium thiosulfate (32).

RT-PCR (Real Time Polymerase Chain Reaction): Total RNA isolation was performed according to the manufacturer's protocol (Thermo GeneJet) on the 7th, 14th and 21st days of the osteogenic induction. Following isolation, RNA samples were transformed into cDNA. For this purpose, iScript cDNA synthesis kit (BioRad) was used. The relative gene expressions were determined by reverse-transcriptase polymerase chain reaction method using QPCR Green Master Mix Kit (Biotechrabbit). The reaction mixture was prepared to 20 µl total volume by using 10 µl master mix, 7.2 µl nuclease-free water, 0.4 µl forward primer, 0.4 µl reverse primer and 2 µl cDNA for each sample. The Cq results were obtained from RT-PCR device (CFX96 Touch Real-Time PCR Detection System) with protocol of 3 minutes at 95 °C, 15 seconds at 95 °C and 30 seconds at 60 °C for 40 cycles. Relative gene expression differences were calculated using the 2-ΔCt formulas and primers were designed for this study (Table 1). The beta actin (ACTB) gene was used for the normalization of the values (27).

Statistical Analysis: Two-way analysis of variance was used to evaluate the effect of group and time on the measurements obtained from MTT, ALP and RT-PCR analyses. In the ANOVA model, group (B-TCP, B-TCP/Col and OC between-subject factor) and time (days 1, 7, 14 and 21 between-subject factor) and the interaction term of these two factors were included. Tukey test was used as an advanced test for the factors that were found to be significant. Analysis was done with GraphPad Prism software and data are presented as mean ± standard deviation. Statistical significance was expressed as * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

Table 1. Primers used for RT-PCR analyses.

	Forward (5'-3')	Reverse (5'-3')
RUNX2	CCCAGAAGGCACAGACAGAA	CATCTGGCTCAGGTAGGACG
SPARC	TTCCTGTTGCCTGGCTCTAA	GGTTCTGGCAGGGGTTTTCA
ACTB	TCCATGAAACTACCTTCAACTCC	AACGCAACTAAAGTAACAGTCC

Results

Characterization of Ad-MSCs: For mesenchymal stem cell characterization, cells were induced for osteogenic, chondrogenic and adipogenic differentiation. Cell colonies were observed from day 9 in cells induced for osteogenic differentiation. The number of these colonies reached its highest level at the end of the second week. Von Kossa staining was performed to show osteogenic differentiation on day 14 and the calcium deposits were formed (Figure 2A). In the second week of chondrogenic differentiation, changes began to be seen in the morphology of the cells. On the 19th day, secreting proteoglycan specific to cartilages was observed. Therefore, Alcian Blue staining was performed to show cartilage differentiation on day 21 (Figure 2B). Adipose vacuoles were observed from day 14 in adipogenic differentiation. At day 21, adipogenic differentiation was demonstrated by staining the oil vacuoles in the cytoplasm of the cells with Oil red O staining (Figure 2C).

The cells isolated from adipose tissue were analyzed with seven antibodies for mesenchymal stem cell characterization and Figure 3A shows the control. It was shown by flow cytometry analysis that CD 44^+ (Figure 3G), CD 73^+ (Figure 3H), CD 81^+ (Figure 3F) and CD 90^+ (Figure 3E) were expressed from these antibodies and CD 29^- (Figure 3B), CD 34^- (Figure 3C) and CD 271^- (Figure 3D) were not expressed.

