Vertical Bone Augmentation Using Bone Marrow–Derived Stem Cells: An *In Vivo* Study in the Rabbit Calvaria

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Purpose: *To evaluate the bone regeneration capacity of bone marrow–derived stem cells (BMSCs) in vertical guided augmentation of bone tissue.*

Material and Methods: *The calvaria of 20 rabbits were vertically augmented with autogenous bone graft (ABG); collagen/beta-tricalcium phosphate (β-TCP) linked scaffold transplanted with 15 × 10⁴ BMSCs; or scaffold alone (control). The augmentation materials were covered with stainless steel domes. BMSCs were isolated with Ficoll-Paque technique and applied directly without* in vitro *expansion. The newly formed bone was evaluated using radiodensitometric, histomorphometric, histological, and micro computed tomographic (micro-CT) analyses after a 12-week healing period. The data excluding micro-CT assessments were compared statistically.*

Results: *Radiodensitometric and bone volume parameters demonstrated increased bone formation in both BMSC group and ABG group compared with control group ($P < 0.01$), but difference between the BMSC and ABG groups was not significant ($P > 0.05$). The mean histological scores for the BMSC, ABG, and control groups were 7.44 ± 1.03, 8.44 ± 0.81, and 6.00 ± 1.10, respectively, indicating significant difference among the groups ($P < 0.05$).*

Conclusion: *BMSCs delivered with a collagen/β-TCP linked scaffold can provide improved new bone formation that is comparable with autogenous bone block graft through vertical guided bone regeneration technique. (Implant Dent 2015;0:1–9)*

Key Words: stem cell, vertical bone augmentation, bone regeneration, rabbit, calvarium

Augmentation of the edentulous alveolar ridges in the vertical direction is considered as one of the most challenging surgical interventions in implant dentistry.[1] Current techniques used for vertical bone augmentation are usually associated with a high complication rate and inconsistent results.[2] It has been claimed that this low success rate is mainly because of inadequate supply of the osteoprogenitor cells and limited blood supply.[3] Thus, among all the various graft materials, autogenous bone grafts (ABGs) still remain as the gold standard graft material, despite their significant disadvantages for example, need of a donor site.[4] Alternative graft materials and techniques that could replicate osteogenic features of ABGs have been studied in many animal and clinical studies.

Bone marrow–derived stem cells (BMSCs) are progenitor cells of the skeletal tissue components such as bone, cartilage, and the hematopoiesis-supporting stroma. They have the ability to differentiate into osteogenic, neural, and myogenic cells.[5,6] Mesenchymal stem cells (MSCs), which are the main stromal cells residing in the bone marrow, may contribute to bone formation directly by differentiating into osteoblasts or indirectly by releasing growth factors, proteins, and cytokines that take part in the bone regeneration process.[3] There are 3 minimum criteria to define the MSC. First, the cells must be plastic adherent, when maintained in standard conditions; second, expressions CD105, CD73, and CD90 surface molecules must be confirmed; and third, the MSCs must differentiate to adipocytes, osteoblasts, and chondroblasts *in vitro*.[7] The CD34

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expression confirms hematopoietic stem cells, which are progenitors of endothelial and hematopoietic cells. Among the adult progenitor/stem cells, the hematopoietic system has traditionally been considered at the top of the hierarchic system and the $CD34^+$ cells have been shown to induce osteogenesis.[8]

Because of their osteogenic differentiation capacity, the number of studies that evaluate the success of MSCs in bone augmentation procedures has increased rapidly. Promising results have been demonstrated using MSCs for various augmentation procedures such as sinus floor elevation,[9] horizontal and vertical bone augmentation,[10] guided bone regeneration,[11] socket preservation after tooth extraction,[12] and repair of the alveolar cleft defects.[13]

The aim of this study was to evaluate the success of guided bone regeneration using autogenous BMSCs in vertical bone augmentation. A rabbit calvarium model was used and the outcomes have been compared with the autogenous bone block grafts.

MATERIAL AND METHODS

The study was reviewed and approved by the Ethical Review Committee of Çukurova University Medical Scientific Research Center. A total of 20 skeletally mature New Zealand rabbits weighing 2.3 to 3.7 kg (mean, 2.9 kg) were used. Each rabbit underwent the same study protocol and bone augmentation method. Three different augmentation materials were applied in the calvarial bone of each rabbit by vertical guided bone regeneration technique.

Bone Marrow Aspiration and Preparation and Characterization of BMSCs

Initially, bone marrow aspirations were made from the iliac bones of the rabbits. The rabbits were anesthetized using a combination of intramuscular 35 mg/kg ketamine (Ketasol; Richterpharma, Wels, Austria) and 3 mg/kg xylazine (Xylazin; Bioveta, Ankara, Turkey). The posterior iliac crest region was shaved and prepared with 10% povidone-iodine solution. Bone marrow aspiration was performed using a 16-gauge bone marrow aspiration needle that was coated with 5 IU heparin/mL. Subsequently, 4 mL of bone marrow was collected.

