Case Series

Histologic Evaluation of a Stem Cell–Based Sinus-Augmentation Procedure

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Background: Predictability has been demonstrated for the long-term success of dental implants placed simultaneously with or after a sinus-augmentation procedure. However, the time required to obtain optimal bone formation can be from 6 to 9 months or longer with grafting materials other than autogenous bone. For this reason, there is interest in a surgical technique that does not require the harvest of autogenous bone but still results in sufficient bone formation within a relatively short time frame.

Methods: The purpose of this case series was to evaluate the bone formation following sinus-augmentation procedures using an allograft cellular bone matrix containing native mesenchymal stem cells. Biopsy and histologic evaluation were performed after ~4 months of healing.

Results: Histomorphometric analysis revealed an average vital bone content of 33% (range, 22% to 40%) and an average residual graft content of 6% (range, 3% to 7%) for the five cases reported that had an average healing period of 4.1 months (range, 3 to 4.75 months).

Conclusion: The high percentage of vital bone content, after a relatively short healing phase, may encourage a more rapid initiation of implant placement or restoration when a cellular grafting approach is considered. J Periodontol 2009;80:679-686.

KEY WORDS

Bone regeneration; case series; osteoblasts; ridge augmentation; stem cells; tissue engineering.

The repair of clinically significant bony defects has always posed a therapeutic challenge in clinical dentistry. Often, there is failure of bone defects to heal, or bony defects may fill through the process of repair or fibrous tissue in-fill rather than the desired in-fill by organized bone tissue. Healing of bone defects is dependent on the presence of osteogenic precursor cells in the surrounding tissues to invade the defect and differentiate into osteoblasts that produce the bone matrix. Bone formation will be limited in the absence of adequate quantities of these differentiating precursor cells or osteoblasts. Osteoblasts contain the cellular machinery for the production of bone matrix, but they are unable to undergo further division and have limited migratory capacity.

There is a diversity of opinion regarding what materials should be used for typical clinical applications, the rationale for their use, the rationale for using combinations of materials, and the percentages of each material used in combination.[1-6] A variety of materials have been used over the years to promote and stimulate bone formation.[7] The use of these materials in regenerative procedures is based on the assumption that they possess osteogenic potential (contain bone-forming cells), are osteoinductive (contain bone-inducing substances), or are simply osteoconductive (serve as a scaffold for bone formation). Autologous cancellous bone is the gold standard for bone grafting because it possesses osteogenic capacity through the presence of appropriate cellular content.[1] However, grafts of autologous bone and marrow origin exhibit high variability in the numbers of cells with osteogenic potential between harvest sites and patients, which often fall below the threshold required for regeneration of a defect of significant proportion. Hence, although it is recognized that autologous grafts possess the appropriate osteogenic, osteoconductive, and osteoinductive properties, less than desirable results are sometimes the outcome of therapy. In addition, drawbacks with its acquisition, the associated morbidity, and a limited intraoral supply have prompted the development of

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alternate materials for the repair of oral and maxillofacial bony defects.[8]

Synthetic osteoconductive grafts, as well as allografts and xenografts, provide the scaffold for the ingrowth of capillaries, perivascular tissues, and osteoprogenitor cells from the adjacent recipient bed.[9,10] However, they lack osteogenic capabilities. Osteoinductive graft materials, including allografts, such as demineralized bone matrices[11] and bone morphogenetic proteins (BMPs), in purified and recombinant forms[12,13] have been used in animal and human experimental models to achieve bone formation. However, successful bone regeneration with these grafts is dependent on the presence of progenitor cells that are subsequently induced to undergo differentiation and bone matrix production.

Based on our current understanding of graft healing and the prerequisites for optimal bone regeneration, tissue-engineering research has focused on providing the necessary cellular machinery, i.e., the mesenchymal stem cells (MSCs), directly in sites that require bone regeneration.[14] Stem cell therapy has a broad base of current applications under investigation that include the repair and regeneration of heart muscle,[15] cartilage,[16] and bone tissues.[17] Pluripotential MSCs have the unique capability to differentiate into a variety of cell types based on the inducing signals received from the recipient tissue. Of great interest to osseous reconstruction for implant dentistry is the appropriate stimulation of implanted MSCs that can differentiate along the osteoprogenitor cell lineage[18] with osteogenic properties that would result in bone formation for reconstructive implant therapy.

