Vertical Bone Augmentation With Simultaneous Implant Placement Using Particulate Mineralized Bone and Mesenchymal Stem Cells: A Preliminary Study in Rabbit

Arash Khojasteh, DMD, MS
Mohamadreza Baghaban Eslaminejad, PhD
Hamid Nazarian, MSc
Golnaz Morad, DDS
Seyyedeh Ghazaleh Dashti, DDS
Hossein Behnia, DMD, MS
Mark Stevens, DMD, MS

This study aimed to assess vertical bone augmentation with simultaneous implant placement in rabbit tibiae using particulate mineralized bone/fibrin glue/mesenchymal stem cell. Bone marrow was aspirated from tibiae of five 10-week-old New Zealand White male rabbits. Right and left tibiae of each rabbit were prepared, and a 3-mm protruding implant from tibial bone was placed in each side. Particulate allogenic bone/fibrin glue/mesenchymal stem cell combination was placed around test implants and particulate bone graft/fibrin glue around controls. Two months postoperatively, the animals were euthanized, and sections were prepared for histological analysis. The mean amount of vertical bone length was higher in the experimental group than the control group (2.09 mm vs 1.03 mm; \( P < .05 \)). New supracrestal trabecular bone formation was also significantly higher in the test group (28.5 ± 4.5% vs 4.3 ± 1.8%; \( P < .05 \)). Mesenchymal stem cell/particulate allograft/fibrin glue appears to be a promising combination for vertical bone augmentation around simultaneously inserted implants in rabbit tibia.

**Key Words:** mesenchymal stem cell, bone regeneration, dental implant, vertical augmentation

**INTRODUCTION**

The growing science of hard-tissue engineering has introduced various principles and solutions to confront the challenge of bone reconstruction. The process of bone augmentation is mandatory for successful placement and integration of root form dental implants in atrophic jaws. Although different techniques have achieved a predictable amount of horizontal bone gain, vertical augmentation has proven to be a consistent challenge, with often less than desirable results. This is especially evident in the posterior mandible, where the inferior alveolar nerve further accentuates the limited amount of available bone height. Because of this lack of predictability, a large range of reconstructive techniques and materials have been used: onlay bone grafting, guided bone regeneration (GBR), and distraction osteogenesis to list a few. The advantages and disadvantages of each technique impart its applicability to particular circumstances. The gold standard, autogenous bone graft, has several shortcomings, namely, its availability, its occasional poor quality, and the need for a second surgical site.
which is associated with donor-site morbidity. For this reason, autogenous bone is usually combined with allografts, xenografts, or synthetic graft materials to provide more structure and help minimize resorption. However, even these additional variations have not drastically improved predictability. The application of each material also has limited inherent properties and host response. Different studies have shown that adding a membrane, that is, the GBR technique, can enhance the reconstructive effect. Recently, tissue engineering methods have been applied to improve the results of vertical bone augmentation. Investigators have documented the promotional effects of growth factors in bone grafting. Mesenchymal stem cells (MSCs) have also demonstrated the potential to differentiate into osteoblast-like cells, and the efficacy of MSC-seeded scaffolds in bone regenerative procedures has also been verified by assay. The challenge of implementing this new methodology is the ability of a delivery system that provides an equal and viable distribution of cell seeding throughout the scaffolds and/or carriers. Fibrin glue has been used on delivery systems to improve these types of tissue engineering techniques.

Several authors have documented the MSCs/growth factor/scaffold triad to be the best combination in achieving bone regeneration. However, careful application of viable, unaccompanied MSCs through special techniques may obviate the need for additional factors, such as platelet-rich plasma and other more costly growth factors. Besides the importance of the quality and quantity of regenerated bone in atrophic ridges, it is desirable to accomplish the optimal result with fewer surgeries. Thus, the concept of simultaneous implant placement has also been advocated, although most studies have reported no greater predictable results. The aim of this study was to assess vertical bone augmentation with simultaneous implant placement in rabbit tibia using particulate mineralized bone/fibrin glue in combination with MSCs.

Methods and Materials

Animal

In this study five 10-week-old New Zealand White male rabbits, weighing 2.5–3.0 kg were used according to the guideline approved by Ethic Committee of Royan Institute, Tehran, Iran. Each rabbit was housed in an individual aluminum cage and fed a standard commercial rabbit chow. Water and food were available ad libitum. To isolate MSCs, marrow aspirates obtained from each animal tibia were plated and culture expanded through a few successive subcultures. Before transplantation experiments were performed, the isolated cells were differentiated along 3 mesenchymal cell lineages, including bone, cartilage, and adipose, to verify their MSC identity.