The aspirate was diluted with the same amount of phosphate-buffered saline (PBS). Mononuclear fractions were isolated by density-gradient centrifugation at 400*g* for 30 minutes at room temperature using Ficoll-Paque solution (d = 1.077 g/mL; GE healthcare, Piscataway, NJ). The opalescent layer present between the Ficoll and the plasma was transferred to another test tube, diluted with PBS until a total volume of 10 mL was reached, and then centrifuged at 400*g* in 18°C for 10 minutes. Next, the supernatant was discarded and the cell sediment at the bottom of the tube, where there is a larger number of nucleated cells, was obtained. The material was further diluted with 1 mL of PBS to be characterized and later to be installed onto the scaffolds. A total of 10 μL sample was collected from the material for cell count and cell viability test. Cell viability was quantified with trypan blue staining by standard methods.[14] The cell count was determined with a blood count device (Cell DYN 1800; Abbot Laboratories, Chicago, IL).

Immunohistochemical Analysis

Immunophenotyping was performed on the paraffin-embedded tissue blocks of bone marrow clots from 4 animals. One section was prepared from each animal and used for the analysis. The 4- to 5-micron thick sections were prepared and immunohistochemical analysis was performed using standardized laboratory protocol for formalin-fixed paraffin-embedded tissue sections on the Ventana, BenchMark XT automated platform (Ventana, Tuscon, AZ) using the basic AEC Detection Kit (Ventana 52660441-760-020). The antibody used was mouse monoclonal antibody against CD34 (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom). Control tissue was tonsil. The slides obtained after the staining were evaluated using light microscope under ×400 magnification. The number of positively stained bone marrow nucleated cells was counted on 10 high-power fields of each slide by a senior pathologist excluding the cortical and trabecular bone, periosteal connective tissue, hemorrhagic areas, and fat tissue occupied areas. The ratio of positively stained nucleated cells to all included cells was calculated for each field. The mean proportion of the $CD34^+$ cells was calculated for each section. No statistical analysis was performed because of limited sample size.

Scaffold

The scaffold used in this study was a highly porous strip for defect filling, and it was prepared from a collagen slurry and beta-tricalcium phosphate (β-TCP) mixture (SupraFlex; BMT Calsis Co., Ankara, Turkey). The production of the material was conducted by freeze-drying and dehydrothermal (DHT) processing. Freeze-drying was performed at −80°C for 24 hours, whereas DHT processing was conducted for 5 days (Monograph; BMT Calsis Co.). The graft material was sterilized by gamma-irradiation (25 kGy). Micro-computerized tomography revealed a porosity of around 65.63% (with an open porosity percentage of 65.54%). The average pore size of the scaffolds calculated from SEM analysis was around 35 μm. The manufacturer designed blocks of 4 × 25 × 100 mm for this study.

Bone Augmentation Surgery

On the same day of collection and isolation of the BMSCs, bone augmentation procedure was performed. After the general anesthesia that had been maintained with intramuscular 35 mg/kg ketamine (Ketasol; Richterpharma) and 3 mg/kg xylazine (Xylazinbo; Bioveta), the animal was secured in a supine position. The forehead region was shaved and draped under aseptic conditions. Articaine with 1:200,000 epinephrine (Ultracain DS; Hoechst Marion Roussel, İstanbul, Turkey) was administrated supraperiosteally to provide anesthesia and hemostasis. A linear, sharp incision was made toward the bone at the middle of the forehead. The mucoperiosteum was reflected and the bone was exposed. Under copious saline irrigation, 3 circular 0.5-mm-deep slits were prepared in the bone on each side of the midline using a trephine bur with an inner diameter of 8 mm. Using the same trephine drill, a bicortical bone block was harvested for using it as an ABG. The bone block had a cylindrical shape with 8 mm diameter and 1.5 mm height. For obtaining an even thickness of 1.5 mm, the bone blocks

