STEM CELL AND TISSUE ENGINEERING

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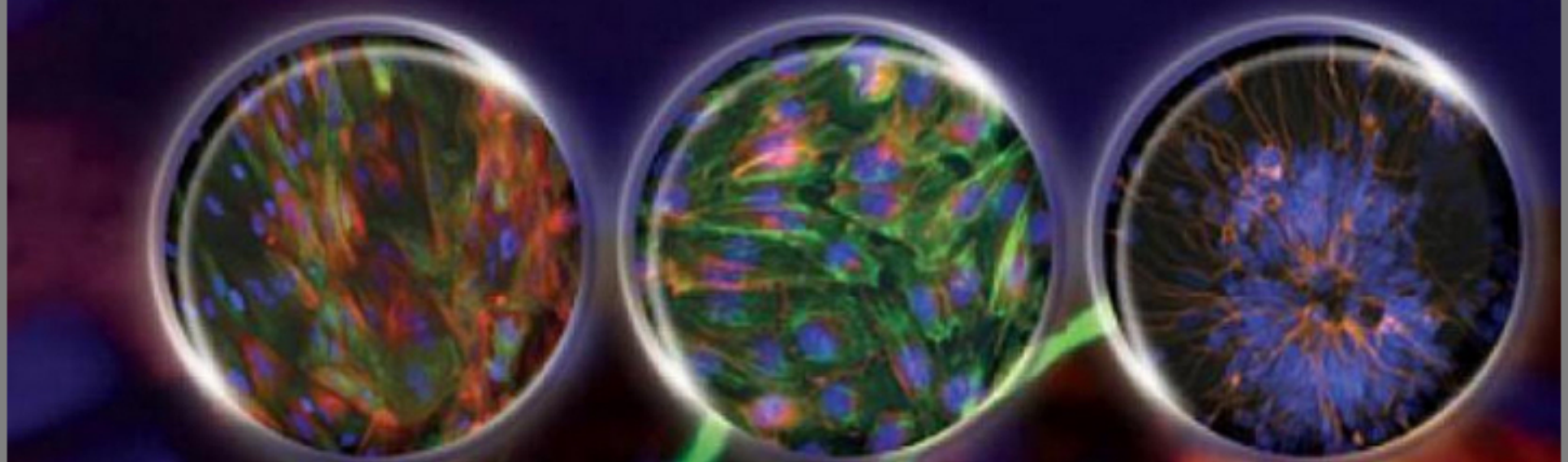

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Clinical Applications of a Stem Cell Based Therapy for Oral Bone Reconstruction

Bradley McAllister and Kamran Haghighat

1. Introduction

We have observed a rapid evolution in both surgical techniques and materials utilized in the promotion of regenerative therapy for oral reconstructive procedures. In particular, bone augmentation has been promoted through different methods which include the use of growth and differentiation factors, particulate and block grafting materials,[1–4] distraction osteogenesis,[5–7] as well as membrane-assisted guided bone regeneration (GBR).[8] A central theme to all of these oral reconstructive technologies is the need for rapid and complete revascularization.

Early research that introduced us to the concept of using barrier membranes in what is referred to as GBR outlined the need for exclusion of undesirable soft tissue cellular contents and provision of a secluded space into which osteogenic cells from various sources can migrate for successful bone healing.[9–11] The pattern of bone regeneration involves angiogenesis and ingress of osteogenic cells from the defect periphery towards the center to create a well-vascularized granulation tissue. This provides a

scaffold for woven bone proliferation and bone apposition within the defect.[12] The size of the defect influences the bone healing capacity. In circumstances where the defect size is too large to generate a biomechanically stable central scaffold, bone formation will become limited to the marginal stable zone with a central zone of disorganized loose connective tissue. Critical to the outcome of GBR is maintenance of primary wound closure throughout the healing period.[13,14]

Perforation of the cortical bone layer has been advocated in GBR as it has been postulated that this will increase the vascularity of the wound and release growth factors and cells with angiogenic and osteogenic potential. Aggressive recipient bed preparation with decortication, intramarrow penetration has also been supported due to increases in the rate of revascularization, the availability of osteoprogenitor cells and the increased rate of remodeling.[12,15] In addition to the surrounding bone, the periosteum is also widely considered as an important source of cells with osteogenic capability. Despite the desirable graft containment and cellular exclusion characteristics of most barrier membranes, limiting the access of periosteal-derived osteogenic cells during the early phases of wound healing may not be of benefit to the healing of larger sized defects.

The autograft, allograft, alloplast and xenograft materials all have reported success either alone, or in combination, for particulate bone augmentation.[16] The particulate autograft is currently recognized as the gold standard for most bone grafting, including the treatment of dental implant related defects. Several studies have demonstrated the effectiveness of particulate autograft due the availability of cells with osteogenic potential, as well as osteoinductive and osteoconductive properties.[17,18] However, autografts have recognized limitations, such as donor site morbidity, increased cost, potential resorption, size mismatch, an inadequate volume of graft material, as well as the unpredictability in the quantities of osteogenic precursor cells.

Allografts have the advantage of being available in higher quantities and eliminate the morbidity associated with a second surgical site. Biochemical extraction techniques have shown that growth and differentiation factors are present in demineralized freeze-dried bone allograft (DFDBA) preparations; however, quantities have been shown to be variable from lot to lot indicating a potential variation in performance.[19–21]

Thus, allografts primarily act as a scaffold for the in-growth of capillaries, peri-vascular tissues, and osteoprogenitor cells from the adjacent recipient bed.

The absence of differentiating precursor cells or osteoblasts in adequate quantities will ensue in limited bone formation. Osteoblasts contain the cellular machinery for production of bone matrix, but they are unable to undergo further division and have limited migratory capacity. This limits the expected benefits of cells contained in oral derived autografts, for example, which exhibit high variability in the numbers of cells with osteogenic potential.

Based on our current understanding of graft healing and the prerequisites for optimal bone regeneration, tissue-engineering research has been focused on providing the necessary cellular machinery, namely the mesenchymal stem cells (MSCs) and osteoprogenitor cells, directly in sites that require bone regeneration. It is this concept that has been utilized in the processing of the commercially available graft material that will be discussed here. Historically the majority of efforts for bone grafting with MSCs and osteoprogenitor cells have focused on the concept of harvesting these cells followed by *in vitro* culture expansion for later implantation.[22,23] The following methodology section describes a novel approach that leaves the MSCs and osteoprogenitor cells found within allogeneic bone and substantially depletes unwanted cells.