Cell culture

Under general anesthesia, 0.5 mL bone marrow was aspirated from the tibia of each rabbit using 19 gauge needles. Bone marrow aspirate was then mixed with 5 mL Dulbecco’s modified Eagle medium (DMEM) containing 15% fetal bovine serum, (FBS), 100 IU/mL penicillin, and 100 IU/mL streptomycin (all from Gibco, Paisley, Germany). Bone marrow cells were washed by centrifugation for 3 minutes at 1200 rpm, after which the supernatant was discarded. The pellet was suspended in 1 mL DMEM and plated in 75 cm² culture flasks at a density of 10⁵ cells/mL. Each culture flask contained 15 mL DMEM with 15% FBS and the aforementioned antibiotics. The cultures were incubated in an atmosphere of 5% CO₂ at 37°C. Three days after culture initiation, the medium was replaced by DMEM supplemented with 50 mg/mL L-ascorbic-2
phosphate, 10 nM dexamethasone, and 10 mM β-glycerol phosphate (all from Sigma, St Louis, Mo).

**Alizarin Red Staining**

Three weeks after inducing differentiation, the cultures were fixed by methanol for 10 minutes and then subjected to alizarin red solution for 2 minutes.

**Adipose Differentiation**

For adipogenesis, DMEM containing 100 nM dexamethasone and 50 mg/mL indomethacin (both Sigma, St Louis) was added to the confluent culture of passage-3 cells.

**Oil Red Staining**

Three weeks after culture initiation, the cells were fixed with 4% formalin at room temperature, washed by 70% ethanol, and stained by oil red solution in 99% isopropanol for 15 minutes.

**Cartilage Differentiation**

To induce cartilage differentiation, a micro mass culture system was used. For this purpose, 2.5×10^5 passage-3 cells were pelleted under 300g for 5 minutes and cultured in DMEM supplemented by 10 ng/mL transforming growth factor-β (Sigma), 10 ng/mL bone morphogenetic protein-6 (Sigma), 50 mg/mL insulin transferrin selenium plus premix (Sigma), 1.25 mg bovine serum albumin (Sigma), and 1% fetal bovine serum.

**Toluidine Blue Staining**

Three weeks after culture initiation, the pellets were removed and subjected to the following: fixing in 10% formalin, dehydrating in ascending concentrations of ethanol, clearing in xylene, embedding in paraffin wax, and sectioning at 5 μm by microtome. The sections were then stained by toluidine blue for 30 seconds at room temperature.

**Reverse transcriptase polymerase chain reaction analysis**

All differentiations were also evaluated by reverse transcriptase polymerase chain reaction (RT-PCR) analysis for specific gene expression in induced cells.

**RT-PCR Procedure**

Total RNA was isolated from differentiated cells using RNX-plus solution (RN7713C, CinnaGen Inc., Tehran, Iran). Before reverse transcriptase (RT), a sample of the isolated RNA was treated with 1 U/mL of RNase-free DNase (EN0521, Fermentas, Leon-Rot, Germany) per 1 mg of RNA to eliminate residual DNA in the presence of 40 U/mL of ribonuclease inhibitor (E00311, Fermentas) and 1×reaction buffer with MgCl₂ for 30 minutes at 37°C. To inactivate the DNase, 1 mL of 25 mM ethylenediaminetetraacetic acid (EDTA) was added and incubated at 65°C for 10 minutes. Standard RT reactions were performed with 2 μg total RNA using oligo(dt) as primer and the RevertAid First Strand complementary DNA (cDNA) Synthesis Kit (K1622, Fermentas) according to the manufacturer’s instructions. To provide a negative control in the subsequent polymerase chain reaction (PCR) for every reaction set, one RNA sample was prepared without RevertAid M-MuLV Reverse Transcriptase (RT-negative reaction). To minimize variations in RT reaction, all RNA samples from a single experimental setup were reverse transcribed simultaneously. Reaction mixtures for PCR included 2 mL cDNA, 1×PCR buffer (AMS, CinnaGen Co, Tehran, Iran), 200 mM deoxynucleotide triphosphate (dNTPs), 0.5 mM of each antisense and sense primer (Table 1), and 1 U Taq DNA polymerase.