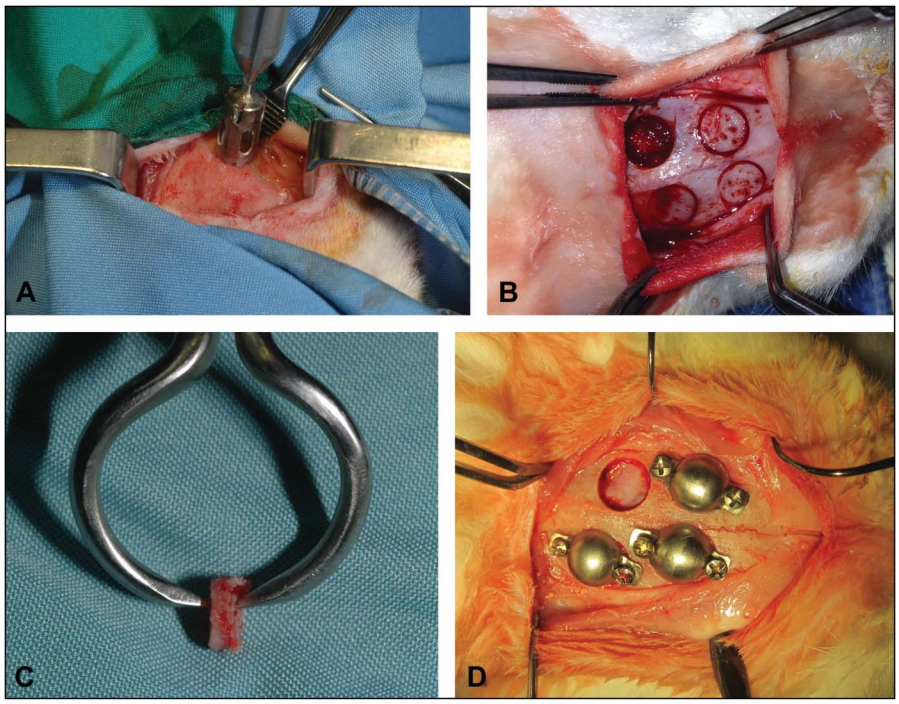

Fig. 1. A–D, Intraoperative photographs showing phases of the guided vertical bone augmentation. **A**, Trephine bur was used to prepare slits on calvarial bone and obtain a block graft. **B**, Three circular slits were prepared on each side of the midline. The inner area of each slit was decorticated with a round bur. **C**, The thickness of bone block obtained from the calvarial bone was measured with a caliper. **D**, The domes, 1 grafted with autogenous bone, 1 with scaffold alone, and 1 scaffold + BMSC were positioned on the slits and tightly fixed to the calvarial bone by means of 2 miniscrews.

were trimmed using a low-speed round carbide bur and measured with a thickness gauge. The recipient bony surfaces were decorticated with a round bur to induce bleeding from the marrow spaces within the circles. The scaffolds were cut and shaped into the same dimensions as the autogenous block using the same trephine bur. Preformed stainless steel domes were used for guided bone regeneration. The domes had the same inner diameter as the trephine bur that was used in the preparation of the slits. The height of the domes was 5 mm. They had 2 slots on their margins for fixation to the recipient surface by means of miniscrews. Three different augmentation materials were placed on the recipient surfaces of the calvaria; a scaffold loaded with BMSCs, an autogenous bone block graft, and a scaffold alone. The number of the cells transplanted onto the scaffolds was 15×10^4. The domes were placed above the graft materials and then fixed with two 4-mm miniscrews (Synthes, Oberdorf, Switzerland) on the calvaria. Figure 1, A–D display the details of the bone augmentation surgery. The flap was positioned and sutured primarily with resorbable sutures.

The rabbits were housed in separate cages postoperatively and were fed *ad libitum*. Analgesics (Tramadol 1 mg/kg) and antibiotics (Cefazolin 25 mg/kg) were administered intramuscularly preoperatively and twice per day over 4 postoperative days. Food and water intake and weights of the subjects were monitored and recorded daily. At the end of the 12-week healing period, the animals were killed with intraperitoneal injection of 100 mg/kg thiopental (Pental; IE Ulagay, Istanbul, Turkey). The calvaria were harvested and prepared for histological and radiographical evaluations.

Table 1. Histological Scoring System Used for Evaluation of the Newly Formed Bone

Score (A): characterization—inflammation
Intense presence of inflammatory cells
Low-moderate presence of inflammatory cells
Score (B): characterization—formation and quality of bone tissue
Defect filled with connective tissue containing blood capillaries fibroblasts, macrophages, and newly formed collagen fibers
Dense connective tissue suggesting bone tissue differentiation with presence of a large number of cells andorganizing fibers
New bone formation in which the connective tissue is differentiating to form a bone matrix or osteon
Presence of bone tissue
Score (C): characterization—collagen maturation
No evidence of bone union, filling of the surgical wound with connective tissue—isotropy (absence of birefringence)
Osteon (formation of connective tissue in bone with osteoprogenitor and osteogenic cells)—low anisotropy
Isolate immature bone spicules—moderate anisotropy
Compact bone formation—intense anisotropy (total polarization)

The sum of scores A, B, and C is considered for the comparisons.
Modified from Pretel et al, 2007. Adaptations are themselves works protected by copyright. So in order to publish this adaptation, authorization must be obtained both from the owner of the copyright in the original work and from the owner of copyright in the translation or adaptation.

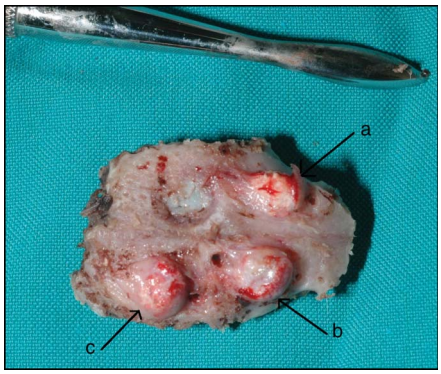

Fig. 2. Photograph showing a specimen harvested 12 weeks after the augmentation and after the removal of the domes. Note the presence of newly formed tissue in each recipient site; Arrows showing (**A**) scaffold only, (**B**) autogenous graft, (**C**) scaffold loaded with BMSC.

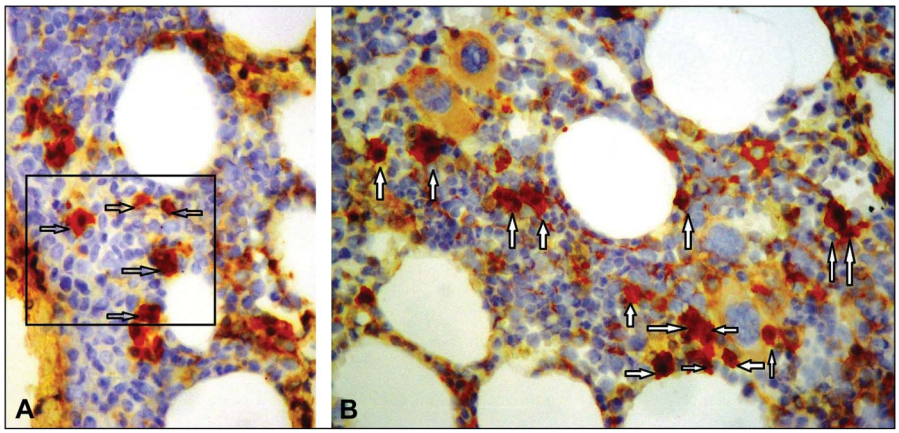

Fig. 3. Paraffin immunohistochemical analysis of bone marrow clot used for cell phenotyping. Photographs of light microscopy images under magnifications ×400 (**A**) and ×200 (**B**) show positive reaction (arrows) to monoclonal antibody against CD34.