The evolution and refinement of techniques for harvesting, ex vivo culture expansion, and in vivo reimplantation of adult stem cells have led to the production of biomaterials for clinical application. Stem cells are characterized by their ability to renew themselves through cell division and differentiate into a diverse range of specialized cell types. The two broad types of mammalian stem cells are embryonic stem cells, found in blastocysts and adult stem cells found in adult tissues, such as the marrow. In adult organisms, stem cells give rise to progenitor cells that act as a repair system for the body, replenishing specialized cells and tissues. The term adult stem cell refers to those found in a developed organism; thus, in research and therapy, their use is not as controversial as embryonic stem cells, which entails the destruction of an embryo. Despite the extensive research into their potential use,[19] there are no commercially available clinical applications using embryonic stem cells.

Techniques to expand cell numbers in vitro, for later implantation to regenerate bone, have been an area of pursuit for many years.[20,21] The use of culture-expanded MSCs for the regeneration of osseous defects showed superior results compared to those achieved with fresh marrow.[20] Animal studies using these cell-based therapies showed excellent bone regeneration in long bone critical size defects,[17,22-25] as well as in their oral application in the extraction socket model.[26] A hydroxyapatite/tricalcium phosphate porous cylinder used as a delivery vehicle provides a suitable osteoconductive scaffold for supporting *de novo* vascularization and bone formation.[20] Early findings in samples containing MSCs include the rapid differentiation of osteoblastic cells and bone formation, which were significantly higher than in cell-free samples or in those that contained fresh marrow alone.[20] With regard to regenerative potential, MSCs also showed superior outcomes compared to BMPs loaded on the same delivery vehicle.[27] This further elucidates the limitations of growth factors used in the absence of adequate cellular components required for the production of bone.

When considering implant therapy in the posterior maxillary region, a combination of postextraction alveolus resorption and continued sinus pneumatization limits the vertical volume of available bone. Sinus augmentation with a variety of bone grafts has repeatedly produced high success rates when used to increase the height of bone required for implant placement.[28,29] Histologic data on the healing patterns of grafts placed in the sinus showed that bone primarily forms from osteogenic cells originating from the osseous floor and walls.[30,31] Thus, cellular infiltration, vascularization, *de novo* bone formation, and graft replacement often require long healing times to produce adequate bone formation for implant placement.

It is possible to maintain the adult MSC population in allograft tissue. Techniques for processing the graft material have been refined and include the selective depletion of immunogenic cells as well as the preservation of a rich source of viable multipotential stem cells and osteoprogenitor cells. The graft material used in this case series was a combination of cellular cancellous bone and a particulate demineralized bone matrix obtained from the same donor. The cellular graft retains its cell viability and multipotential characteristics through cryopreservation. This potentially expedites the healing process by directing bone formation from within the graft material, which results in a larger quantity of available vital bone at an earlier time point. This case series evaluated this hypothesis.

MATERIALS AND METHODS

Patient Selection

Patients (one male and four females) between the ages of 25 and 75 years requiring maxillary sinus augmentation were screened, and they provided written and oral consent. Patients were accepted

for this case series if none of the criteria outlined was present and if they desired an implant-supported reconstruction of the posterior maxilla. The patients were enrolled in the study from August to October 2007, and the study was conducted in accordance with the Helsinki Declaration of 1975, as revised in 2000. The case-selection criteria included the absence of sinus pathology or history of chronic sinus inflammatory disease; the presence of ≤6 mm posterior maxillary alveolar height in the future implant site; women who were not pregnant or intended to become pregnant during the study period; no history of cancer or human immunodeficiency virus; no untreated periodontal disease, including periapical disease, especially with teeth adjacent to the sinus floor; and the absence of any medical condition or therapeutic regimen that alters soft and/or hard tissue healing (i.e., osteoporosis, hyperparathyroidism, autoimmune diseases, chemotherapeutic or immunosuppressive agents, steroids, bisphosphonates, or similar type drugs).

