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Is bone transplantation the gold standard for repair of alveolar bone defects?

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Abstract
New strategies to fulfill craniofacial bone defects have gained attention in recent years due to the morbidity of autologous bone graft harvesting. We aimed to evaluate the in vivo efficacy of bone tissue engineering strategy using mesenchymal stem cells associated with two matrices (bovine bone mineral and α-tricalcium phosphate), compared to an autologous bone transfer. A total of 28 adult, male, non-immunosuppressed Wistar rats underwent a critical-sized osseous defect of 5 mm diameter in the alveolar region. Animals were divided into five groups. Group 1 (n = 7) defects were repaired with autogenous bone grafts; Group 2 (n = 5) defects were repaired with bovine bone mineral free of cells; Group 3 (n = 5) defects were repaired with bovine bone mineral loaded with mesenchymal stem cells; Group 4 (n = 5) defects were repaired with α-tricalcium phosphate free of cells; and Group 5 (n = 6) defects were repaired with α-tricalcium phosphate loaded with mesenchymal stem cells. Groups 2–5 were compared to Group 1, the reference group. Healing response was evaluated by histomorphometry and computerized tomography. Histomorphometrically, Group 1 showed 60.27% ± 16.13% of bone in the defect. Groups 2 and 3 showed 23.02% ± 8.6% (p = 0.01) and 38.35% ± 19.59% (p = 0.06) of bone in the defect, respectively. Groups 4 and 5 showed 51.48% ± 11.7% (p = 0.30) and 61.80% ± 2.14% (p = 0.88) of bone in the defect, respectively. Animals whose bone defects were repaired with α-tricalcium phosphate and mesenchymal stem cells presented the highest bone volume filling the defects; both were not statistically different from autogenous bone.

Keywords
Stem cell, bone, bone tissue engineering, biomaterial, α-tricalcium phosphate, alveolar defect, osseous defect

Introduction

Autologous bone transfers have been considered the standard method for approaching congenital and acquired bone defects in humans.[1] However, the morbidity of the procedure remains a drawback that has driven development of new strategies based on bone engineering, which may induce bone formation with less morbidity and similar efficiency.[2,3] The concept of tissue engineering was described by Langer and Vacanti[4] as an "interdisciplinary field that applies the principles of engineering and life sciences toward the development of biologic tissues that restore, maintain, or improve tissue function." Conceptually, it can be divided into three interconnecting elements: (1) the biomaterial matrix carrier/scaffold, (2) the cells in the matrix/scaffold, and (3) an environment or recipient site where this association resides.[5]

There are constant efforts to maximize the role of each of these elements to enhance the capacity of a chosen strategy

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to regenerate needed tissue. Concerning bone tissue engineering (BTE), advances in technology of biomaterials and characterization of mesenchymal stem cell (MSC) immunophenotype have contributed tremendously to this area.[6] As strategies of BTE continue to gain more solid scientific footing, they may fulfill an important gap in plastic and reconstructive surgery and may create opportunities to heal osseous defects with high efficiency and low morbidity.

At this point, there are at least six stages necessary to achieve effective BTE: (1) selection of the extracellular matrix scaffold, (2) isolation and characterization of the stem cells in vitro, (3) cell expansion and characterization, (4) inoculation of stem cells into extracellular matrix scaffold, (5) surgical application of the chosen BTE strategy into an osseous defect, and (6) evaluation of the effectiveness of the BTE strategy.[5,7–9]

Calcium phosphate matrices have been used in animal models to replace bone in critical-sized defects.[10,11] The main representatives of this group are α-tricalcium phosphate (TCP) and β-TCP.[12] Although they have similar chemical structures (α-$Ca_3(PO_4)_2$ and β-$Ca_3(PO_4)_2$, respectively), they differ in their crystalline structure,[13] namely, the solubility of α-TCP is higher than β-TCP.[13] Alpha-TCP can be added to calcium sulfate hemihydrates to increase solubility capacity, and it provides a higher porosity to facilitate cell adherence and ease osteoconductivity.[13] The bovine bone mineral, Bio-Oss collagen® (Geistlich Pharmaceutical®, Wolheisen Switzerland), possesses osteoconductive and biocompatibility properties.[14] It is characterized by a spongy structure and interconnected pore system that may facilitate cell adherence.[15–17] These biomaterial properties have driven researches to use them in the development of BTE strategies. The next stage of developing a BTE strategy is to define the source of cells to be used.