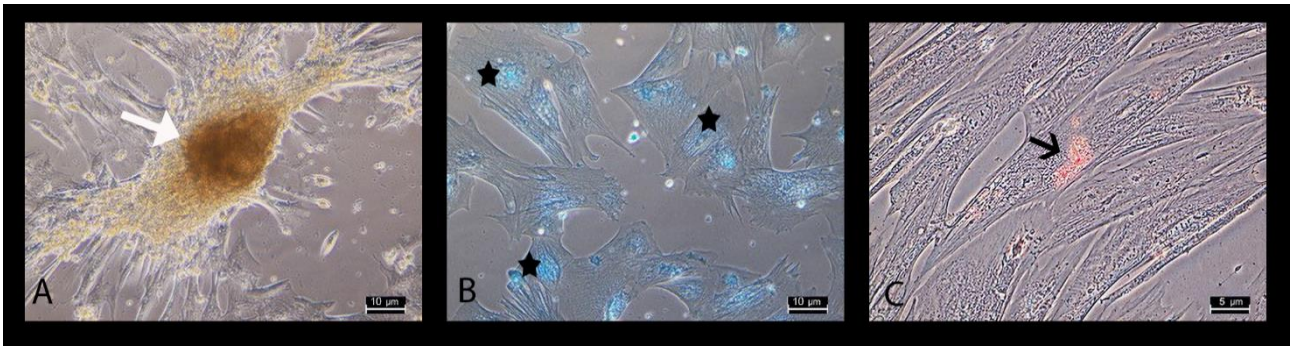

Figure 2. A. Osteogenic differentiation of Ad-MSCs. Von Kossa staining was used to detect calcium deposit at 14 days after osteogenic culture (white arrow), scale bar: 10 µm.**B.** Chondrogenic differentiation of Ad-MSCs. On the 21st day, chondrogenic differentiated cells were observed by Alcian blue staining (stars), scale bar: 10 µm. **C.** Adipogenic of differentiation of Ad-MSCs. On the 21st day, oil vacuoles were observed in the cytoplasm of the Ad-MSCs by Oil red O staining (black arrow) scale bar: 5µm.