Bone Graft Preparation

The bone graft material[§] used in this study is commercially prepared from cadavers within 24 hours of death. Rigorous safety testing and donor screening is performed. Cortical bone is separated and processed into demineralized bone particles. The selective immunodepletion removes unwanted cells from the remaining cell-rich cancellous bone. Fluorescence-activated cell sorting (FACS) testing is performed to confirm that nearly all remaining cells are positive for cluster differentiation (CD)105 and CD166 and negative for CD45 (Fig. 1).[32] This marker profile is indicative of MSCs and osteoprogenitor cells.[33] The remaining viable MSCs and osteoprogenitor cells remain attached to the cancellous bone matrix. In addition, the demineralized bone particles are added back to the cell-containing cancellous bone component. A cryopreservation solution is added, and the product is stored at –80°C ± 5°C, permitting a 5-year shelf life. Quality testing is performed on every lot to validate a minimum cell count of 50,000 cells/ml and a minimum cellular viability of 70%. The average cell viability count for the material used in this case series was 88.9% (range, 86.4% to 90.7%). The cell count and cell viability are determined by a trypan blue dye exclusion test.[34] The cellular activity of each lot is also validated by performing in vitro alkaline phosphatase assays.[35]

The bone graft material was shipped to the clinic on dry ice and prepared as per the manufacturer's recommendation. The graft was thawed using a water bath at room temperature. Because the graft contains vital cells, the maximum temperature of the water bath during thawing was 37°C. After the cryopreserved cells
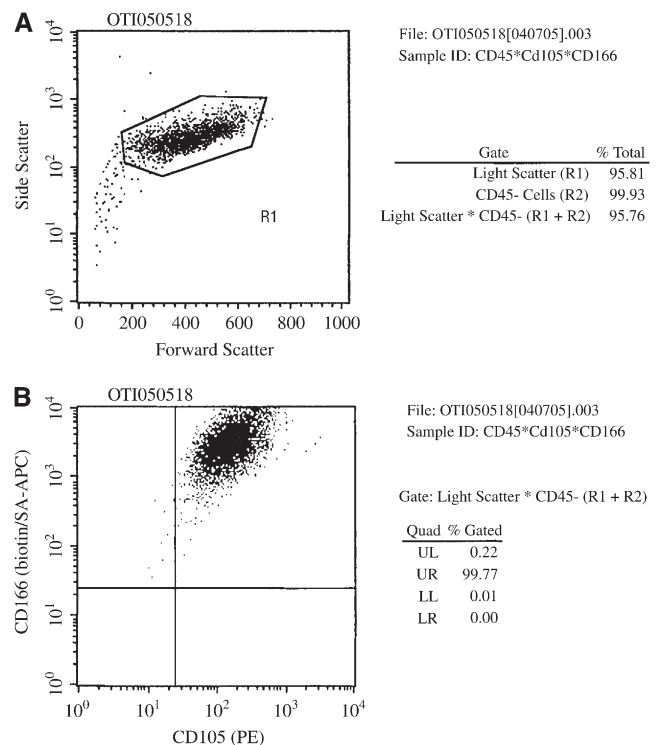

Figure 1.
FACS evaluation with a representative dot graph showing cells gated for CD45 negative (99.93%) **(A)** and the occurrence of positive expression of CD105 and CD166 (99.77%) in cells extracted from the allograft cellular bone matrix **(B)**.

were thawed, the liquid was decanted, and the cell-containing graft was ready to be implanted, with a working window of 4 hours (Fig. 2). If the particle size (1 to 3 mm) was too large, rongeurs were used to carefully reduce the particle size.