MSCs are adult clonogenic, non-hematopoietic cells. According to the *Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy*, the following criteria define MSCs:[15–19] (1) They are plastic-adherent when maintained in standard culture conditions. (2) They express the surface markers CD73/CD90/CD105 and lack expression of CD14/CD34/CD45, CD11b/CD79 and CD19/HLA-DR. (3) They are capable of differentiation into osteoblasts, chondrocytes, and adipocytes in vitro. (4) They present positive surface reaction for the CD73, CD90, and CD105 markers, all of which are key for defining MSCs.

Our group characterized the immunophenotype of muscle-derived stem cells from the *orbicularis oris* of patients born with cleft lip and palate.[20] BTE strategies based on these cells may represent a promising alternative to autogenous bone transfers.

The objective of this study was to evaluate the in vivo efficacy of a BTE strategy based on α-TCP and bovine bone mineral matrices associated with muscle-derived MSCs. We also aimed to compare its efficacy with autologous bone transfer. For this purpose, we used a critical-sized alveolar osseous defect in a rat model[21,22] that shows the anatomical parameters and its relation to teeth, maxillary sinus, and nasal cavity that could resemble a patient born with cleft deformity. These current BTE strategies have not yet been tested, and we aimed to bring evidence for the use of BTE in the rehabilitation of cleft patients.

Methods and materials

Study design

Our experimental protocol was approved by the Ethical Committee of the Institute of Biosciences at the University of São Paulo (USP), Brazil (permit numbers 076/2008; 588/2009). Rats were kept according to guidelines in the Guide for the Care and Use of Laboratory Animals (Institute for Laboratory Animal Research, 1996) and according to the ethical principles of the Brazilian College on Animal Experimentation (COBEA). Adult, male Wistar rats weighing 300–370 g were individually housed in a temperature-, light-, and humidity-controlled environment.

A total of 28 adult, male non-immunosuppressed Wistar rats underwent a 5-mm round critical-sized osseous defect in the alveolar region.[21,23] Previous studies using these particular types of MSCs and other types of MSCs have demonstrated that BTE strategies with human MSCs used to repair osseous defects in rats do not provoke immunological response or suffer rejection.[20,24–29]

Animals were divided into five groups (Figure 1). In Group 1 (n = 7), our reference group defects were repaired with autogenous bone grafts; in Group 2 (n = 5), defects were repaired with bovine bone mineral free of cells; in Group 3 (n = 5), defects were repaired with bovine bone mineral loaded with MSCs; in Group 4 (n = 5), defects were repaired with α-TCP free of cells; and in Group 5 (n = 6), defects were repaired with α-TCP loaded with MSCs. Due to technical difficulties and following the recommendation of the Ethical Committee Board of Animal Research of the Institution, it was not possible to have the planned seven animals per group.

Isolation of stem cells from the orbicular oris muscle of cleft patients

Stem cells were isolated from small fragments of the *orbicularis oris* muscle, which was discarded after the primary correction of cleft lip repair. This study included tissue samples from three 3-month-old cleft lip and palate patients, randomly selected among 20 patients, who were enrolled by a consent form signed by their parents, in accordance with the guidelines of the Ethical Committee of the Institute of Biosciences at the USP (permit number

Figure 1. Illustration of the alveolar osseous defect of animals.

037/2005). We only included donors who did not present systemic diseases and/or oral infections and who were not syndromic.