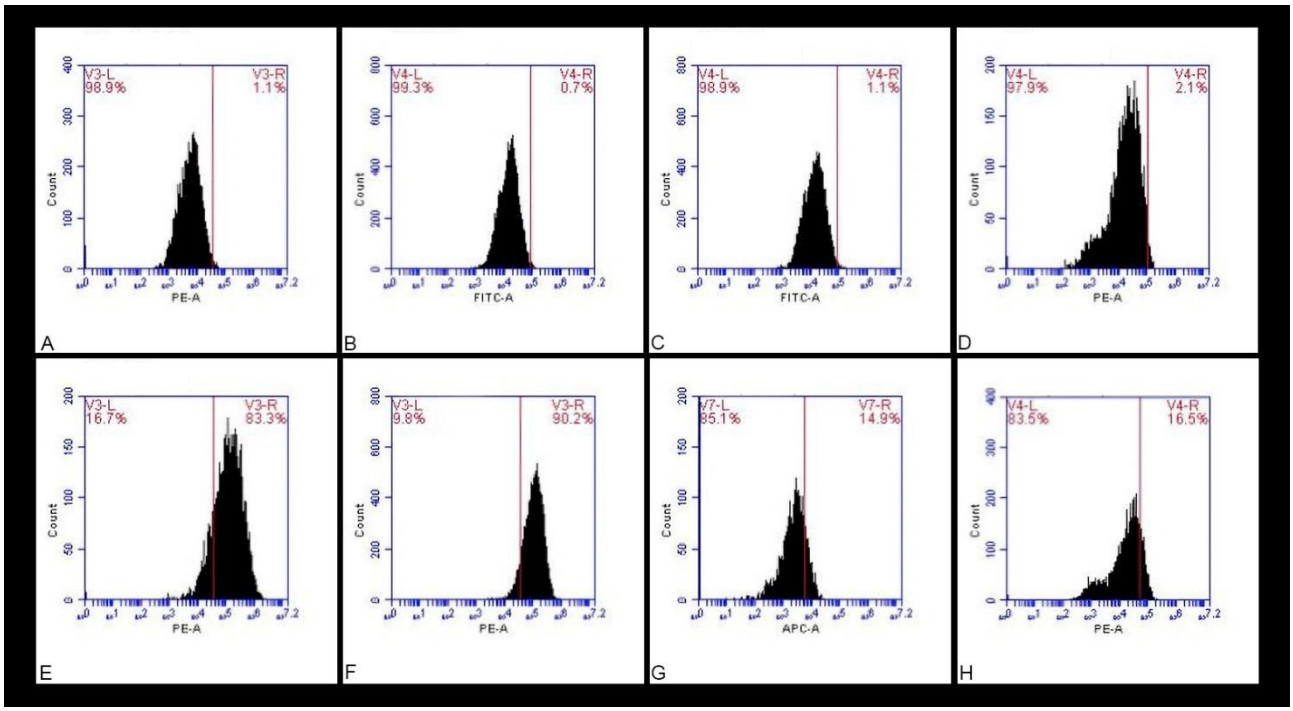

Figure 3. The expression of cell surface molecules in canine Ad-MSCs was detected by flow cytometry: **A.** Control, **B.** CD 29^-, **C.** CD 34^-, **D.** CD 271^-, **E.** CD 90^+, **F.** CD 81^+, **G.** CD 44^+, **H.** CD 73^+.

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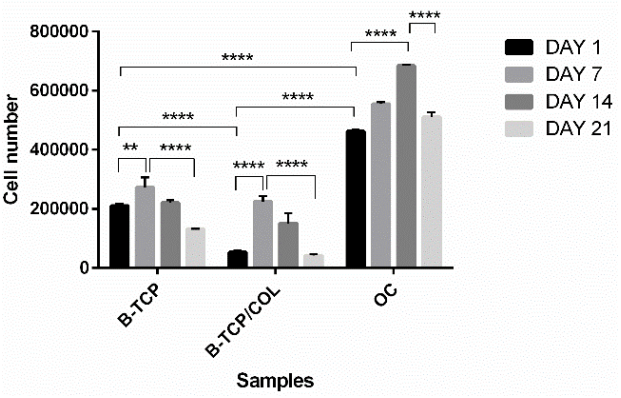

Figure 4. Ad-MSC cell numbers in cell-seeded B-TCP, B-TCP/Col and Only cell (OC) groups obtained from absorbance values of MTT test on days 1, 7, 14 and 21 (** P<0.01, **** P<0.0001).

MTT Assay: Colorimetric MTT test was performed on the 1st, 7th, 14th and 21st days on B-TCP and B-TCP/Col based biomaterials and groups of only cells (OC) (Figure 4). According to the calibration curve, the cell numbers cultivated on biomaterials were calculated. Although the number of cells in the B-TCP material was significantly higher than the B-TCP/Col combination on day 1, there was no significant difference between the two materials on the other days. The cell number of the OC group was statistically higher than cell proliferation in both materials on days 1, 7, 14 and 21. The increase in cell number from day 1 to day 7 in both materials was statistically significant. The number of cells adhering to the materials decreased significantly from day 7 to day 21, while the proliferation of cells grown in tissue culture plates decreased from day 14 to day 21.

Scanning Electron Microscopy (SEM): As a result of scanning electron microscopy, the general surface properties of cell seeded materials were photographed. Ad-MSCs attached to the porous surface of B-TCP was demonstrated in cell seeded biomaterials (Figure 5A). Biomaterials containing β-TCP/collagen were shown by SEM to have fewer pores than β-TCP. The collagen structure and location of Ad-MSCs were photographed in the cell-planted β-TCP/collagen-containing biomaterial (Figure 5B).

Alkaline Phosphatase Assay: It was observed that ALP activity significantly increased from day 1 to 7 days in B-TCP and B-TCP/Col based biomaterials and groups of OC. Enzyme activity of ALP significantly decreased from day 14 to day 21 in all groups. Cells seeded in B-TCP/Col scaffolds possessed higher ALP enzyme activity on days 1 and 7 than B-TCP. ALP enzyme activity of cells seeded in B-TCP/Col scaffolds was significantly higher than OC groups on the 1st day. On the 14th and 21st days, there was no significant difference between the two materials. The ALP activity of wells with OC increased from day 1 to day 14 and decreased on day 21 (Figure 6).