Surgical Procedures and Computed Tomography (CT) Scans

A classic lateral window approach sinus-augmentation procedure was performed (Fig. 3). Care was taken not to tear the Schneiderian membrane. No membrane was used to cover the lateral wall access window. Following the placement of bone graft material, primary closure was achieved using 4-0 polyglactin 910[||] sutures. Routine postoperative instructions and medications were given, and care was taken so that no prosthesis contact with the area occurred during the first 2 weeks of healing. CT scans[¶] were taken prior to surgery to screen for sinus pathology and to better understand the sinus anatomy. Follow-up scans were taken immediately following flap closure and at the time of implant placement ~4 months later (Fig. 4).

Figure 2.
Stem cell–containing allograft after the thawing process and decanting of the liquid cryopreservative.

Figure 3.
A) Right side sinus access after a classic lateral window approach.
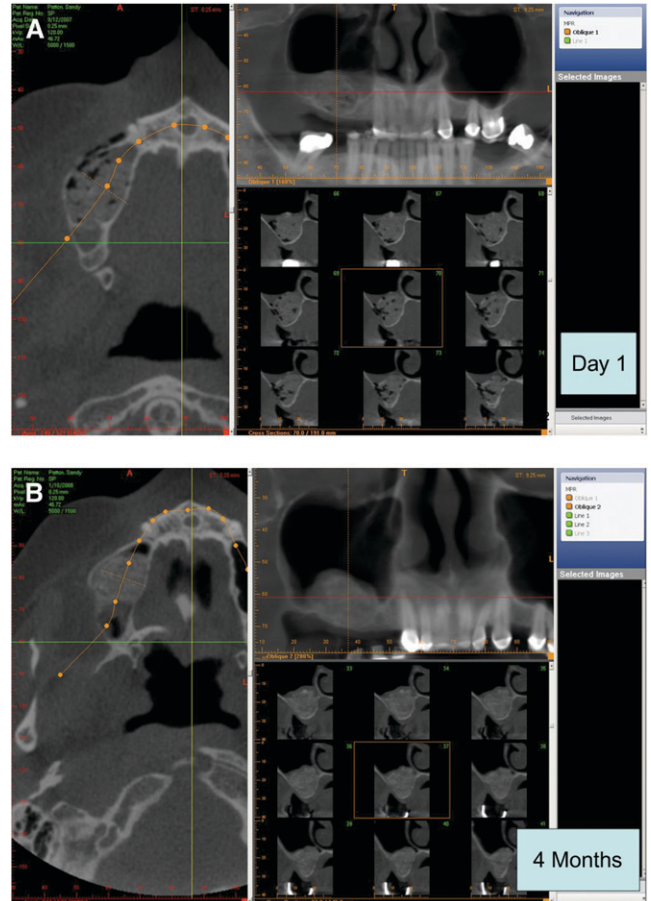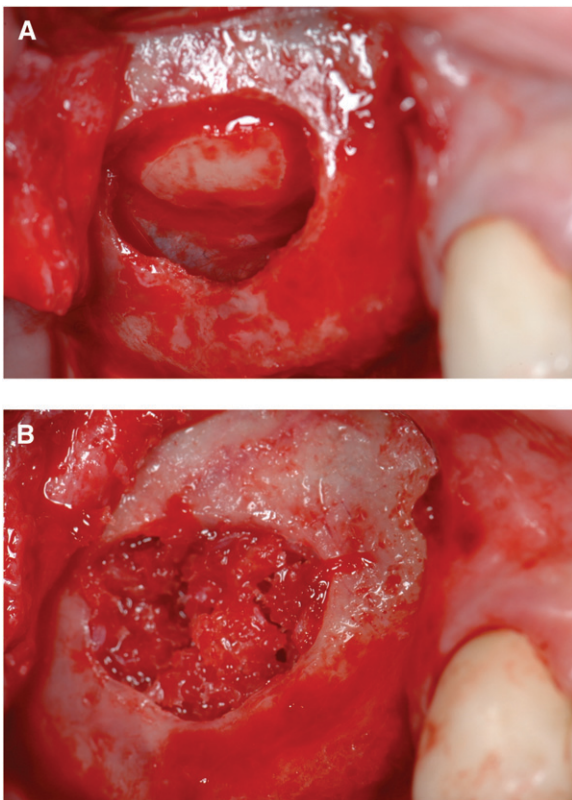*B) Sinus after grafting with allograft cellular bone matrix. No membrane was used to cover the bone graft and lateral wall access window.*

Figure 4.
A) CT scan of a grafted sinus immediately after graft placement. B) CT scan of the same grafted sinus 4 months later.