Cell culture

MSCs were obtained using a pre-plating technique, previously described by our group.[20] They were cultured in a Dulbecco's modified Eagle medium (DMEM)/F12 medium (Invitrogen®, São Paulo, Brazil) with 15% fetal bovine serum (FBS; Hyclone, Hyclone Laboratories, Logan, UT, USA), 2 mM L-glutamine, 2 mM nonessential amino acids (Invitrogen), 100 units/mL penicillin, and 100 mg/mL streptomycin (Invitrogen) with medium changes every 3 days. The target concentration of cells after a 15-day culture used to seed the matrices was 1.0×10^6 cells/mL, obtained at third passage.

Immunophenotype of the MSCs from the orbicularis oris muscle

The osteogenicity of MSCs from the *orbicularis oris* muscle has been studied by our laboratory and has been previously shown to induce in vitro *and* in vivo bone formation in critical-sized calvarial osseous defects in rats.[20]

Flow cytometry analysis was performed using a Guava Easy Cyte microcapillary flow cytometer (Guava Technologies, Hayward, CA, USA) utilizing laser excitation and emission wavelengths of 488 and 532 nm. Cells were pelleted and resuspended in phosphate-buffered saline (PBS; Gibco-Invitrogen®, Carlsbad, CA, USA) at a concentration of 1.0×10^6 cells/mL and stained with saturating concentrations of antibodies. After a 45-min incubation in the dark at room temperature, cells were washed three times with PBS and resuspended in 0.25 mL of cold PBS. In order to analyze cell surface expression of typical protein markers, adherent cells were treated with the following primary anti-human antibodies: CD29-PE-Cy5, CD31-PE, CD45-FITC, CD73-PE, CD90-R-PE, and CD105-PE (Becton Dickinson, Franklin Lakes, NJ, USA). Unstained cells were gated on forward scatter to eliminate particulate debris and clumped cells. A minimum of 5000 events were counted for each sample.

These cells presented high expression of MSC markers (CD73-PE, CD90-R-PE, CD105-PE) and lack of expression of hematopoietic and endothelial markers (CD29-PE-Cy5, CD31-PE, CD45-FITC).

Preparation of matrices

A quantity of 100 mg of Bio-Oss collagen, a bovine bone mineral matrix with spongy consistency in commercial form, was equally divided into three parts of 33 mg each. Each part was used to seed 1.0×10^6 cells/mL of undifferentiated MSCs and placed on a 35-mm plate (six-well plate; Corning, NY, USA). Prior to transplantation, the cells were supplemented with 2.5 mL of medium and incubated at 37°C and 5% CO_2 for 24 h in order to adhere to the spongy surface of the Bio-Oss collagen. The same type of medium culture was used with the Bio-Oss collagen, free of cells, and transplanted into animals from the control group.

Alpha-TCP matrix (Labiomat®, Brazil), consisted of a powder (75% of α-TCP and 15% of calcium sulfate hemihydrate), and a solution containing an accelerator (2.5% of Na_2HPO_4) was added until a solid consistency was formed. The α-TCP matrices were prefabricated into a 5-mm round matrix, and then leached and seeded with 1.0×10^6 cells/mL of undifferentiated MSCs using the same protocol as previously described.[20] After removing both matrices containing the MSCs from the 35-mm plate, the remaining cells that did not attach with the matrices were counted using a cell counter (Invitrogen) and ranged from 300 to 500 cells. These leftover cells were considered insignificant in comparison to 1.0×10^6 cells/mL included on the matrices. The same medium culture was used with the customized α-TCP implants, free of cells transplanted into animals of the control group.

Surgical model

Preoperatively, all animals were shaved and weighed and aseptic techniques were used. Animals were anesthetized with an intraperitoneal injection (0.3 mL/100 g body weight) of ketamine hydrochloride (5%) combined with xylazine (2%). Supplemental local anesthetic (2% lidocaine with 1:100,000 epinephrine, 7 mg/kg lidocaine, 5 μ/kg epinephrine) was also used.