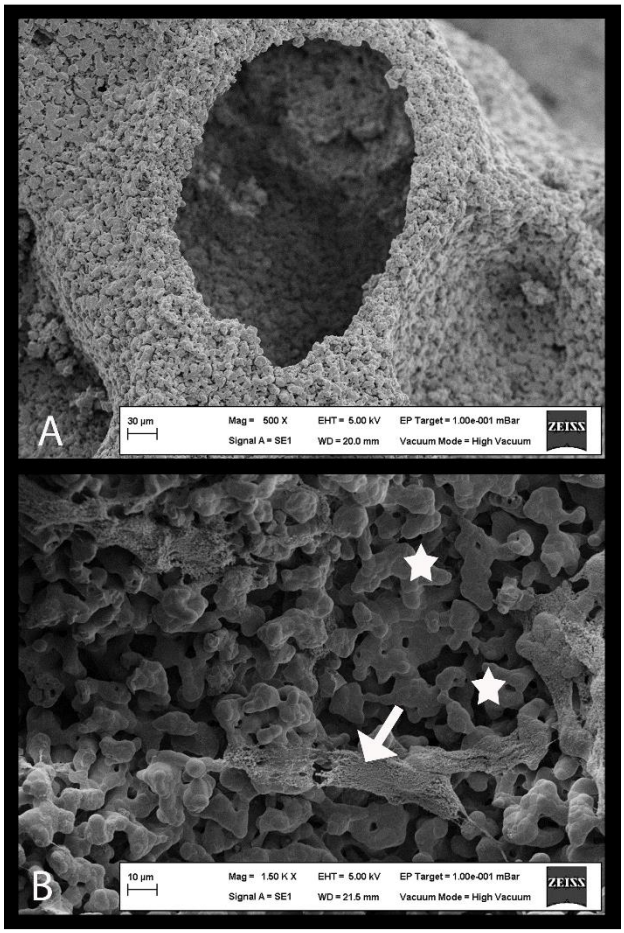

Figure 5. A. SEM image of Ad-MSCs seeded B-TCP material. Ad-MSCs covered the entire porous surface of the material. **B.** SEM image of Ad-MSC seeded B-TCP/Col material. The white arrow indicates pieces of material and stars point out cells.

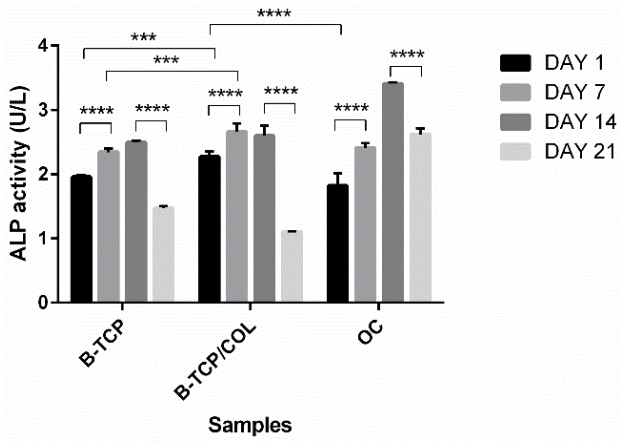

Figure 6. ALP activity of Ad-MSCs in cell-seeded B-TCP, B-TCP/Col and Only cell (OC) groups on days 1, 7, 14 and 21 (*** P<0.001, **** P<0.0001).