Core Biopsies

A biopsy core was taken with a 2.7-mm internal diameter (3.5 mm external diameter) trephine at the implant placement appointment. Biopsies were left within the trephine and placed in 10% neutral buffered formalin for fixation. Implants were placed in the osteotomies created following trephine core biopsies.

Histologic Preparation

All histologic preparations were performed by the Division of Pathology, University of Minnesota, Minneapolis, Minnesota. The specimens were retrieved and placed in 10% neutral buffered formalin. Upon receipt in the Hard Tissue Research Laboratory, specimens were dehydrated with a graded series of alcohols for 9 days. Following dehydration, the specimens were infiltrated with a light-curing embedding resin.# Following 20 days of infiltration with constant shaking at normal atmospheric pressure, the specimens were embedded and polymerized by 450-nm light; the temperature of the specimens never exceeded 40°C. Then the

Technovit 7200 VLC, Kulzer, Wehrheim, Germany.

specimens were prepared by the cutting/grinding methods of Donath and Breuner[36] and Rohrer and Schubert.[37] Specimens were prepared in an apico-coronal direction (parallel to the long axis) and were cut to a thickness of 150 μm on a cutting/grinding system.** The cores were polished to a thickness of 45 to 65 μm with a series of polishing sandpaper disks from 800 to 2,400 grit, using a microgrinding system, followed by a final polish with 0.3-μm alumina polishing paste. The slides were stained with Stevenel's blue and Van Gieson's picro fuchsin and coverslipped for histologic analysis using brightfield and polarized microscopy.

Histomorphometry

Following non-decalcified histologic preparation, the cores were evaluated morphometrically. The cores were digitized at the same magnification using a microscope[††] and a digital camera.[‡‡] Histomorphometric measurements were completed using a combination of programs.[§§‖] Parameters evaluated were total area of the core, percentage of

Figure 5.

A) A representative mineralized core. **B** and **C)** Higher magnification views. Red-stained tissue is mineralized allograft (lighter red, no cells visible, non-vital bone) or newly formed bone (darker red, cells visible, vital bone). Green-stained tissue is the demineralized allograft (no cells visible, non-vital bone). (Original magnification: A, ×15; B, ×100; C, ×200.)

new bone formation, and percentage of residual graft material. The remainder of the area was considered soft tissue, void, or osteoid. The primary slide evaluated for each specimen was from the most central region of the obtained core. No comparison was made between the apical and coronal sections.

RESULTS

Histologic evaluation of the samples revealed an average vital bone content of 33% (range, 22% to 40%). A residual graft content of 6% (range, 3% to 7%) was found for the five cases following a healing time of 4.1 months (range, 3.0 to 4.75 months). Figures 5 and 6 show histologic cores taken from the stem cell–grafted sites.