Radiographical Assessment

The domes were removed and each augmented region was sawed out separately before the radiographic assessments. Digital direct radiograms of the specimens were obtained from the lateral aspect with an aluminum step wedge attached to the sensor of the digital radiography device (RVG; Trophy Radiologie, Vincennes, France). The aluminum step wedge consisted of 14 steps with a thickness ranging from 0.5 to 7 mm. The same aluminum step wedge was used for all radiograms. The x-ray unit (Philips Densomat, Eindhoven, the Netherlands) was set at 65 kvp, 300 mA, and 0.12 milliseconds. The x-ray cone was directed perpendicularly to the sensor from a distance of 20 cm. The digital images were analyzed using an image analyzing software (ImageJ, Wayne Rasband; National Institutes of Health, Bethesda, MD). The gray level of each step of the aluminum step wedge was measured and used for calibration of the software. Aluminum equivalent bone density at the newly formed bone and the pristine bone was measured. The results were expressed in millimeters of aluminum. The ratio of the bone density of the newly formed bone to the pristine bone was calculated and used for statistical analysis. After the completion of the radiographic assessments, the samples were fixed in 10% formalin for further histological evaluation.

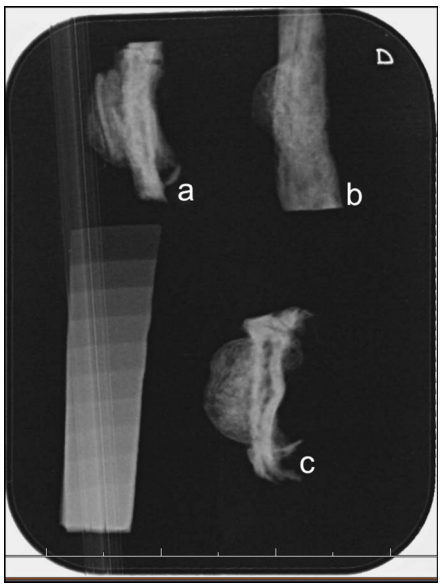

Fig. 4. Direct digital radiograph of a specimen. An aluminum step wedge was used for the calibrations in the densitometric analyses. The recipient sites in the image; (**A**) autogenous graft, (**B**) scaffold only, (**C**) scaffold loaded with BMSC.

Histology and Histomorphometry

Each specimen was cut into 2 equal portions: one piece underwent undecalcified histological sectioning, whereas the other piece underwent decalcified histological sectioning. For the preparation of the undecalcified sections, the specimens were fixed in 10% buffered formalin, dehydrated in increasing concentrations of ethanol, from 70% to 99% during 12 days, and embedded in methylmethacrylate (Technovit 7200; Haerus Kulzer GmbH, Wehrheiam/Ts, Germany). Afterward, the 50-μm-thick sagittal sections were prepared using an electric diamond saw and grinding system (Exakt Vertriebs; Exakt, Norderstedt, Germany) and stained with toluidine blue. Digital images of the sections were obtained by a digital camera (Camedia C4040; Olympus Corp., Tokyo, Japan) attached to an Olympus BX50 microscope (Olympus Corp.) at a magnification rate of ×10. The images were transferred to a personal computer, and measurements were made by histomorphometry software (TAS V 1.2.9, Steve Paxton; University of Leeds, Leeds, West Yorkshire, United Kingdom). The parameters used for the histomorphometric evaluations were bone volume, trabecular separation, and trabecular thickness.

For the preparation of the decalcified histological sections, the specimens were fixed in 10% formalin and decalcified with 10% nitric acid. After embedding in paraffin, 5-μm-thick longitudinal sections were prepared and stained with hematoxylin-eosin or Masson trichrome. Two slides from each specimen, 1 stained with hematoxylin-eosin and the other stained with trichrome, were examined. A single-masked, senior, pathologist (G.G.) scored the stained sections regarding the mineralization amount of the newly formed bone, using a grading system developed by Pretel et al[15] and modified by our group. According to this scoring method, the pathologist gives a score between 1 and 10 regarding the ossification level of the newly formed bone. The descriptions of the histological scoring used in this study are given in Table 1. The mean scores of each group were calculated and statistically analyzed.

Micro Computed Tomographic Evaluation

Micro computed tomographic (micro-CT) images of 2 specimens from each group were obtained. The specimens, containing the newly formed bone and pristine bone with 0.5 cm surrounding bone, were examined with a micro-CT system (SkyScan 1172; Bruker, Kontich, Belgium). The image matrix used was 1024 × 1024 pixels. The images were qualitatively evaluated regarding ossification status of the newly formed tissue at the augmentation sites.