**Surgical procedure**

General anesthesia was induced by xylazine (5 mg/kg; Alfasan, Woerden, Holland) and ketamine (50 mg/kg; Alfasan) intramuscular injection. The posterior aspect of both left and right tibiae were shaved and scrubbed with betadine, which was followed by a full-thickness skin incision and gentle flap elevation. Right and left tibiae were prepared to receive endosseous implants with minimal surgical trauma, and initial stability was obtained to carry out the requirements for osseointegration. In every specimen the right side was regarded as the test group; the left side was held as the control. Dental implants (Astra Tech AB, Mölndal, Sweden) of 3 mm diameter and 8 mm length were placed partially, the microthread section (3 mm) protruding from the bone surface (Figure 1a). A combination of particulate mineralized bone (Puros Allograft, Tutogen Medical U.S, Inc, Alachua, Fla), fibrin glue (Tisseel VH, Vienna, Austria), and autogenous MSCs was placed around test implants, and particulate bone graft and fibrin glue were place around the control group. All implants were protected with
nonresorbable expanded polytetrafluoroethylene (ePTFE) membrane (Gore Tex, W.L.Gore and Associates, Flagstaff, Ariz) (Figure 1b). The flaps were repositioned and sutured with vicryl 3.0 and nylon 3.0 (Ethilon, Brussels, Belgium).

The postoperative management was carried out with 10 mg/kg diazepam and 5000 U/kg gentamicin (both Daroupakhsh, Tehran, Iran) injections to control pain and infection, respectively. The sutures were removed after 2 weeks.

**Histological evaluation**

Two months postoperatively, the animals were euthanized with an anesthetic overdose, and the tibiae were removed. The specimens were immediately placed in 10% formalin for 48 hours. They were then dehydrated in a graded series of ethanol baths and embedded in light curing plastic resin (Technovit 7200 VCL, Kultzer & Co, Wehrheim, Germany). The saw machine (Struers A/S, DK-2616, Copenhagen, Denmark) cut through the center of the implants, and 3 sections were arranged sagittally. The sections were polished to a thickness of 15 μm using a grinding system (EXAKT Apparatebau, Norderstedt, Germany) for histological examination under light microscopy. Sections were stained with hematoxylin and eosin. Quantitative and qualitative computer-based analyses were performed on a microphotographed plate of bone ingrowth in contact with implants. The resulting high and low

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*PCR-RT indicates reverse transcriptase polymerase chain reaction; PCR, polymerase chain reaction; PPAR, peroxisome proliferator activated receptor.

**Figure 1.** (a) Partially inserted implant in rabbit tibia, with the microthread parts placed supra-tibial. (b) The application of mesenchymal stem cells/fibrin glue/particulate mineralized bone material around partially inserted implant supported with expanded polytetrafluoroethylene.
magnification images were scanned into a computer and subjected to image analysis using NIH Image 1.57 software (National Institutes of Health, Bethesda, Md). The amount of the new bone length over the original crest of tibia (vertical bone length [VBL]) was measured from the most inferior point of the microthread part of the implants to the most superior level of the newly formed bone. Bone formation (BF%) was also calculated within the aforementioned limits.

**Statistical analysis**

The groups were compared using nonparametric Mann-Whitney U test. The results and statistical analysis were investigated with SPSS 13.0 computer software (SPSS, Chicago, Ill). Statistical analysis was considered significant if \( P < .05 \).

**Results**

**Culture**

Rabbit bone marrow cell cultures were daily observed with invert microscopy. According to these observations, marrow primary culture grew with colony formation (Figure 2a). At the end of day 8 of culturing, a homogenous monolayer of fibroblastic cells was formed (Figure 2b). Cells maintained their fibroblastic appearance during the passages. The cell proliferation rate tended to increase at subcultures so that the confluency was achieved within 6 days.

**Differentiation**

**Bone Differentiation**

Nodule-like structures that developed in some areas were heavily stained red following the alizarin red staining method, indicating the deposition of mineralized matrix at osteoinductive cultures (Figure 3a). Differentiation was further demonstrated by RT-PCR analysis of the osteocytic markers. After a 3-week induction period, osteocalcin, osteopontin, and collagen I messenger RNA were largely produced (Figure 3b).