DISCUSSION

We have entered a new era in the surgical reconstruction of the deficient maxilla and mandible; molecular enhancement and cell-based therapies can be used to improve and expedite our outcomes.[7] All three critical
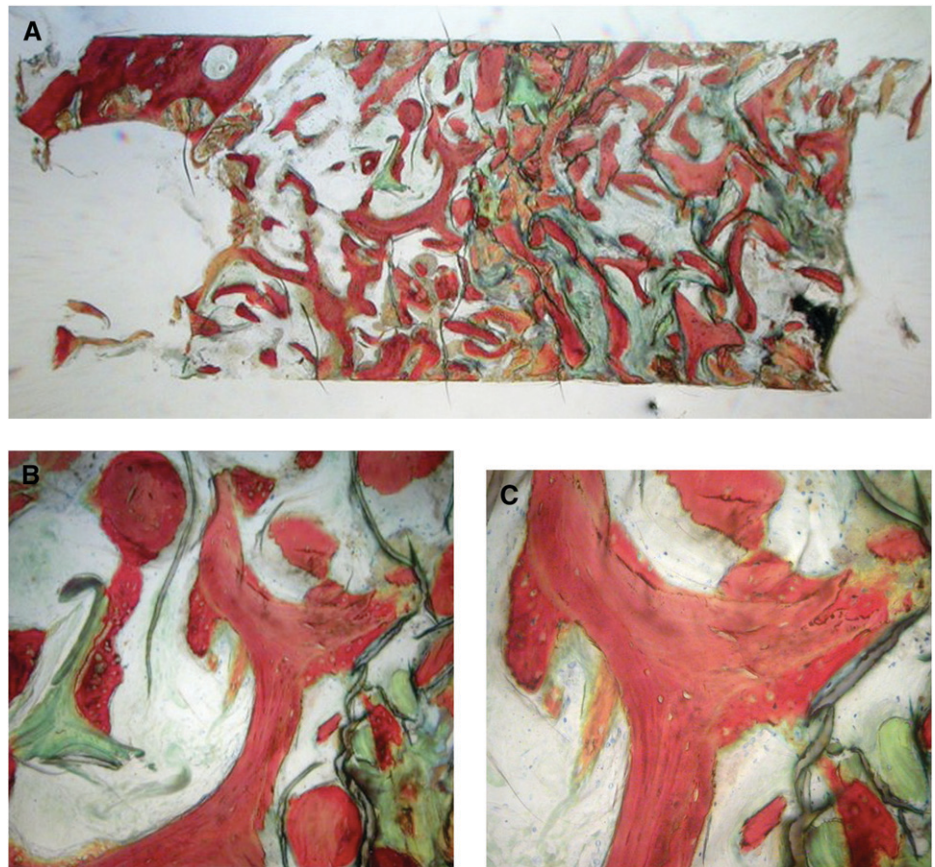
aspects of bone formation, osteoconduction, osteoinduction, and osteogenesis, are now readily available from sources other than autogenous bone. The use of growth and differentiation factors, such as recombinant human platelet-derived growth factor and recombinant human BMP (rhBMP)-2, results in bone formation in such applications as extraction socket defects,[38] vertical bone growth in the posterior mandible,[39] sinus augmentation,[40] and horizontal ridge augmentation.[41] This article described how a commercially available allograft cellular bone matrix containing native MSCs can be used for rapid bone formation in the sinus-augmentation procedure. The percentage of new bone formation with the graft material used in the cases reported at 4 months compares favorably with that achieved with other graft materials at a later healing time-point.[28,42]

** EXAKT Technologies, Oklahoma City, OK.
†† Zeiss Axiolab, Carl Zeiss MicroImaging, Thornwood, NY.
‡‡ Nikon Coolpix 4500, Nikon, Melville, NY.
§§ Adobe PhotoShop, Adobe Systems, San Jose, CA.
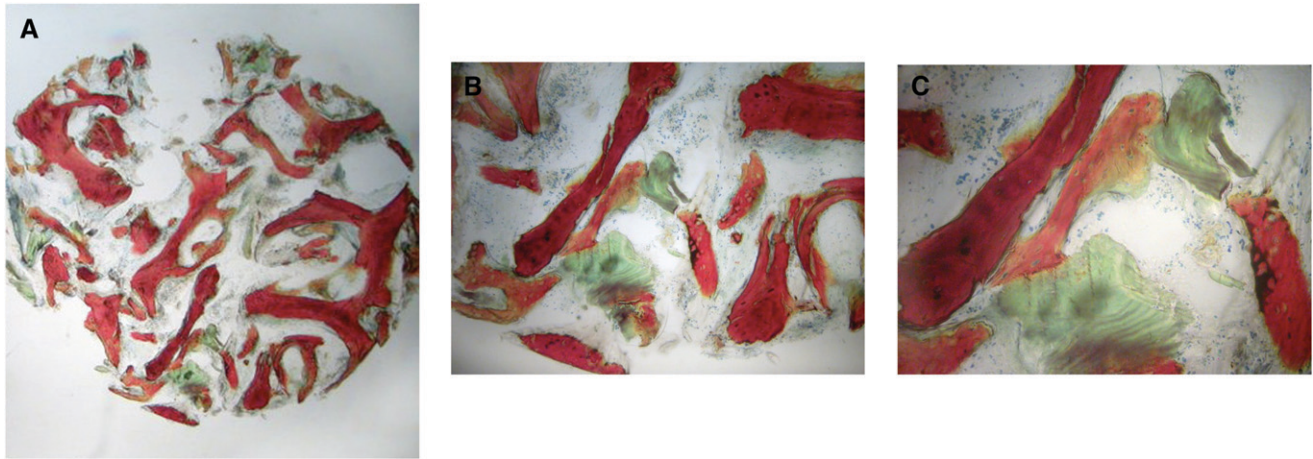‖ NIH Image, National Institutes of Health, Bethesda, MD.