Table 2. Comparisons of Bone Densities Among the Groups

	Density of Newly Formed Bone (mm Al)			Density of Pristine Bone (mm Al)			Density Ratio; Newly Formed Bone/Pristine Bone (%)		
Group (n)	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD
BMSC (18)	0.81	1.07	0.94 ± 0.07	0.97	1.51	1.21 ± 0.18	70.0	93.0	79.1 ± 7.7
ABG (18)	0.75	1.17	0.93 ± 0.10	1.10	1.42	1.25 ± 0.11	65.0	99.0	79.0 ± 10.5
Control (18)	0.54	0.90	0.70 ± 0.11*	0.97	1.49	1.15 ± 0.16	43.0	70.0	59.5 ± 7.5*

Aluminum (Al) equivalent bone density at the newly formed bone and the pristine bone was measured using an aluminum step wedge. BMSC group and ABG group had significantly higher bone density and bone density ratios at the newly formed bone region than the control group.
*Control group had significantly lower bone density and bone density ratio than BMSC and ABG groups ($P < 0.01$, Mann-Whitney *U* Test and Kruskal-Wallis Analysis).

Table 3. Comparisons of Histomorphometric Parameters Obtained From Undecalcified Histological Sections

	Bone Volume (%)			Trabecular Separation (μm)			Trabecular Thickness (μm)		
Group (N)	Min	Max	Mean ± SD	Min	Max	Mean ± SD	Min	Max	Mean ± SD
BMSC (18)	71.06	95.69	83.80 ± 6.23	6.79	46.16	13.52 ± 8.48	8.1	55.3	16.2 ± 10.1
ABG (18)	71.24	92.48	84.09 ± 6.41	4.42	31.30	15.24 ± 7.34	5.3	31.3	15.2 ± 7.3
Control (18)	60.03	84.70	77.01 ± 6.1*	6.60	19.14	12.73 ± 3.42	7.9	22.9	15.2 ± 4.6

Among the groups, the bone volume parameter was significantly lower in the control group than the BMSC and ABG groups. The differences in the other parameters were not statistically significant among the groups.
*Control group had significantly lower bone density and bone density ratio than BMSC and ABG groups ($P < 0.01$, Mann-Whitney *U* Test and Kruskal-Wallis Analysis).

Statistical Analysis

The data obtained from the radiological assessments, histomorphometric analyses, and histological scorings were statistically analyzed using Kruskal-Wallis Analysis and Mann-Whitney *U* tests. The significance levels were set at $P = 0.05$. The data were analyzed in SPSS 15.0 software for Windows (SPSS, Chicago, IL).

RESULTS

Of 20 rabbits, 2 have died (one in 3rd and the other in the 5th week) because of systemic conditions manifesting with the loss of appetite and dehydration. The remaining 18 animals were considered for evaluation. Gross evaluations of the specimens revealed the presence of a newly formed bone tissue under the domes in all samples (Fig. 2).

Immunophenotyping and Cell Viability

Immunohistochemical analysis demonstrated positive reaction to CD34 in all samples (Fig. 3, A and B). The mean proportion of $CD34^+$ cells was 7.6 ± 1.9%. The mean of cell viability was found to be 90 ± 5%.

Radiographic Analysis

The aluminum equivalent of bone densities was 0.94 ± 0.07 mm for the BMSC group, 0.93 ± 0.10 for the ABG group, and 0.70 ± 0.11 for the control (scaffold only) group. Bone density ratio values of newly formed bone/pristine bone were 79.13 ± 7.77, 79.06 ± 10.54, and 59.56 ± 7.58 for the BMSC group, ABG, and control groups, respectively (Fig. 4). According to the intergroup comparisons performed with Mann-Whitney *U* tests, both BMSC group and ABG group had significantly higher bone density and bone density ratio at the newly formed bone region than the control group ($P < 0.01$). There was no significant difference between the BMSC and ABG groups regarding bone density and bone density ratio parameters. For comparisons of the mean values among the 3