2. Procurement Methodology for Stem Cell Containing Allograft

The allogeneic bone graft material described in this chapter (Osteocel®) is commercially prepared for NuVasive, Inc.™ from cadavers recovered by licensed tissue procurement agencies (AlloSource) and distributed into the dental market by ACE Surgical Supply. Cadaver tissues are rushed to the processing facility on wet ice and processing is begun within 24 hours of the donors' death. In parallel rigorous safety testing, donor screening and evaluation for bacterial, fungal and spore contamination begins. Screening measures consist of physical examination and evaluation of both medical and social history, including a next of kin interview. Comprehensive serological and microbial testing are also performed

which includes nucleic acid testing (NAT) for Hepatitis-C and HIV. Donor assessment culminates with a complete medical record review by a licensed physician. Cortical bone is separated and processed into demineralized bone particles for adding back to the cellular graft component. A process of selective immunodepletion, that involves several extensive wash steps, is initiated to remove undesirable cells, such as red blood cells and lymphocytes that can provoke an immune response. These unwanted cells are substantially depleted leaving the remaining cell rich cancellous bone matrix. The cellular cancellous bone component then undergoes a broad-spectrum antimicrobial treatment (vancomycin, gentamicin sulfate and amphotericin) designed to eliminate potential contamination while preserving the viability of the cells. These remaining viable MSCs and osteoprogenitor cells remain attached to the cancellous bone matrix. Approximately 20% demineralized cortical bone particulate from the same donor is then combined to the cell containing cancellous bone matrix component. A standard cryopreservation solution containing 10% DMSO with human serum albumen (HSA) is added and the product is stored at −80 ± 5 degrees Celsius (°C), permitting a five-year shelf life.

During the product processing validation, FACS (fluorescence activated cell sorting) testing was performed to confirm the retention of MSCs that are positive for cluster of differentiation 105 (CD105) and CD166 while being negative for CD45.[24] Figure 1 depicts a representative FACS Scatter Plot of the cellular allograft. While there is not a single identifiable surface marker for MSCs, this marker combination profile is indicative of MSCs and osteoprogenitor cells. Quality testing is performed on every lot of Osteocel® to validate a minimum cell count of 50,000 cells/cc, and a minimum cellular viability of 70% of the enzymatically released cells. Another iteration of the cellular allograft product (Osteocel® Plus), which was not utilized in the clinical and histological aspect of this chapter, has quality testing for a minimum cell count of 250,000 cells/cc. The cell count and viability are determined on released cells by a Trypan Blue dye exclusion test with a hemocytometer. Cellular osteogenic activity of each lot is also validated by performing *in vitro* cell differentiation and alkaline phosphatase assays (Fig. 2).

The cellular bone graft material is stored at −80°C, shipped to the clinic on dry ice where it is prepared as per the manufacturer's recommendation.

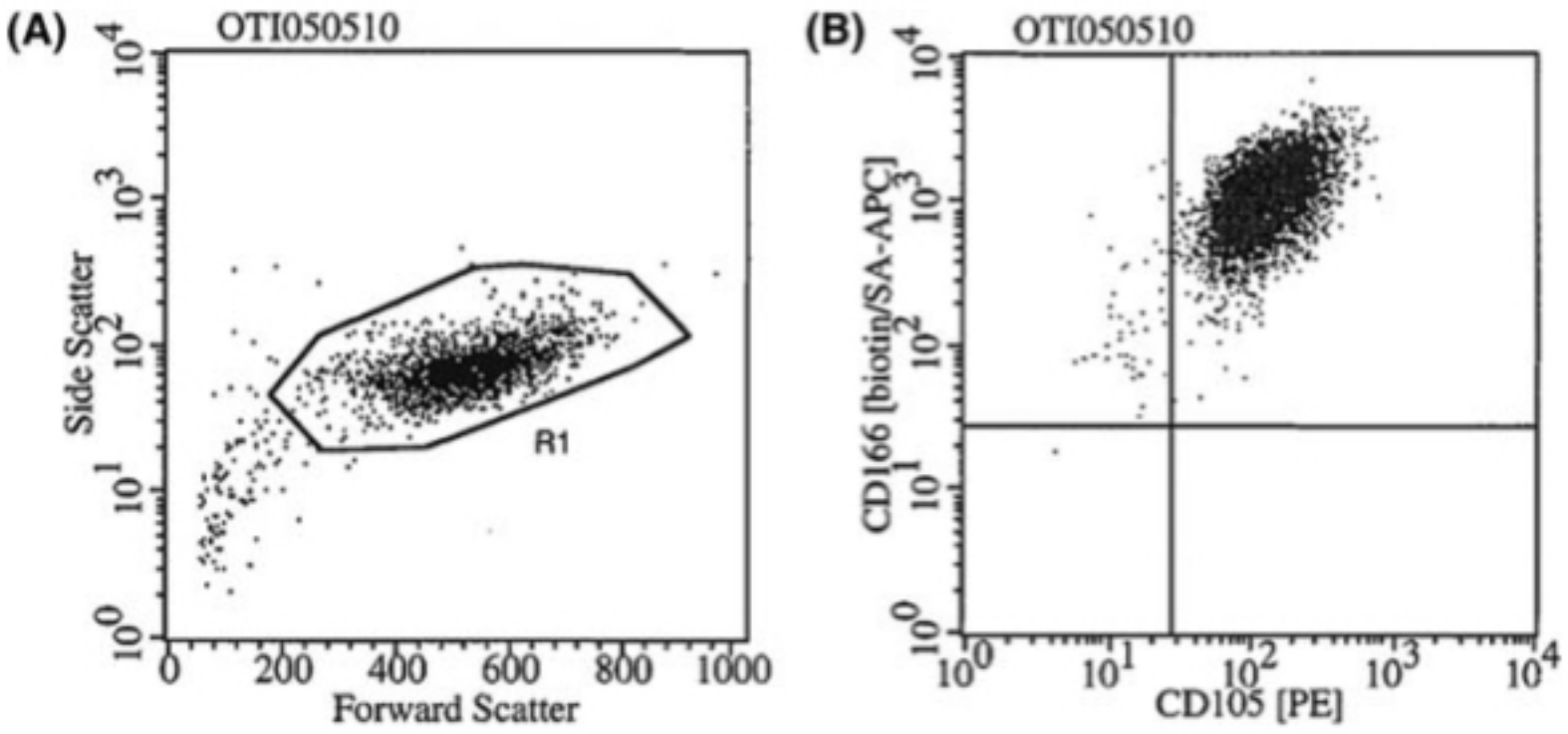

Fig. 1. Representative FACS Scatter Plot of Osteocel®. (**A**) Forward Scatter and Side Scatter dot plot from the donor 2 population of Osteocel® Plus cells with [R1] 94.44%, [R2] 99.93% and [R1 + R2] 94.42%. (**B**) Dot plot showing positive expression of CD105 and CD166 markers, after gating for CD45- from the donor 2 cells. Quadrant gating UL 0.82%, UR 99.14%, LL 0.04% and LR 0.00%.