**Adipose Differentiation**

Nodular structures were not observed at adipogenic culture. Small lipid droplets appeared within the cytoplasm of the cells first on days 3–4 and occupied the whole cells by day 21. The lipid droplets turned red upon oil red O staining (Figure 3c). According to RT-PCR analysis, the peroxisome proliferator activated receptor alpha (PPAR alpha), the PPAR gamma, and lipoprotein lipase genes were expressed at adipogenic cultures (Figure 3d).

**Cartilage Differentiation**

As a result of matrix production and secretion, the size of the pellet tended to increase during the
Figure 3. Differentiation potential of the rabbit bone marrow–derived mesenchymal stem cells. (a,b) Alizarin red staining of osteogenic culture and reverse transcriptase polymerase chain reaction (RT-PCR) analysis of bone-specific genes. (c, d) Adipogenic culture stained with oil red and the RT-PCR analysis for adipogenic gene markers. (e, f) Toluidine blue staining of the sections prepared from chondrogenic cultures and RT-PCR analysis of cartilage-related genes.
chondrogenic culture period. This matrix tended to be metachromatic after the toluidine blue staining (Figure 3e). The RT-PCR analysis indicated that cartilage-specific genes, including aggrecan and collagen II, were largely expressed at chondrogenic cultures (Figure 3f).

**VBL measurement**

The height of newly formed bone was calculated for each implant in the coronal 3-mm part (Table 2). The mean amount of VBL was 2.09 mm in the MSC-treated group compared 1.03 mm in the control group (*P* < .05).

**Histomorphometric measurement**

Eight-week specimens contained a part of dental implant, the cross section of the native tibia, the new regenerated bone around the extra bony part of the implants, and the fibrous sheath just beneath the skin. None of the grafted areas extended over the extra-bony part of the implants (Figures 4 and 5a–c). The new regenerated bone had more density around the microthread part of the implants. A distinct borderline was seen between the grafted and the recipient bone. Percent of new supracrestal trabecular bone formation was statistically higher in the test group than the control group (28.5 ± 4.5% vs 4.3 ± 1.8%, *P* < .05).

**DISCUSSION**

There are many advantages to immediate implant placement with simultaneous augmentation, the primary one being the elimination of a second surgical procedure. Previous studies have achieved small vertical augmentations, varying from 2 mm to 4 mm, using membranes around partially submerged implants.23–25 For example, de Macedo and colleagues25 demonstrated an increase in bone formation from 0.43 mm (without ePTFE) to 2.42 mm (with ePTFE) around partially inserted implants in rabbit tibia, using nonporous membrane and the GBR technique. Other investigators have consistently demonstrated the efficacy of GBR and membranes, irrespective of the graft materials.26 This experimental study also expands on GBR techniques by means of the coapplication of particulate mineralized bone with fibrin glue and MSCs to enhance bone formation around partially submerged implants in the tibia of rabbits. Allogenic bone grafts have a long and well-documented successful history as an alternative material to autografts.4 Concomitant application of structured particulates and/or blocks of allogenic bone with implant placement has further improved the favorable outcome of augmentation.27,28 Recently, the endeavor to further manipulate bone formation has encouraged the concept of tissue engineering through the use of growth factors.29

Furthering the concept of modulating bone formation, this study seeks to advance the science of bone augmentation with the use of MSCs with allografts in a simultaneous implant placement model. This model has already been shown to be efficacious for defect grafting. Shih and colleagues30 successfully used marrow stromal cells and partially demineralized bone matrix in a canine non-weight-bearing model to fill bone defects. In a clinical study, Filho Cerruti Cerruti and colleagues31 enhanced bone gain in the anterior and posterior maxilla with a combination of mononuclear cells (made up of MSCs and hematopoietic precursors), platelet-rich plasma (PRP), and allograft. This study supports the enhancing effects of MSCs/allograft on osteoregeneration around simultaneously inserted implants.

Although there have been several efforts to determine the specific marker for MSCs in animals, no single epitope has been yet identified. Research in humans, however, has been productive for several markers, including CD133, low-affinity nerve growth factor receptor, and STRO-1.32–34 For this reason, the MSC committee of the International Society for Cell Therapy has proposed two specific criteria for identifying MSCs with an animal source:

<table>
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<th>Group 1 (Test)</th>
<th>Group 2 (Control)</th>
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<td>Vertical bone length (mm)</td>
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<tr>
<td>Bone formation at 8 weeks (%)</td>
<td>28.5 ± 4.5</td>
<td>4.3 ± 1.8</td>
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**Figures 4 and 5.** Figure 4. Histological sections of the control group after 8 weeks. (a) The whole implant body length revealing the partially inserted implant in the tibia (hematoxylin & eosin stain; original magnification ×8). (b, c) Fibrous connective tissue over the augmented area (original magnification ×12). Figure 5. Histological sections of the group treated with mesenchymal stem cells after 8 weeks. (a) The emergence of the new bone over the native tibia could be seen. The trabecular bone was regenerated around the microthread part of the implant, tibia, and overlying connective tissue (hematoxylin & eosin stain; original magnification ×8). (b, c) Original magnification ×12.
They must be adherent cells, and they must be able to produce bone, cartilage, and adipose cell lineages. Because the isolated cells of the present study grew as adherent cells and subsequently differentiated into bone, cartilage, and adipose cell lineages, this study meets the MSC criteria set by the committee. The use of MSCs in the current study enhanced the amount of bone formation around the partially submerged implants from 4.3% to 28.5%. The 8-week postoperative results also demonstrated improvement of the overall vertical height from 1.03 mm to 2.09 mm.

The other aspect in maximizing the effectiveness of MSCs in bone healing is the coapplication of fibrin glue in the MSC delivery system. This tissue adhesive is a biocompatible combination of fibrinogen and thrombin. The major disappointment of previous experiences regarding the use of MSCs in treating bone defects was the method of cell delivery and stabilization, which may have affected further stimulation of existing stromal cells and proper bone regeneration. Lee and colleagues inserted implants in surgically reduced canine mandibular ridges. The simulated supralveolar peri-implant defects were simultaneously treated with a combination of particulate autogenous bone graft and platelet-enriched fibrin glue. The resultant bone augmentation was significantly higher in the treated group. New bone height in the created defect was increased from 9% (empty control group) to 63% for the test group. This study design proved that fibrin glue may eliminate the need for a membrane and can still sufficiently support the graft’s particulates.

In our experiment, the use of fibrin glue with the addition of ePTFE membrane further enhanced the stable environment and subsequent vertical bone apposition. The other advantage of fibrin glue is that it promotes wound healing by providing a cross-linked structure for cell adhesion and proliferation. The use of these previously proven tissue-engineering methods and the addition of MSCs to the mixture demonstrated significant enhanced bone formation from 4.3% (control) to 28.5%. However, there is still some controversy about whether fibrin glue has a positive or negative effect on cell proliferation and differentiation. Omitting the bone substitute, Lee and colleagues compared the osteogenic capacity of MSCs/fibrin glue with MSCs/macro-porous biphasic calcium phosphate in cranial defects of rabbits, and the former resulted in a more mature bone. The addition of PRP to MSCs/fibrin glue by Ito and colleagues also showed enhanced bone formation in a canine simultaneous implant placement model. In a recent study, Kim and colleagues tested the triple effect of polycaprolactone tricalcium phosphate/MSCs/recombinant human bone morphogenetic protein-2 with fibrin glue. Their study claimed that the combination was highly effective in bone growth of surgically created defects in dog scapulas. Although the addition of growth factor was not incorporated in this study, improved novel methods in cell-delivery systems may lessen the need for additional factors, ultimately reducing the cost and complexity. Further clinical studies are required to investigate whether direct addition of MSCs can predictably promote bone regeneration around simultaneously grafted implants. It is noteworthy that rabbit tibia is not the perfect model for simulating the deficient alveolar ridge. More valid animal models for this purpose have been applied. Nevertheless, this model is easily transferable to a more reliable animal and/or human cohort so that the efficacy of the presented combination could be further investigated.

Based on the results of this present study, an MSCs/particulate mineralized bone/fibrin glue combination appears to be promising for vertical bone augmentation around immediately placed and partially submerged implants in the tibia of rabbit. The criteria set by the International Society for Cell Therapy for the identification of animal-derived MSCs as a graft source was met in this study.

**CONCLUSION**

**ABBREVIATIONS**

cDNA: complementary DNA  
DMEM: Dulbecco’s modified eagle medium  
ePTFE: expanded polytetrafluoroethylene  
FBS: fetal bovine serum  
GBR: guided bone regeneration  
MSC: mesenchymal stem cell  
PCR: polymerase chain reaction  
PPAR: peroxisome proliferator activated receptor  
PRP: platelet rich plasma  
RT: reverse transcriptase
RT-PCR: reverse transcriptase polymerase chain reaction

VBL: vertical bone length

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