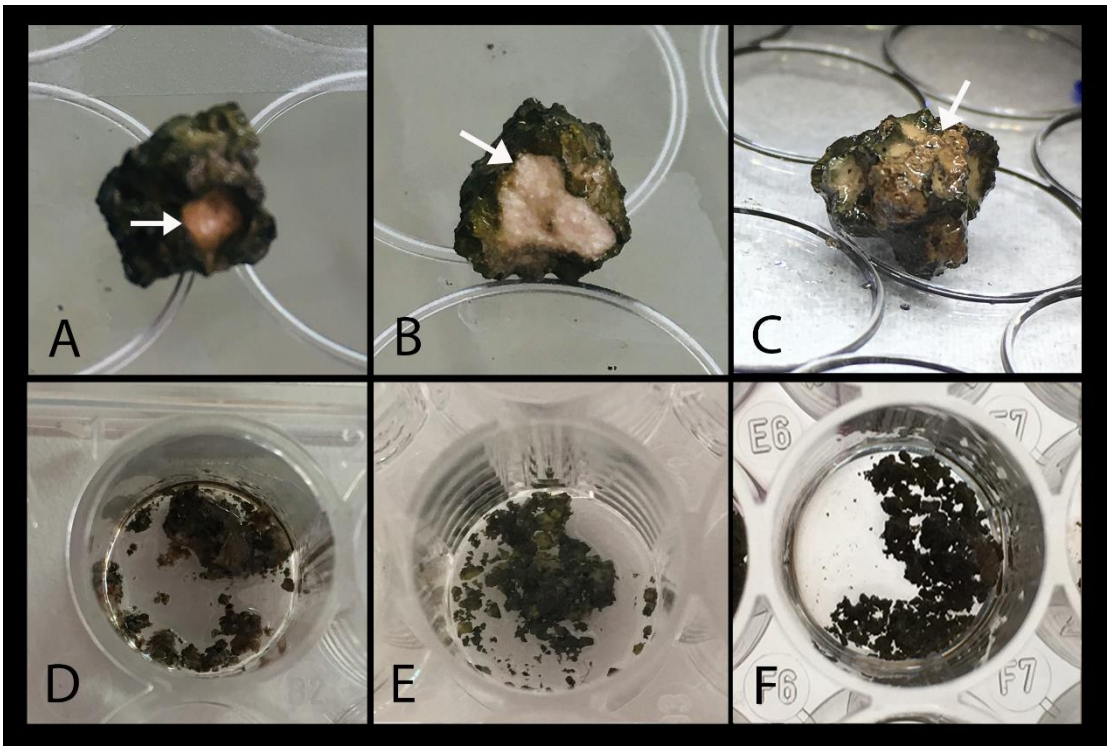

Figure 7. On days 7 (**A.**), 14 (**B.**) and 21 (**C.**), an increased amount of calcium deposition was demonstrated by Von Kossa staining as brown staining areas (arrows). However, on days 7, 14 and 21 (**D., E., F.**), the amount of calcium deposition could not be demonstrated because of the high biodegradation level of B-TCP/Col scaffolds.

Von Kossa Staining on Biomaterials: Von Kossa staining results showed that the number of calcium deposits increased from the 7th day to the 21st day in the B-TCP and B-TCP/Col scaffolds (Figure 7). These deposits were demonstrated by the increase in areas of brown staining on the material after 60 minutes of UV light exposure. However, due to the high biodegradation rate of B-TCP/Col scaffolds, degradation started after the seventh day and calcium deposits could not be demonstrated.

Real Time PCR: The relative expressions of osteogenic marker genes; RUNX2 (Runt-related transcription factor 2) and SPARC (Secreted protein acidic and cysteine-rich) by cells inside B-TCP and B-TCP/Col containing materials and the group with OC were examined. RUNX2 gene expressions significantly increased from day 7 to day 14 in both biomaterials. Furthermore, from the 14th to the 21st day, the gene expressions of RUNX2 were significantly higher in B-TCP and B-TCP/Col containing materials and the OC group. 14th-day expressions of RUNX2 in both biomaterials were higher than in the OC group. There was no statistically significant difference between the two biomaterials regarding RUNX2 expressions. SPARC gene expressions significantly increased from day 7 to day 21 in B-TCP and B-TCP/Col containing materials and the OC group. Cell-seeded B-TCP biomaterial possessed significantly higher SPARC gene expression than the BTCP/Col and OC groups on day 21 (Figure 8).