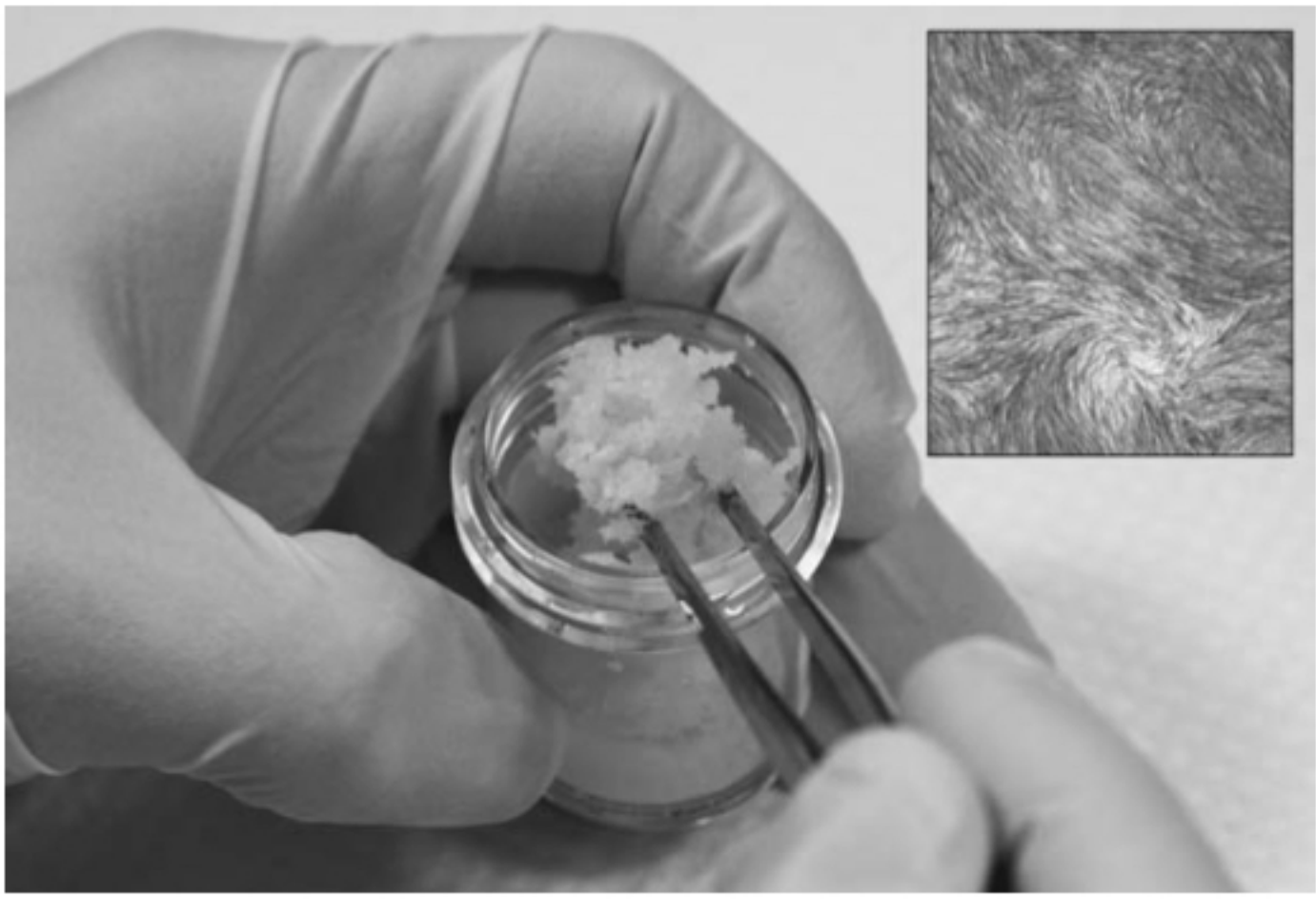

Fig. 2. Macroscopic aspect of the Osteocel® product. Following the appropriate thawing of the cellular allograft, there is a recommended four-hour window for its use. (*Inset*) Cellular image from the Osteocel® derived cells demonstrating positive osteogenic activity (staining positive for alkaline phosphatase activity).

Since the graft contains vital cells the maximum temperature of the water bath used during the thawing process should not exceed 37°C. After the cryopreserved cells are thawed, the liquid is decanted and the cells are rinsed with sterile saline. The cell containing graft is then ready for implantation, with a working window of four hours (Fig. 2). Depending on the defect treated, the particle size (1–3 mm) is often found to be too large for oral reconstructive procedures. In such instances rongeurs can be used to carefully reduce the particle size.

Scanning Electron Microscopy (SEM) images from this particulate graft have consistently revealed the cellular component of the allograft together with the extracellular matrix surrounding them. Figure 3 contains example SEMs at different magnifications. The SEM preparation starts with a 0.1M cacodylate buffer containing 5% sucrose rinse. The cells are fixed with 2.5% gluteraldehyde for one hour. Following three rinses with cacodylate buffer, the samples are soaked in 1% osmium tetraoxide solution in water for one hour at 4°C. The samples are subsequently dehydrated step-wise in increasing concentrations of ethanol starting at 50% and continuing to 100% for approximately two minutes at each step. A critical point dryer is used to dry the samples. The bone particles are attached to the SEM plates with adhesive and silver paint followed by a gold/platinum sputter coating prior to imaging. Images were captured utilizing a Quanta Model 600 (FEI, Hillsboro, OR) with a Tungsten filament at high vacuum mode and Soft Image Solutions (Olympus, Inc., Germany) software was employed for image collection.