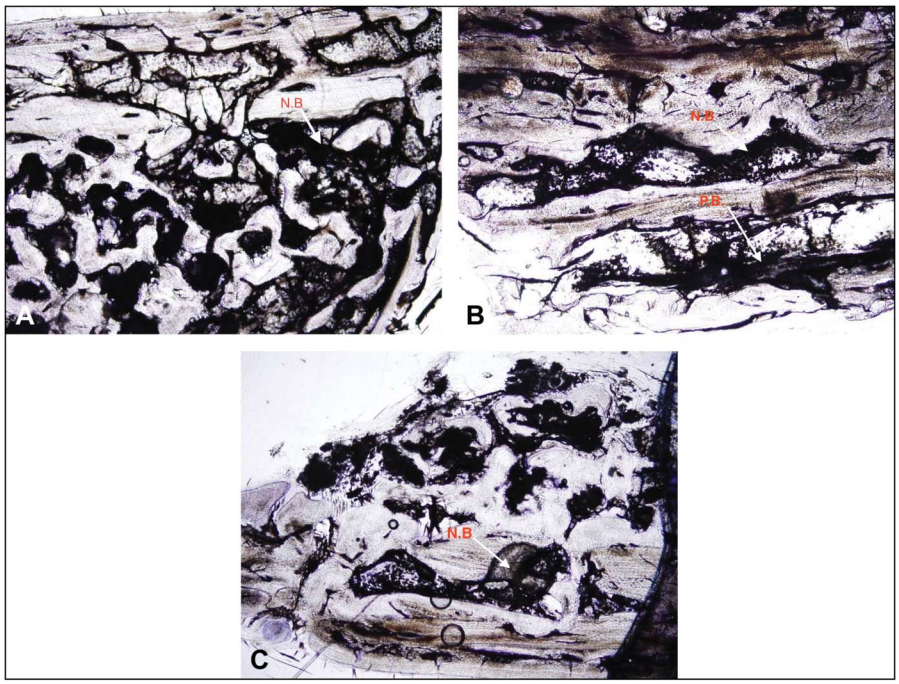

Fig. 5. **A**–**C**, Undecalcified histological slides obtained from the specimens (Magnification rate ×4, stained with toluidine blue). **A**, Sample from BMSC group. **B**, Sample from ABG group. **C**, Sample from scaffold only group. N.B. indicates New bone; P.B., pristine bone.

groups, the Kruskal-Wallis analysis was applied. After the Bonferroni correction, the significance level was considered as $P < 0.0167$. The results of the Kruskal-Wallis analysis showed that both bone density and bone density ratio parameters were significantly lower in the control group than the BMSC and ABG group (Table 2).

Histomorphometric Analyses

Data from the histomorphometric analyses is shown in Table 3. The mean bone volume values for the BMSC, ABG, and control groups were 83.80 ± 6.23%, 84.09 ± 6.41%, and 77.01 ± 6.15%, respectively. This parameter was significantly lower ($P < 0.01$) in the control group than the BMSC and ABG groups, both for intergroup comparisons with the Mann-Whitney *U* test and the 3-group comparison with the Kruskal-Wallis Analysis. The differences in the other parameters were not statistically significant among the groups ($P > 0.05$) (Table 2) (Fig. 5, A–C).

Histological Evaluation

Histological observations revealed mild foreign body reaction in 2 samples (one from the control and another from the BMSC group). There was no sign of infection in any of the specimens. There was a newly formed bone tissue in all samples. The mean mineralization scores for the BMSC, ABG, and control groups were 7.44 ± 1.03, 8.44 ± 0.81, and 6.00 ± 1.10, respectively. Data from the histological evaluation is shown in Table 4. The differences were statistically significant between each group according to the Mann-Whitney *U* test ($P < 0.01$). The Kruskal-Wallis analysis also showed that the differences among the 3 groups were significant ($P < 0.001$) (Fig. 6, A–C).

Table 4. Comparisons of the Mean Histological Scores Among the Groups

Group (n)	Min	Max	Mean ± SD
BMSC (18)	5.00	8.00	7.44 ± 1.03*
ABG (18)	8.00	10.00	8.44 ± 0.81*
Control (18)	4.00	7.00	6.00 ± 1.10*

The mean mineralization score in the BMSC group was significantly higher than the ABG and significantly higher in ABG than the control group.
*All differences were significant ($P < 0.01$) according intergroup comparisons between each group (Mann-Whitney *U* Test) and 3-group comparison (Kruskal-Wallis Analysis).

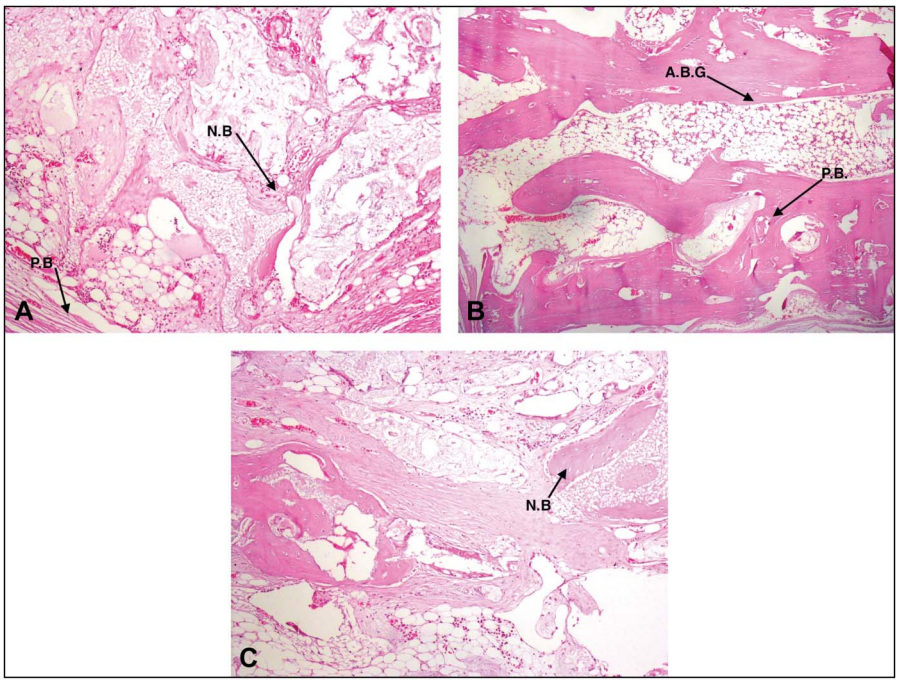

Fig. 6. A–C, Decalcified histological slides obtained from the specimens (Magnification rate ×4, stained with hematoxylin and eosin). **A**, Sample from BMSC group. **B**, Sample from ABG group. **C**, Sample from scaffold only group. A.B.G. indicates autogenous bone graft; N.B., new bone; P.B., pristine bone.