The rats were placed in a lateral position for the surgical procedure. Lidocaine with adrenaline (0.3 mL) was used

for anesthesia of the oral mucosa. A 2-cm incision was made in the transitional zone of dry to wet oral mucosa. The underlying muscle and maxillary periosteum were elevated. The maxilla and zygomatic bones were completely dissected. A high-speed trephine burr of 5 mm in diameter was used with constant irrigation to create the osseous defect in the alveolar region. The same trephine was used to harvest a full-thickness calvarial bone grating from the parietal region, which was inserted into the created osseous defects of Group 1.

The matrices were inserted into animals of Groups 2, 3, 4, and 5 after the creation of alveolar osseous defects, according to the distribution of the groups. The matrices were secured in place using local muscle flaps. Skin incisions were closed by layers of superficial sutures using nylon 5-0 (Ethicon, Brazil).

Animals were fed a standard soft diet for 1 week after surgery and a regular diet for the subsequent weeks. After 8 weeks, the rats were killed by carbon dioxide narcosis, and histomorphometric analysis was performed on the alveolar region of all animals to quantify bone and fibrosis formation in all groups. Before being killed, five animals of each group underwent a computerized tomography (CT) examination.

Radiographic analysis

The rats' craniofacial skeletons were immediately imaged after surgery and postoperatively at 8 weeks by CT using a Shimadzu® SCT-7800TC (n = 5 in each group). Serial 1-mm-thick coronal sections of full craniofacial segments yielded 56 slices per sample. Scans were reconstructed as three-dimensional isosurfaces using Osirix Dicom Viewer® software (Apple Inc. (Website)). Radiological analysis assessed the remaining osseous defects and the amount of bone tissue like in the osseous defects based on three possibilities: partial bone healing scored 1 point, complete heterogeneous bone healing scored 2 points, and complete homogeneous healing scored 3 points. The minimal remaining full-thickness osseous defects classified the animals' defects as partial bone healing. Flat bone tissue like that filled the entire osseous defect seen in the lateral, basal, and oblique tomographic profiles classified the animals' defects as homogeneous bone healing. Any type of exacerbation of bone-like tissue shown in the lateral, basal, and oblique tomographic profiles classified the animals' defects as heterogeneous bone healing. A maximum of 15 points were possible per group and determined complete homogeneous healing of all animals in the group. The average scores were given in each group by two blinded observers, who analyzed 25 samples (five samples for each of the five groups).

Histomorphometric analysis

The regions of osseous defects were dissected and cut using a micro saw (Aesculap®, Germany). They were cut into a square shape preserving the proximal segment of the zygomatic arch as an anatomic landmark to orient subsequent histomorphometric cuts.

The specimen was fixed in 70% dehydrated ethanol, embedded in methylmetacrylate, and sectioned longitudinally using a Polychrome S microtome (Reichert-Young®, Heidelberg, Germany). We obtained 5-μm sections from the original osseous defects of the specimens. The 5-μm sections were stained with 0.1% toluidine blue at a pH of 6.4, and at least two non-consecutive sections were examined for each sample. The static indices of the bone structure were bone volume as a percentage of the tissue volume (BV/TV) and fibrosis volume as a percentage of the total volume (Fb.V/TV).[30] Assessment of fibrosis volume was used to quantify the amount of scarring tissue induced by the BTE strategies.[23]

All analyses were performed under a light microscope using 125× of magnification (Nikon®, Japan) and a semiautomatic method (Osteometrics Inc®, Atlanta, GA). Histomorphometric indices were reported according to the standard nomenclature recommended by the *American Society of Bone and Mineral Research*.[30] Sequential alternated markings with short intervals using tetracycline (20 mg/kg) and calcein (10 mg/kg), two doses of each in alternating weeks, were performed to study the overall healing distribution and to complement the imaging obtained by CT and histomorphometric evaluation.