3. Ridge Augmentation

With the greater acceptance and awareness of dental implant therapy as the strongest method of tooth replacement, amongst practitioners and patients alike, it is not uncommon to encounter reconstructive scenarios that require bone augmentation in the overall treatment plan. This is particularly seen in cases with long-standing edentulism, trauma and infection. Augmentation of the alveolar ridge for the ideal placement of an implant thus becomes necessary for an optimal esthetic outcome.[25] The various techniques and materials employed in ridge augmentation procedures have been discussed elsewhere and is beyond the focus of this chapter.[4] Of relevance, Osteocel®,

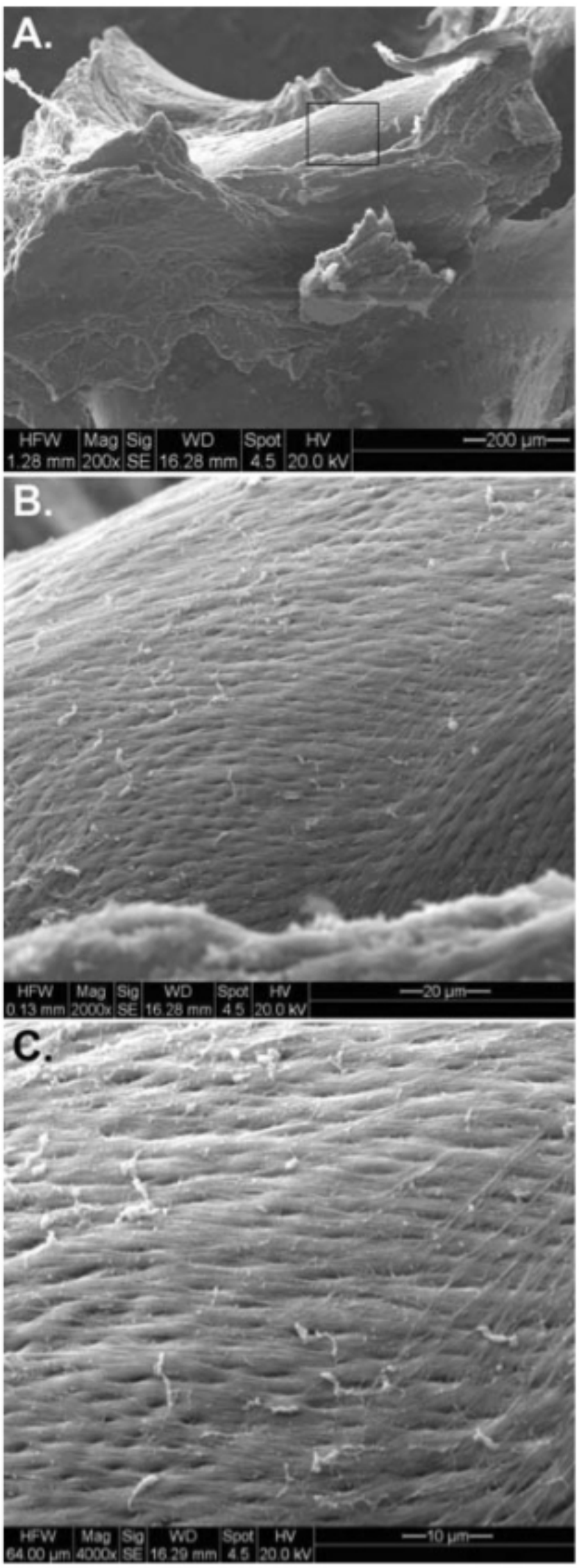

Fig. 3. **(A)** SEM image showing the cancellous bone coated with native cells. The cells are covered by extracellular matrix. **(B)** Higher magnification (2000×) of the box in **(A)**. **(C)** Higher magnification (4000×).

as a cellular based grafting material, provides an attractive option for ridge augmentation, particularly in larger defects.

It is hypothesized that the cellular contents within Osteocel® would benefit from a rapid revascularization. This revascularization process takes place at a much faster rate from the periosteal source than bone. To exploit this notion, use of a *classic* barrier membrane should ideally be avoided, when possible, to facilitate this process during the initial stages of healing. Other graft materials containing molecular enhancement products have likewise been shown in certain studies to undergo a slower healing rate when used in conjunction with a barrier membrane.[26,27] Figure 4 shows the successful use of Osteocel® alone for a small defect. The use of barrier membranes, however, may be deemed necessary when treating larger non space-maintaining defects for graft containment. In larger sized defects, as well as in cases where control of the location of the graft is critical, such as grafting against dental implants, a space-maintaining device in the form of a titanium mesh has been successfully employed.[4,28,29]

Titanium mesh offers resistance to bone graft collapse without the compromise in revascularization found with many products. However, when faced with a thinner tissue biotype caution is necessary when using any non-resorbable device as ensuing soft tissue dehiscences can lead to a compromised outcome.[14] Recently Pieri and colleagues demonstrated titanium mesh use in combination with a mixture of intraoral autogenous bone and xenograft on 16 partially edentulous patients.[30] They reported a mean horizontal augmentation gain of 4.2 mm. Only one of the cases showed early exposure of the mesh device. In cases where it was desired to regenerate more than 3 mm of horizontal bone we have predictably used the graft in conjunction with a titanium mesh for both space maintenance and graft containment preventing the cellular allograft from collapsing (Fig. 5).

4. Sinus Augmentation

The posterior maxilla represents an area that has historically posed a challenge for treatment with dental implants. These range from comprising sites with poor bone quality to unfavorable bucco-lingual resorption patterns and inadequacy in the vertical dimension of available bone following extraction of the teeth.[31,32] In addition, bone regeneration within