Micro-CT Assessments

The micro-CT images obtained from 2 rabbits revealed the presence of a newly formed bone in all samples. The autogenous blocks grafts were well integrated with the recipient site without any loss in their volumes. The specimens from the BMSC group had well-organized and relatively dense bone tissue, which was formed up to the peripheral edges of the augmentation sites. The amount and the density of the newly formed bone were apparently lower in the samples from the control group than that of in the other groups. The proximal parts of the augmentation area, which are in close contact with the pristine bone, had more bone formation compared with the distal parts (Fig. 7, A and B).

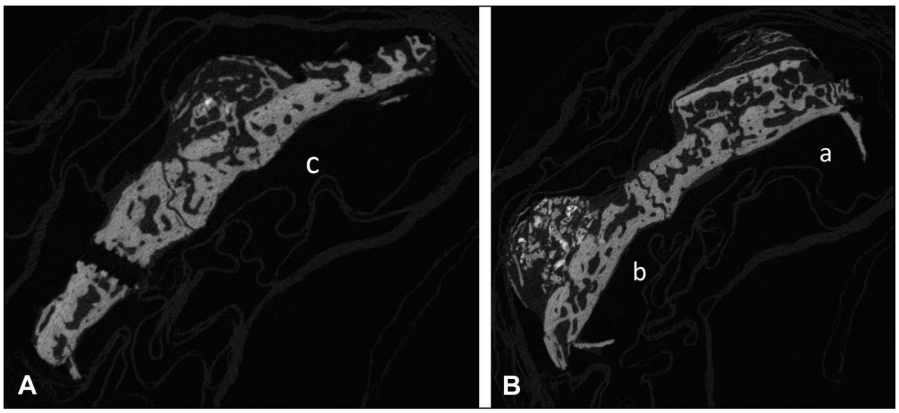

Fig. 7. A and **B**, Micro-CT images of 3 recipient sites from a specimen. Note the presence of various amounts of newly formed bone tissue in all sites. The recipients sites in the images are; (**A**) ABG, (**B**) scaffold loaded with BMSC, (**C**) scaffold alone. The defect due to bicortical bone block is apparent in Figure 7, A.

Discussion

This study demonstrated that vertical guided bone regeneration with the aid of BMSC is a feasible alternative to autogenous bone block grafting method. The current techniques used in the clinical practice for vertical bone regeneration include guided bone regeneration with synthetic or xenogenic grafts, distraction osteogenesis, interpositional grafting, and onlay ABGs. These techniques are associated with significant drawbacks such as low success rate, unpredictable results, donor site requirement, technique sensitivity, and unknown long-term outcomes. Among the current methods, onlay grafting with autogenous bone blocks is the most frequently used technique, thus considered the gold standard for vertical augmentation of alveolar bones.[16–19]

Utilization of MSCs in alveolar bone augmentation procedures has been proposed to become a significant alternative to autogenous bone blocks, as bone harvesting is avoided with their application. MSCs are undifferentiated cells that have the capacity to differentiate into osteoblasts; thus, the graft material that is applied concomitant with MSCs would have osteogenic capacity as it occurs with ABGs. It is possible to obtain MSCs from several sources including bone marrow, adipose tissue, salivary glands, or dental tissues. They can be applied directly after isolating the cells from the tissue to the recipient site as we did in this study, or they can be applied after *in vitro* expansion and differentiation into osteoblasts. Furthermore with contemporary tissue engineering methods, it is possible to transplant the cells to 3D scaffolds, which are prepared in accordance with the shape of the alveolar defect. Although, using an *in vitro* expansion method, it is possible to obtain differentiated MSCs in desired amounts, it is associated with the risk of contamination, increased cost, and arduous laboratory process. Therefore, avoiding *in vitro* processing of the cells would provide significant advantages such as shortened treatment time, reduced cost, reduced workload, and low risk of contamination. If the surgeon prefers to apply MSCs without *in vitro* expansion, the most commonly used source of MSCs is the bone marrow as it contains high amount of MSCs, which are well-characterized cells among the clinically available stem cells with proven osteogenic ability.[20] In this study, we applied the BMSCs with the Ficoll-Paque isolation and direct application method.

There are various clinical and animal studies, which evaluated the effectiveness of MSCs in onlay bone augmentation procedures. The study by Smiler et al[21] was one of the first clinical studies in which MSCs were used in combination with xenogeneic graft or alloplastic grafts for onlay bone augmentation. The authors demonstrated histological evidence that stem cells aspirated from bone marrow and transplanted into biocompatible scaffolds could successfully regenerate bone in 5 patients. Another clinical study by Filho Cerruti et al[22] evaluated the success of bone marrow–derived mononuclear cells in combination with platelet-rich plasma and allogenic bone graft material for onlay bone augmentation in 32 patients. Results of the study suggested that the graft materials were well integrated with the cortical bone. Additional clinical studies reported a positive contribution of MSCs to onlay bone augmentation procedures.[4,23] There are also significant number of studies, in which MSCs were obtained from other sources such as adipose tissue or periosteum, applied with sophisticated tissue engineering methods, and used successfully for different types of augmentation procedures such as sinus floor augmentation, socket preservation, and simultaneous guided bone regeneration procedures.[19,24,25]