Figure 6.
A) A portion of a mineralized core. B and C) Higher magnifications views. Red-stained tissue is mineralized allograft (lighter red, no cells visible, non-vital bone) or newly formed bone (darker red, cells visible, vital bone). Green-stained tissue is the demineralized allograft (no cells visible, non-vital bone). (Original magnification: A, ×25; B, ×75; C, ×150.)

The cellular content of autogenous bone grafts varies based on the individual patient's medical profile, the harvest technique (aspiration or open harvest), the anatomic location of the harvest (intraoral or extraoral), the type of bone harvested (cortical or cancellous), age, and gender. The cellular content has an effect on the bone graft performance.[43] Therefore, the identification of MSCs and their concentration in different anatomic tissues has been an area of recent investigation.[33,44,45] Evaluation of bone marrow aspirates from the anterior iliac crest revealed a fairly small MSC count, although a higher percentage of cells that tested positive for CD105 was found in the iliac crest aspirates compared to peripheral blood.[33] McLain et al.[44] compared the connective tissue progenitor cell concentrations between iliac crest and vertebral body aspirates. Their findings showed that vertebral aspirates (465 cells/ml marrow) have a higher mean concentration than iliac crest aspirates (356 cells/ml marrow). The MSCs obtained from the aspiration-harvest methods are not all attached to the graft material, indicating that the graft material used in this case series has a significant cell concentration advantage; because the cells are bound to the cancellous graft material, they do not wash out during implantation. The process used to prepare the cellular bone matrix in this study involved the selective removal of immunogenic cells in a hematopoietic lineage from cell-rich cancellous bone, while retaining the osteopotent cells in the mesenchymal lineage. Quality controls ensure that the minimum cell count of commercially available product is 50,000 cells/cm^3 bone graft. A new iteration of the commercially available cellular allograft used in this case series has in-creased cell counts, with a minimum of 250,000 cells/cm^3.

An exciting evolutionary step for optimizing our clinical outcomes could include a combination approach, studying the synergistic effects of MSCs and induction factors, such as BMPs. Lane et al.[46] combined rhBMP-2 with MSCs in an animal model and found an additive effect that was superior to rhBMP-2 or MSC therapy alone. Although it was shown that MSCs have rhBMP-2 receptors and the potential to produce rhBMP-2, the supraphysiologic doses possible with rhBMP-2 delivery may allow for a more robust response, as found by Lane et al.[46]

Studies[15,47-50] on MSCs have characterized many of the surface markers and receptors found and have demonstrated that this subpopulation of marrow cells does not elicit an immune response. Our observation corroborates these studies, in that no clinical or histologic inflammatory response was noted postoperatively or at the time of obtaining the core harvests, respectively.

CONCLUSIONS

Despite the favorable results reported in this case series and in other MSC publications, continued studies are warranted to evaluate the safety and efficacy of MSCs in applied bone regeneration. A multicenter study evaluating the application of MSCs in the sinus-augmentation model is underway.

ACKNOWLEDGMENTS

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