Statistical analysis

Data were expressed as mean ± standard deviation, and statistical significance was determined using the Mann–Whitney test. Cohen's kappa coefficient was used to evaluate interobserver agreement on radiographic analysis. A value of p <0.05 was considered significant. All analyses were completed using Statistical Package for Social Science (SPSS® version 10.0 for Windows, Inc., Chicago, IL, USA).

Results

Radiographic analysis

The mean scores for the five groups rated by the two observers was 2 ± 0 for Group 1, 1.4 ± 0.52 for Group 2, 1.5±0.53 for Group 3, 1.6 ± 0.52 for Group 4, and 1.8 ± 0.42 for Group 5 (Figures 2–4). The comparison of Group 1 with Group 2 (p = 0.051), Group 3 (p = 0.053), Group 4 (p = 0.134) and Group 5 (p = 0.317) did not statistically differ. The observers scored an identical value for all groups, except for Group 3, which presented interobserver variability of ±1 (k = 0.911) (Table 1).

Histomorphometric analysis

Through histomorphometric analysis, at 8 weeks post-surgery, we observed average bone volumes in the alveolar

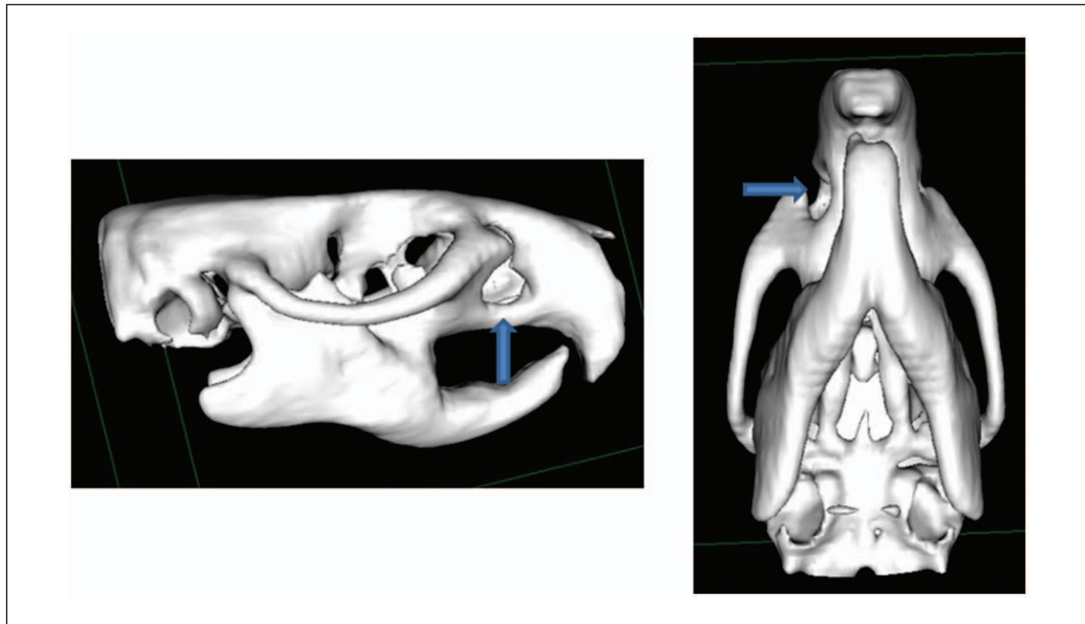

Figure 2. Reformatted computerized tomography imaging showing the initial size of the bone defect as 5 mm in diameter. The blue arrows show the alveolar osseous defect.