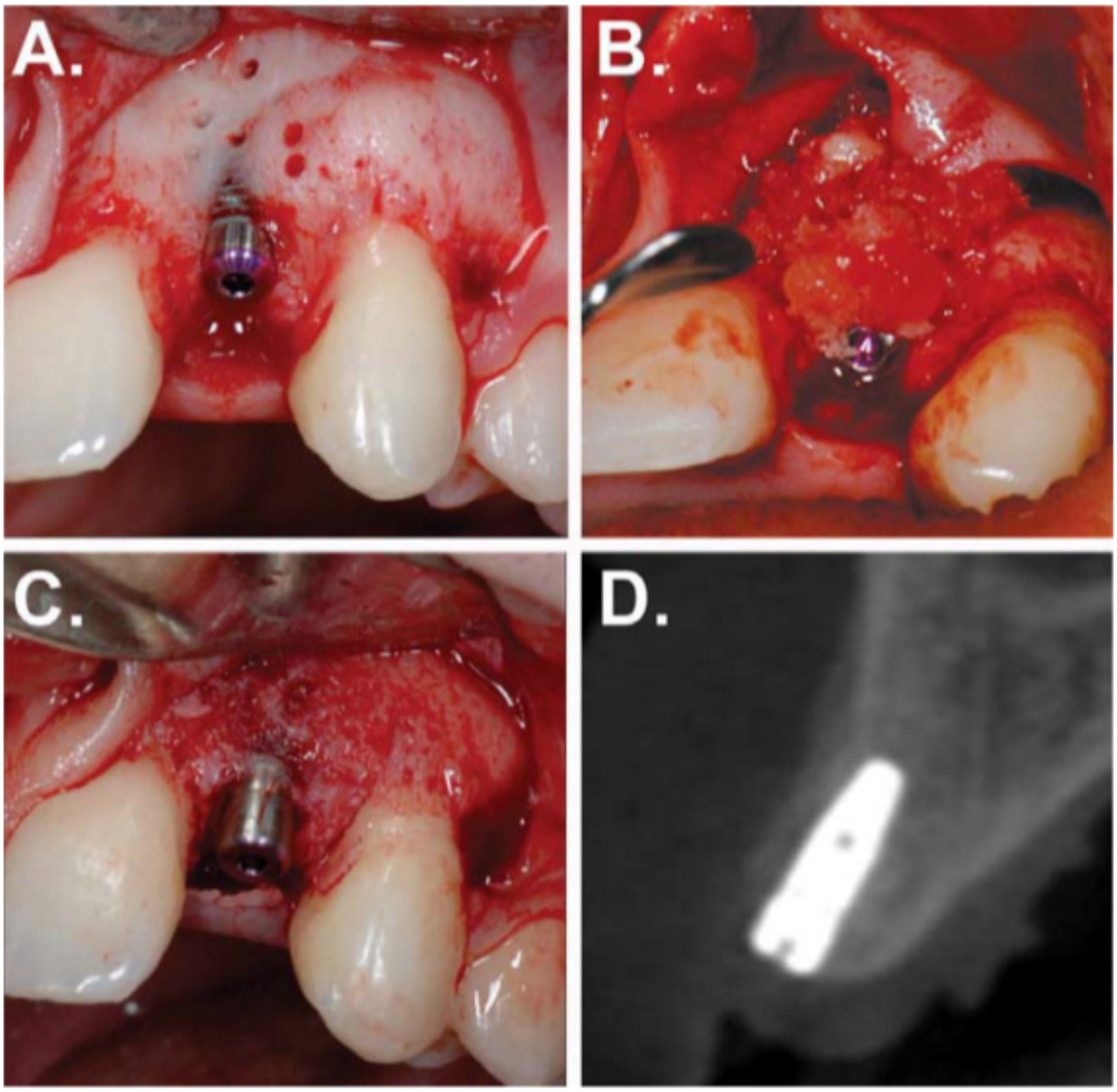

Fig. 4. Horizontal bone augmentation with Osteocel®. **(A)** Clinical view showing the dental implant dehiscence at the time of placement. Note intra-marrow penetrations have been made. **(B)** Clinical view with the cellular allograft in place. No membrane was utilized and the flap was sutured to tension-free primary closure with Vicryl™ sutures. **(C)** The four-month post-operative clinical view showing 2–3 mm of lateral bone augmentation and complete coverage of the implant threads. **(D)** A four-month post-operative CT scan showing the regeneration of a 2–3 mm thick buccal plate of bone over the implant.

the graft material is dependent upon it being populated by osteogenic cells that primarily originate from the osseous floors and walls, and to a smaller degree from the Schneiderian membrane.[33,34] Thus, cellular infiltration, vascularization, *de-novo* bone formation and graft replacement often require long healing times to produce bone of adequate quantity and quality for implant placement in the posterior maxilla.