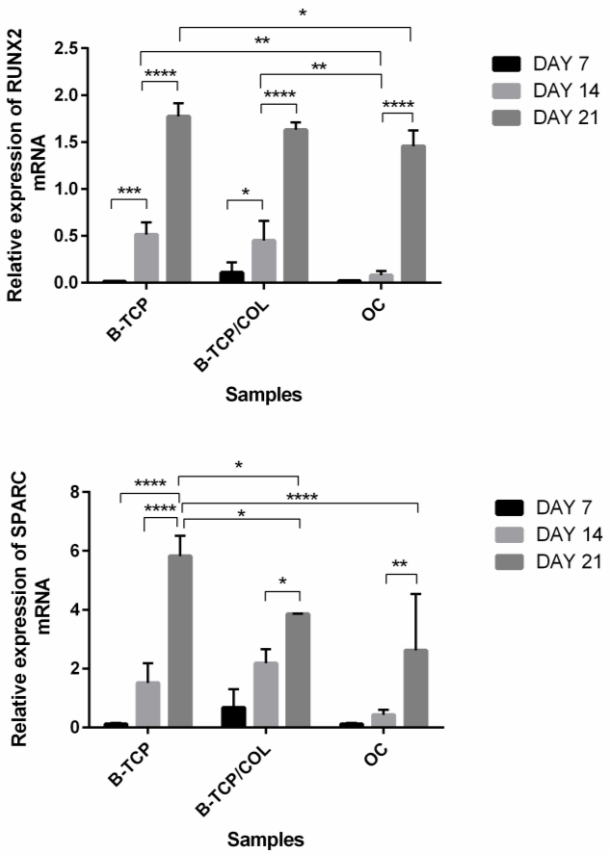

Figure 8. Relative mRNA expressions of RUNX2 and SPARC in cell-seeded B-TCP, B-TCP/Col and Only cell (OC) groups on days 7, 14 and 21. (* P<0.05, ** P<0.01, *** P<0.001, **** P<0.0001).

Discussion and Conclusion

Tissue engineering provides a new approach to the therapy of damaged tissues. The development of biomaterials that can allow mesenchymal stem cell attachment, growth and proliferation are essential for creating tissue engineering scaffolds to support bone regeneration. The ideal biomaterial for bone tissue engineering should be osteoinductive, osteoconductive and biodegradable at the desired rate. Collagen is one of the natural polysaccharides that are biocompatible and biodegradable, the structure of which regenerates natural bone tissue glycosaminoglycans (36). In contrast, B-TCP which is a bioactive ceramic has a low capacity for biodegradation (4). Therefore, we used 3D porous scaffolds containing B-TCP and B-TCP/Col for use as bone repair substitutes. In this study, Ad-MSCs were seeded on mentioned biomaterials and their growth and differentiation capacities were compared on these materials.

Flow cytometry analysis characterized canine Ad-MSCs with antibodies CD 29, CD 34, CD 44, CD 73, CD 90, CD 81 and CD 271. The MSCs isolated from the canine adipose tissue expressed the stem cell markers CD 44^+, CD 73^+, CD 81^+ and CD 90^+, whereas the markers CD 34^- and CD 271^- were negative; these results are consistent with others (25, 39, 40, 42, 46). In the general Ad-MSCs characterization criteria, CD 29 is considered positive (18, 29, 37, 51). The previous characterization studies of Ad-MSCs revealed that CD 29 was also expressed in humans, horses, cats and dogs (28, 42, 45, 47, 49). However in this study the CD 29 surface antigen was not expressed. Canine Ad-MSCs isolation and characterization studies of Marx et al. (28) and Screven et al. (43) support the results obtained in this study. It is thought that this difference may arise from the differences between animal species and further research is needed for species-specific characterization.

MTT assay was performed to specify the number of viable cells on B-TCP and B-TCP/Col on the 1st, 7th, 14th and 21st days after seeding. The number of cells increased the first 7 days and reduced on the 21st day indicating that both biomaterials support the attachment, proliferation and osteogenic differentiation of cells. It is thought that the decrease in the number of cells on the 21st day in both materials and in the OC group is insufficient space for the cells to proliferate. The present findings are supported by previous studies made with different biomaterials containing B-TCP and MSC (11, 34, 50).