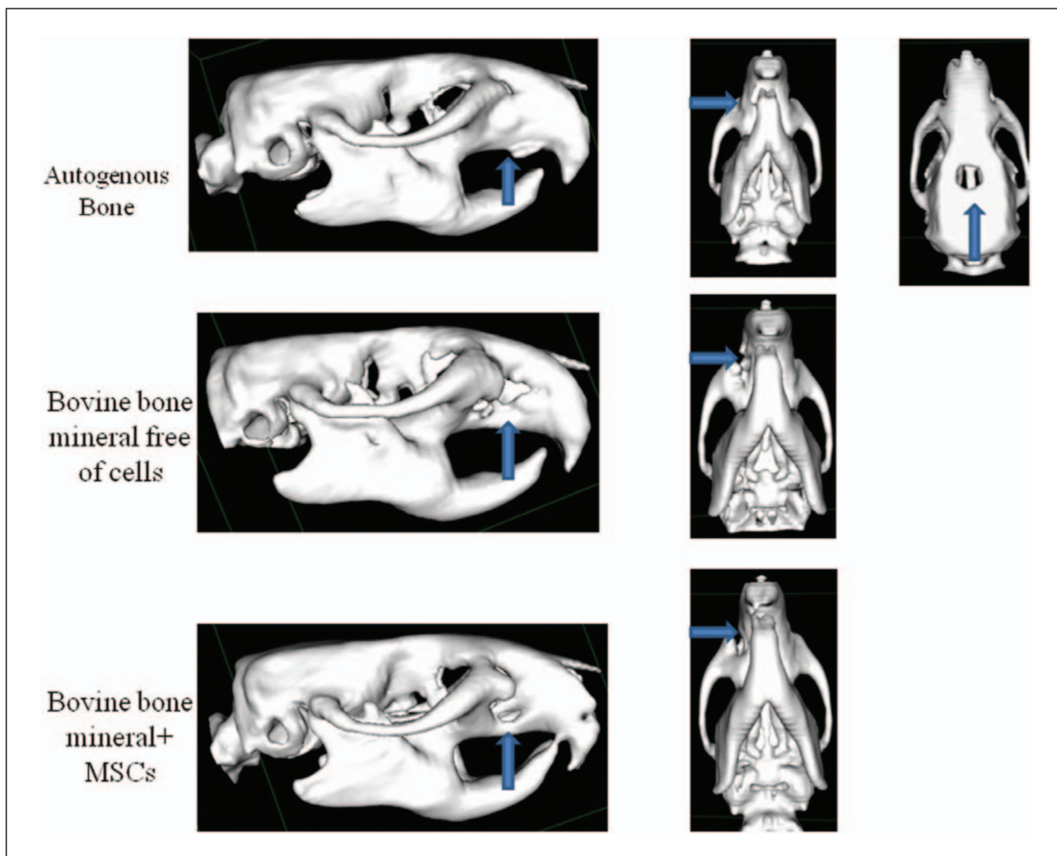

Figure 3. Reformatted computerized tomography imaging showing one example of three modalities of treatment containing the best distribution of bone into the osseous defect at 8 weeks after surgery repaired with autogenous bone graft (*first row*), bovine mineral bone free of cells (*second row*), and bovine mineral bone loaded with mesenchymal stem cells (*third row*). The blue arrows show the region of the alveolar osseous defect repaired with the different modalities, except the donor region of autogenous bone shown in the last column of the first row.
MSC: mesenchymal stem cell.