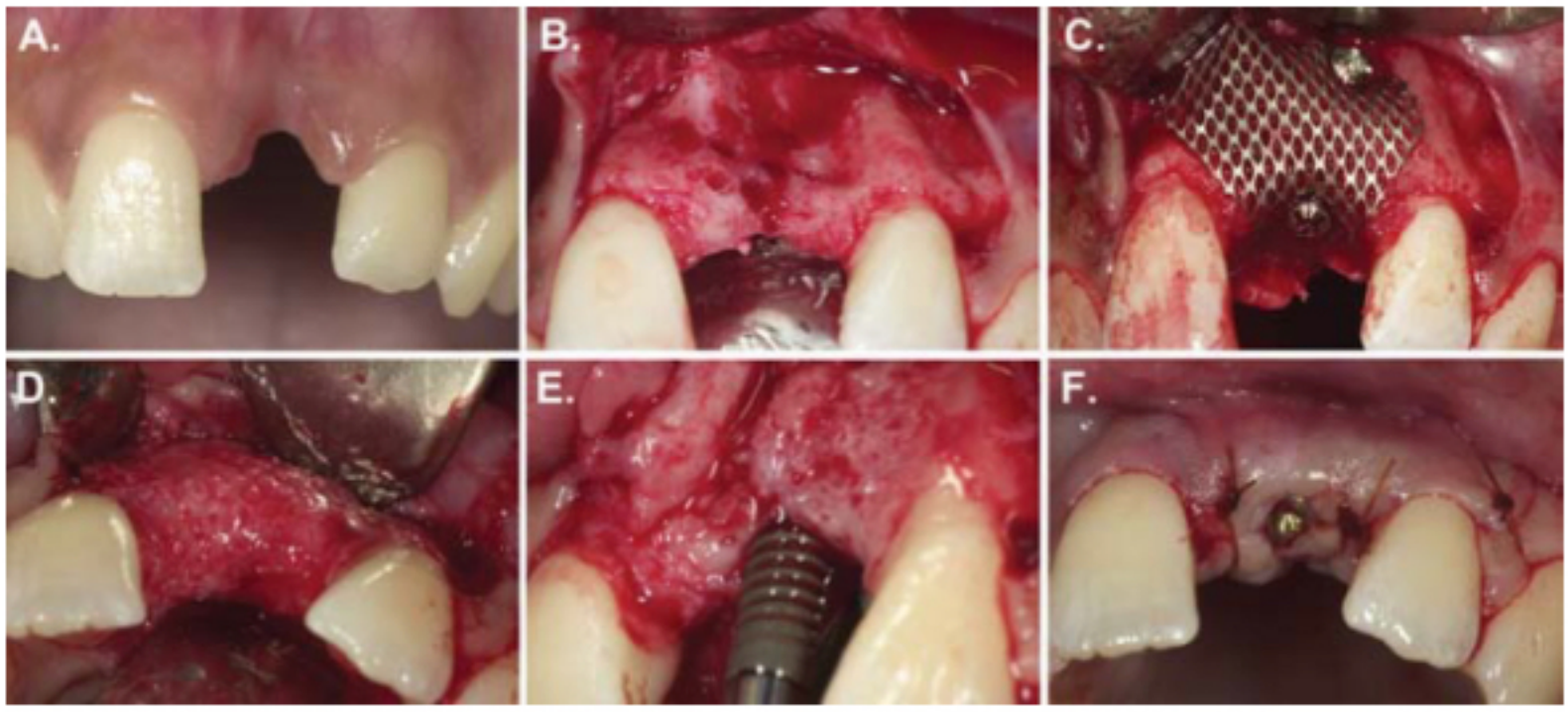

Fig. 5. Horizontal bone augmentation with titanium mesh and Osteocel®. (**A**) Pre-operative clinical view. (**B**) Mucoperiosteal flap elevation revealing a deficient alveolar ridge in the horizontal dimension. Perforation of the cortical layer with intra-marrow penetration has been performed for revascularization of the Osteocel® graft. (**C**) A titanium mesh has been adapted and secured with screws for containment of the cellular allograft material. (**D**) The four-month post-operative view following titanium mesh removal revealing a 3–4 mm increase in horizontal ridge dimensions. (**E**) View of bone regeneration and Straumann implant placement after a partial reflection of the pseudoperiosteum that is often found when using titanium mesh. (**F**) Closure of soft tissues after implant placement, completely within the healed bone graft, showing a normal facial ridge profile.

Ongoing maxillary sinus pneumatization and normal post-extraction bone atrophy has been managed successfully by the sinus augmentation procedure either before or simultaneously with implant placement. The literature is inundated with reports describing this procedure using a variety of grafting materials. The use of a variety of materials has shown a varied bone quality and quantity with reported percentage bone areas that range from as low as 5% to over 40%.[35,36] In addition, studies have demonstrated that it can take in excess of nine months to achieve optimal bone formation for implant stability.[37,38]

Dental implant technology has endeavored for a faster osseointegration period to allow for more rapid restoration of the lost dentition. The concept of molecular enhancement of graft materials with either growth or differentiation factors for a more rapid regenerative outcome becomes desirable.[39,40] Cellular enhanced bone graft materials potentially offer this timing advantage as well. Our experiences with Osteocel® as a sinus