Animal studies, in which MSCs have been used for onlay bone augmentation, usually report positive outcomes. A study by Zigdon-Giladi et al[3] evaluated bone regeneration capacity of bone marrow–derived MSCs in the rat calvarium model. The authors applied guided bone regeneration in a similar method used in our study using gold domes. The MSCs were expanded in cell cultures and positively characterized by CD90 and CD44 antibodies. The results of their study suggest that MSCs in combination with guided bone regeneration significantly enhance bone formation. Another study by Pieri et al[17] evaluated the effects of different doses of adipose–derived MSCs on vertical bone formation in the rabbit calvarium using titanium domes. The authors concluded that the delivery of adipose–derived MSCs effectively increases vertical bone regeneration and implant osseointegration. In a recent study, Khojasteh et al[11] showed that MSCs undergone *in vitro* expansion in cell culture significantly accelerates vertical bone formation in a dog mandible model. The main difference between our study and the previous studies is that we applied the cells directly to the recipient site without *in vitro* expansion. It was important to demonstrate that stem cells that had been isolated and applied without sophisticated laboratory processing can provide successful new bone formation that is comparable with the autogenous block graft, which was one of the study groups.

One important aspect affecting the success of the bone regeneration procedure with the aid of MSCs is the scaffold used to carry the cells to the recipient site. Various types of scaffolds have been used in the previous studies.[11,26] The scaffold used in this study was a semiflexible, biodegradable, composite strip made by a mixture of collagen slurry and β-TCP. Highly porous and flexible structure of the scaffold provided better environment for the cells to be seeded, easier adaptation to the recipient bed, and enhanced ingrowth of the vessels to the newly formed bone. These features are consistent with the characteristics of the ideal scaffold.[27] However, because of its lower biomechanical strength, this scaffold needs to be covered with a stiff material such as titanium mesh or titanium-reinforced membrane to overcome the external forces. Histologically, the scaffold showed good biocompatibility, proved to be a feasible carrier of the cells, and demonstrated osteoconductivity allowing new bone formation.

We assessed the mineralization amount of the newly formed bone quantitatively with radiodensitometric analysis and histomorphometric analysis in this study. The densitometric analysis was performed with digital radiograms, which were calibrated with

the same aluminum step wedge. According to the radiodensitometric results, the amount of the ossification in the BMSC group and the ABG group was equal and approximately 20% higher than the control group, which consisted of unloaded scaffolds. According to the histomorphometric analyses of the undecalcified histological sections, bone volume, which indicates the ratio of the mineralized ossified tissue to the total tissue, was significantly higher in both the BMSC group and the ABG group than the control group. Consistent with the radiodensitometric results, bone volume was not different between the BMSC and ABG groups. Other 2 histomorphometric parameters, namely trabecular thickness and trabecular separation, did not differ among the 3 groups. These parameters mainly evaluate the maturity of the newly formed bone at the augmented region. Therefore, it may be assumed that although there was increased amount ossified tissue in the BMSC and the ABG groups, the maturity of the ossified tissue that had already formed did not differ among the groups. Histological scorings of the decalcified sections regarding the degree of ossification similarly showed that BMSC and ABG group had higher ossification scores than the control group. However, results of this parameter differ from the other assessments as the ABG group had higher ossification scores than the BMSC group. Because the scoring system used in this study evaluates the formation of the well-structured lamellar bone along with the amount of the ossified tissue, it may be concluded that the samples in the BMCS group had an equal amount of the ossified tissue with the ABG tissue. However, the ossified tissue has not reached the level of the maturity of bone in the ABG group at the end of the 12-week healing period.

One of the drawbacks of this study was that the cells were not confirmed to be MSCs. In this study, characterization of the BMSCs was performed only with 1 surface antibody. The gold standard method for determination of the surface antigen expression is flow cytometry technique. In this study, however, we used immunohistochemistry, which has been widely used as well and provided consisted results with flow cytometry.[28] As it is not possible to define the cells used in this study as MSCs under the contemporary guidelines, we avoided calling the cells MSC. Another drawback of this study was that the number of micro-CT evaluations was limited; thus, no statistical analyses could be performed. Previous studies showed that there is a positive and strong correlation between the histomorphometric findings and micro-CT assessments.[29,30] Although the micro-CT assessments did not allow making statistical comparisons among the groups, it helped us to observe the 3D view the newly formed bone tissue in each group and supported our histological assessments.

CONCLUSIONS

The results of the study revealed that BMSCs isolated with Ficoll-Paque technique and delivered with a collagen and β-TCP based scaffold can provide equal amount of new bone formation with an autogenous bone block graft through a vertical guided bone regeneration technique in the rabbit calvarium. The amount of the ossified tissue was similar in the BMSCs group and ABG group and significantly higher than the control group. However, the maturity of the ossified tissue was still higher in the ABGs than the BMSCs group at the end of the 12-week healing period.

DISCLOSURE

The authors claim to have no financial interest, either directly or indirectly, in the products or information listed in the article.

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APPROVAL

The study was reviewed and approved by the Ethical Review Committee of Çukurova University Medical Scientific Research Center.

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