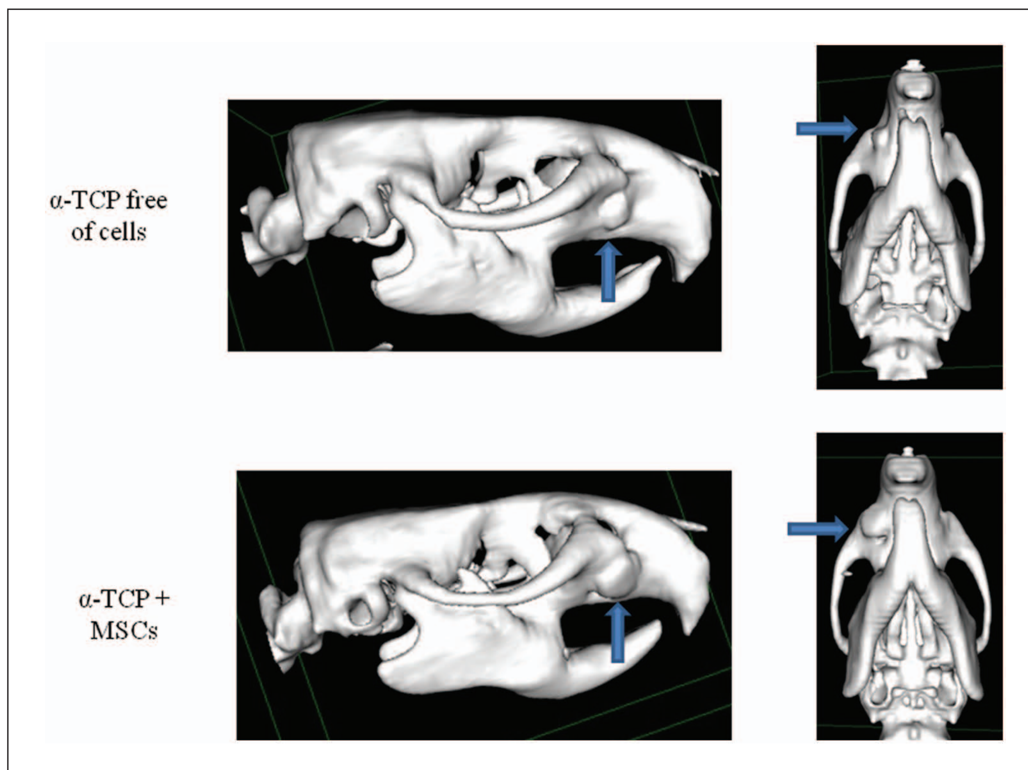

Figure 4. Reformatted computerized tomography imaging showing one example of two modalities of treatment at 8 weeks after surgery using α-TCP free of cells (*first row*) and α-TCP loaded with mesenchymal stem cells (*second row*). The blue arrows show the alveolar osseous defect repaired with the different modalities.
TCP: tricalcium phosphate; MSC: mesenchymal stem cell.

defect of 60.27% ± 16.13% for Group 1, 23.02% ± 8.6% for Group 2, 38.35% ± 19.59% for Group 3, 51.48% ± 11.7% for Group 4, and 61.80% ± 2.14% for Group 5. The bone volume of Group 1 was not statistically different from Group 4 ($p = 0.3$) or Group 5 ($p = 0.88$), whereas it statistically differed from Group 2 ($p = 0.01$) and presented a trend toward statistical difference from Group 3 ($p = 0.06$). The animals of Group 3 presented a trend to generate statistically more bone than Group 2 ($p = 0.06$), whereas Group 5 did not present statistically more bone than Group 4 ($p > 0.05$) (Figures 5 and 6).

Through histomorphometric analysis, at 8 weeks post-surgery, average fibrosis volumes in the alveolar defect were 3.89 ± 10.24% for Group 1, 19.85% ± 7.04% for Group 2, 18.55% ± 12.41% for Group 3, 13.24% ± 12.07% for Group 4, and 0.64% ± 1.56% for Group 5.

Group 1 showed uniform platforms of bone formation labeled by calcein and tetracycline labeling, while Groups 2 and 3 showed an island of bone healing, and Groups 4 and 5 showed bone healing surrounding the α-TCP matrices (Figure 7).

Discussion

This study tested the capability of BTE strategies to heal alveolar bone defects in rats and their negative controls (matrices free of cells) and compared them with autogenous bone transfers. Our results indicate that BTE strategies induced statistically similar bone volume in the defect compared to autogenous bone transfers. Bio-Oss matrix free of cells statistically differed from autogenous bone transfers, while α-TCP free of cells did not differ either from autogenous bone transfers or Group 5 (α-TCP loaded with MSCs).

Several variables may interfere with the success of BTE testing.[5,6,31–33] These include (1) species and age of the chosen animal model, (2) anatomical site of the osseous defect, (3) physical and chemical properties of a chosen matrix and its capacity to regenerate bone free of osteoinductive agents or MSCs, and (4) type and density of an osteoinductive cells. Each of these variables is discussed below.