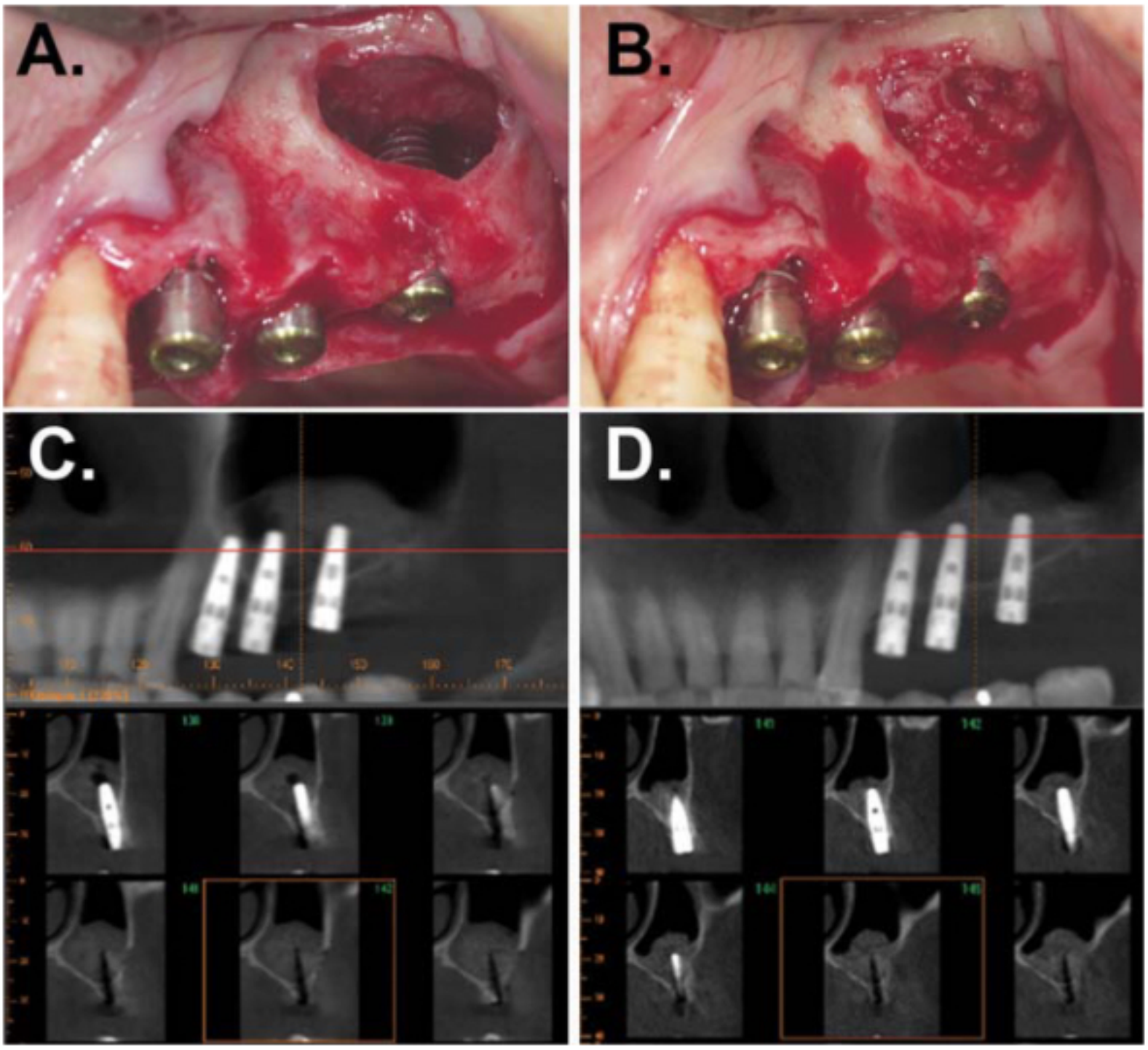

Fig. 6. Sinus augmentation with Osteocel®. **(A)** Sinus access after a classic lateral window approach with simultaneous implant placement. **(B)** Sinus after grafting with the cellular allograft. No membrane was used to cover the bone graft and lateral wall access window. **(C)** A CT scan of the grafted sinus immediately after graft and implant placement. **(D)** A CT scan of the same grafted sinus after four months of healing. Note the radiographic evidence of increased bone density in the bone graft region surrounding the dental implant. The radiographic findings of significant bone formation are consistent with the histologic data showing average percent bone areas in excess of 30%.

augmentation bone graft material has consistently provided promising outcomes.[24] This initial report based on histomorphometric analysis of grafted sinuses with Osteocel® showed an average vital bone content of 33% (range 22%–40%) and an average residual graft content of 6% (range 3%–7%) for cases that had an average healing period of 4.1 months (range from three to 4.75 months). These results were confirmed in a recent

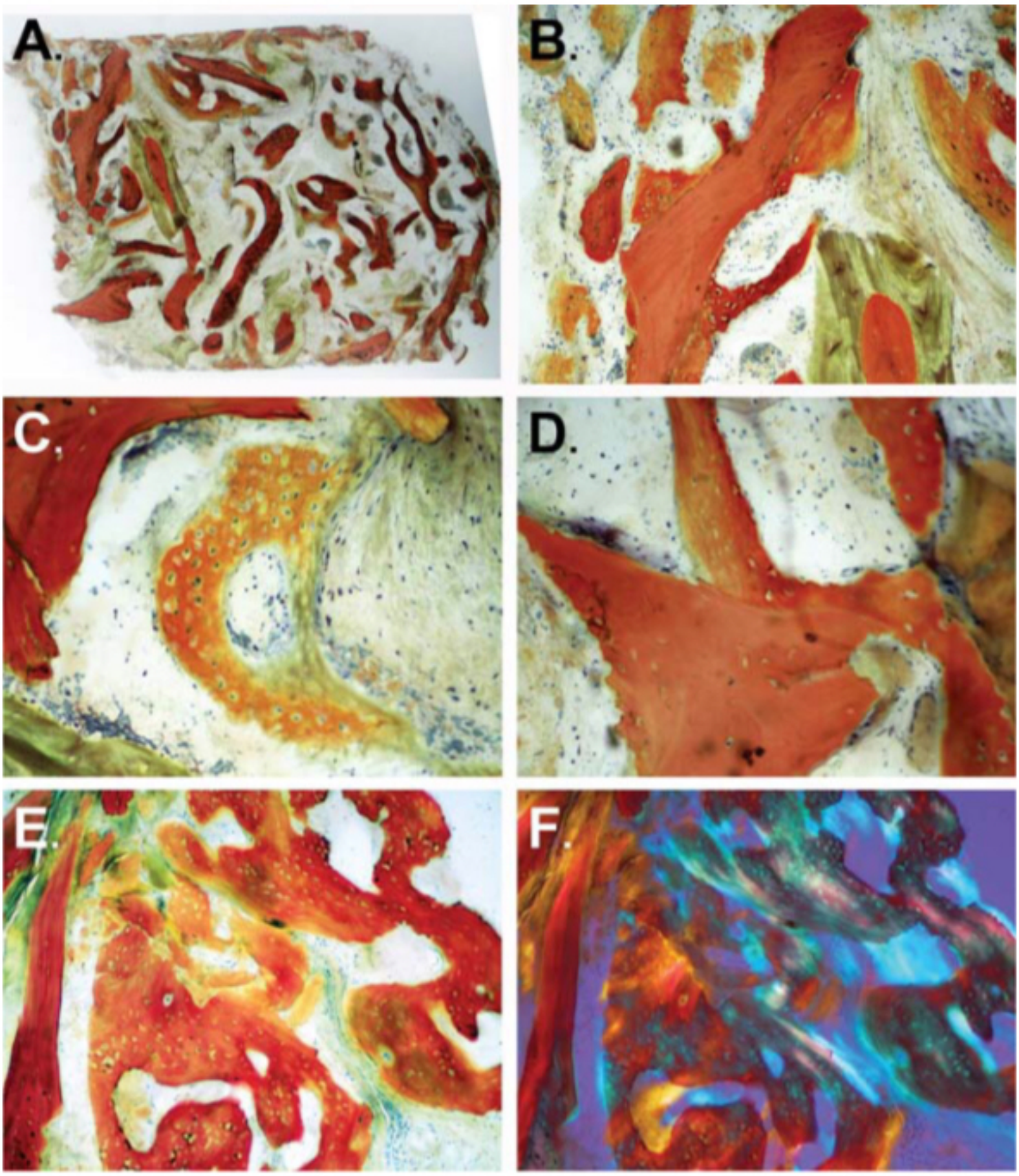

Fig. 7. Histological evaluation of the healed bone after sinus augmentation with cellular allograft residual. **(A)** Representative mineralized histologic core of a cellular allograft residual grafted sinus. The portion of the core shown goes from the most superior aspect (left side) to the original sinus floor (right side). It was harvested four months following the cellular allograft sinus grafting procedure. The red stained tissue is either the residual mineralized cellular allograft material (lighter red, osteocyte nuclei not always visible) or newly formed bone (darker red, osteocyte nuclei visible). The green stained tissue is the residual demineralized allograft material (no cells visible, non-vital bone). New bone formation can be appreciated throughout the core. Bone has formed directly on the residual mineralized and demineralized allograft particulate as well as in areas without residual graft material (original magnification of 20×). **(B)** New bone of varying levels of

larger multicenter study.[41] A faster graft healing time with respect to new bone formation in adequate quantities has encouraged an earlier initiation of implant placement and restoration. A sinus augmentation case with Osteocel® is shown with the radiographic follow-up (Fig. 6) and histological evaluation after four months (Fig. 7).