Alkaline phosphatase is an enzyme that refers to increased osteoblasts activity for new bone formation and growth. Therefore it is used as an early marker for osteogenic activity. The previous findings studied with cell seeded ceramic scaffolds and collagen containing biomaterials have indicated that ALP expression reaches a maximum level after 14 days in culture and declines up to day 21 (15, 22, 31). According to the present findings, the enzyme activity of B-TCP increased from 1 day to 14 days and it decreased on the 21st day. In B-TCP/Col structured biomaterial, ALP activity increased in the first week and decreased after the second week. It is thought that these findings can be the indicator of differentiation from osteoblasts to osteocytes (35).

To compare the qualitative assessment of mineralization of the extracellular matrix, Von Kossa staining was performed on the 7th, 14th and 21st days for both materials. It was determined that calcium deposits increased from 7 days to 21 days in the biomaterial containing B-TCP. However, since the degradation ability of the B-TCP/Col is high, the mineral dissolved after the 7th day and the mentioned calcium deposits could not be shown on the material. The high degradation ability of this material is due to collagen, a natural biomaterial it contains. This high degradation property of the material is a disadvantage for bone tissue engineering (39). Donzelli et al. (15) conducted an in vitro degradation study using MSC derived from rat bone marrow and collagen material. They predicted in vivo studies that collagen could be dissolved before bone tissue healed. Collagen was used in our study in combination with B-TCP to overcome this disadvantage of collagen. Kato et al. (23) demonstrated that the osteoconductivity and biodegradation property of B-TCP/Col composites are superior to B-TCP. However, it was seen that this combination could not sufficiently reduce the degradation properties of the material. Therefore, it is thought that degradation studies about B-TCP/Col should maintain.

RUNX2 also known as core-binding factor subunit alpha-1 is a protein that is an essential transcription factor for osteoblast differentiation (24). SPARC also called osteonectin is a glycoprotein initiating mineralization and promoting mineral crystal formation during bone formation (38). Because they are related to osteoblast activity, both genes are considered an early marker of bone regeneration. However, these genes have a significant role in the early and late phase of osteogenesis (14, 24). In studies with ceramic biomaterials and MSCs, expressions of the SPARC and RUNX2 genes were measured and it was shown that the expressions peak first days and then decrease (2, 50). In this study, Real-time PCR analysis was performed to determine osteogenic markers of RUNX2 and SPARC on days 7, 14 and 21 for materials and the OC control group. Expression of both genes increased in both biomaterials and control groups from 7th day to 21st day. The higher expressions of the RUNX2 and SPARC genes compared to the control group indicated that these materials have the osteoinductive capacity.

In the study, canine Ad-MSCs were propagated and differentiated to the bone on biomaterials containing B-

TCP and B-TCP/Col. The proliferation and osteogenic differentiation capacities of Canine Ad-MSCs were compared in two different biomaterials. Cell adhesion, proliferation and differentiation capacities were tested by performing MTT, Alkaline Phosphatase, Von Kossa and RT-PCR analysis on biomaterials containing B-TCP and B-TCP/Col. It was concluded that both materials were successful in terms of cell attachment, proliferation and osteogenic differentiation. When comparing the biomaterials with each other, there was no significant difference in cell proliferation assay. However, the ALP activity of the B-TCP/Col containing material was significantly higher than the B-TCP. Although there were no significant differences in RUNX2, the SPARC gene expression of the B-TCP material on day 14 was higher than the other material. The results of Von Kossa staining showed that B-TCP/Col has above the desired level degradation capacity. The very high biodegradability of the B-TCP/Col combination is thought to make it possible to work in cartilage tissue engineering rather than bone tissue engineering. More research is needed to increase the absorption rate, mechanical properties and chemical stability of materials prepared in combination with collagen in the body.

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Conflict of Interest

The authors declare no financial or personal conflicts of interest.

Author Contributions

ÖÖÇ and AÖ conceived and planned the experiments. ÖÖÇ carried out the experiments. ÖÖÇ contributed to sample preparation. ÖÖÇ and AÖ contributed to the interpretation of the results. ÖÖÇ took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The data supporting this study's findings are available from the corresponding author upon reasonable request.

Ethical Statement

This study was approved by Ankara University Animal Experiments Local Ethics Committee (2017-5-37).

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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