5. Discussion

Stem cells can be derived from a variety of sources, including bone marrow, and are used in a variety of medical therapies. In the future, medical researchers anticipate being able to use technologies derived from stem cell research to treat a wider variety of systemic diseases, in addition to site specific repair as was described in this chapter. To optimize these exciting applications for stem cells a thorough characterization and understanding of stem cell biology will be required. Although stem cells can be isolated based on a distinctive set of cell surface markers, *in vitro* culture conditions can alter the behavior of cells, making it unclear whether the cells will behave in a similar manner *in vivo*. In fact, debate exists whether some proposed adult stem cell populations are truly stem cells. Because of their combined abilities of unlimited expansion and pluripotency, embryonic stem cells remain a theoretically viable source for regenerative medicine and tissue replacement after injury or disease. Differentiating embryonic stem cells into usable cells and ultimately organs is a challenge that tissue

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Fig. 7. (*Continued*) maturation can be appreciated in this higher magnification view (original magnification of 100×). **(C)** Osteoblasts can be seen lining the newly formed bone (original magnification of 200×). **(D)** Several areas of bone graft resorption can be appreciated in this view. This osteoclastic activity along with the low original packing density is likely responsible for the small percentage area that is the original cellular graft material. Multiple multinucleated cells can be seen (original magnification of 200×). (**E**) The difference in the level of bone maturity with areas of immature woven bone (right side) and areas of mature lamellar bone (left side) can be appreciated (original magnification of 100×). (**F**) Previous field (E) seen under polarized light. The demineralized bone areas (green) did not refract the polarized light to the extent the mineralized bone areas did. The difference in the level of bone maturity with areas of immature woven bone (right side) and areas of mature lamellar bone (left side) can also be appreciated with respect to the level of polarized light refraction (original magnification of 100×).

engineering researchers will face for years to come. Additionally, the use of embryonic stem cells is more controversial than adult stem cells.

Stem cells and progenitor cells act as a repair system for the body, not only replenishing specialized cells, but also maintaining the normal turnover of regenerative organs and tissues. In spite of this important function, pluripotent adult stem cells are rare and generally small in number within the body, with most being lineage-restricted (multipotent). They have also been shown to decrease in number with age. Bone marrow contains numerous cell types from both the hematopoietic stem cell lineage (for example platelets, osteoclasts) and the non-hematopoietic stem cell lineage (for example MSCs, osteoblasts).[42] During the Osteocel® procurement procedures, immunogenic cells and tissues are substantially depleted. The potential for immune response is why typical fresh-frozen bone allografts are not as attractive of a grafting option, even though in a few reports success for oral reconstructive procedures has been demonstrated. One recent study on 21 patients reported high success with dental implant placement into fresh-frozen bone allograft regenerated bone.[43] Since only a disinfection process is performed with typical fresh-frozen bone allografts and not a complete immunodepletion process, there are likely large numbers of undesirable cells remaining in the graft. This could potentially impact the graft performance and the host immune response.

For multiple reasons MSCs enjoy a hypoimmunogenic host response. MSCs lack MHC-II and co-stimulatory molecule expression. MSCs are also immunomodulatory in that they prevent T-cell responses indirectly through modulation of dendritic cells and directly by disrupting NK as well as CD8+ and CD4+ cell function.[44] MSCs also induce a suppressive local microenvironment through cytokine production (prostaglandins and interleukin-10) and expression of indoleamine 2,3-dioxygenase which depletes the local milieu of tryptophan. One of the major proteins produced by MSCs is transforming growth factor-beta (TGF-beta) that regulates the host T-cells by promoting T-regulatory cells.

The cellular content of autogenous bone grafts varies based on the individual patient's medical profile, the harvest technique (aspiration or open harvest), anatomic location of the harvest (intra-oral or extra-oral), type of bone harvested (cortical or cancellous), age and gender. The cellular

content has also been shown to have an effect on the bone graft performance.[45] Therefore, the identification of MSCs and osteoprogenitor cells and determination of their concentrations in different anatomical tissues has been an area of recent investigation.[46–48] Evaluation of bone marrow aspirates from the anterior iliac crest revealed a fairly small count of MSCs, although a higher percentage of cells that tested positive for CD105 was found in the iliac crest aspirates when compared to peripheral blood.[48] McLain and colleagues compared the osteoprogenitor cell concentrations between iliac crest and vertebral body aspirates.[46] Their findings show that vertebral aspirates (465 cells/cc marrow) have a slightly higher mean concentration than the iliac crest aspirates (356 cells/cc marrow). The process used to prepare the cellular allograft bone matrix described in this chapter involves the selective removal of immunogenic cells in hematopoietic lineage from cell-rich cancellous bone, while retaining the osteopotent cells in the mesenchymal lineage. The minimum number of MSCs and osteoprogenitor cells found in the commercially available Osteocel® product is 50,000 cells/cc and for Osteocel® Plus product is 250,000 cells/cc. Clearly these cell counts are a dramatic improvement over the cell counts for aspiration harvests and may result in an enhanced clinical result. Recently Cuomo and colleagues have conducted a preclinical trial evaluating MSCs from human bone marrow aspirates in combination with demineralized bone matrix in athymic rat femur critical size defects.[49] Unprocessed MSC concentrations were found to vary from 64 to 2933 cells/ml with an average of 1010 +/– 960 cells/ml as determined from fibroblast colony forming unit (CFU-F) culture assays. MSC enriched bone marrow aspirate (centrifugation concentration) improved the yield to an average of 6150 cells/ml. Interestingly, the bone forming capabilities were found to be only comparable to the demineralized carrier alone. The authors mention the cell number may be insufficient to stimulate a robust bone formation. The clinical ramification of cell number has also been discussed by Hernigou and colleagues when treating tibia non-unions with marrow aspirates. They have identified 30,000 cells/ml as the minimum number of progenitor cells necessary to induce healing in this indication.[50] Another possibility is that since the MSCs have not had a chance to adhere to the carrier some may wash out of the wound further reducing the MSCs

influence on the healing. All of these considerations could have clinical implications when performing oral reconstructive surgery with bone marrow aspirates as has been presented by some clinicians.[48]

This exciting new cellular allograft technology will further assist us in the management of challenging oral regenerative procedures. Ongoing research is aimed at optimization of the clinical techniques and determining the long-term success of their application.

Acknowledgments

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