First, in terms of species and age, a rat model is easy to manipulate, the animals are easy to house and feed, and it is a less expensive model compared to other bigger models involving rabbits, dogs, and goats, for example.[14]

Second, although a calvarial rat model is a well-documented model for evaluating bone healing,[14] it does not represent a close parallel to the alveolar osseous defects present in patients born with cleft lip and palate. The alveolar region has its own particular characteristics, predominantly consisting of cancellous bone that is thinner

Table 1. Blinded scoring system rated for two observers based on computerized tomography imaging evaluation.

	Animals	Observer 1	Observer 2	Average (M)	Total	Group average (M ± SD)
Group 1	1	2	2	2	10	2 ± 0
	2	2	2	2		
	3	2	2	2		
	4	2	2	2		
	5	2	2	2		
Group 2	1	1	1	1	7	1.4 ± 0.52
	2	2	2	2		
	3	2	2	2		
	4	1	1	1		
	5	1	1	1		
Group 3	1	1	1	1	7.5	1.5 ± 0.53
	2	2	2	2		
	3	2	2	2		
	4	1	2	1.5		
	5	1	1	1		
Group 4	1	1	1	1	8	1.6 ± 0.52
	2	2	2	2		
	3	2	2	2		
	4	1	1	1		
	5	2	2	2		
Group 5	1	2	2	2	9	1.8 ± 0.42
	2	1	1	1		
	3	2	2	2		
	4	2	2	2		
	5	2	2	2		

M: mean; SD: standard deviation; Group 1: autogenous bone graft; Group 2: bovine bone mineral free of cells; Group 3: bovine bone mineral + mesenchymal stem cells (MSCs); Group 4: α-tricalcium phosphate (TCP) free of cells; Group 5: α-TCP + MSCs.

and has higher porosity than the calvarial bone.[34] Thus, it is an ideal site to test less brittle materials: first, because there is no need to protect the underlying neurovascular bundle, and second, because a softer material presents a higher porosity that can ultimately simulate the cancellous bone of this region and also facilitate teeth eruption and mobilization during orthodontia. Therefore, the mechanical strength in this region is of less importance when compared to the calvarial region. De Ruiter et al.[34] repaired alveolar defects in a goat model using β-TCP. De Ruiter et al.[34] emphasized the importance of orthodontic tooth movement while repairing an alveolar defect with these biomaterials. We believe that α-TCP may behave even better than β-TCP for this particular situation, owing to its higher porosity and solubility.[35] However, it may not represent a satisfactory therapeutic option for repairing a calvarial defect because of its weak mechanical strength.

Aalami et al.[31] demonstrated the different regenerative abilities of juvenile versus adult murine models to regenerate bone in the osseous defects of the calvarial regions. Although it has been demonstrated that these differences occur mainly because of the osteoinductive role of the dura mater, the bigger craniofacial skeleton of adult rats allows the osseous defects to be standardized easier than the juvenile model. We also only use one time period to kill all animals because previous studies showed that the ossification rate of alveolar critical-sized defects reaches a plateau at 8 weeks after surgery.[36] This period of time seems to be ideal to test BTE strategies.

Third, in terms of physical and chemical properties, Bio-Oss collagen is a bovine bone mineral with osteoconductive and biocompatibility properties.[16] Mokbel et al.[14] evaluated the capacity of Bio-Oss free of cells, among other matrices, in the healing of critical-sized calvarial bony defects and compared them to an autogenous bone graft. Their data showed 24.6% and 37.1% bone regeneration in the defects for bovine bone mineral and autogenous bone, respectively.[14] Schmitt et al.[37] have also demonstrated their capacity to regenerate bone in a critical-sized bone defect. Pinto et al.[11] compared the capacity of α-TCP free of cells and autogenous bone to repair bone defects in the femurs of animal models and showed a similar regenerative capacity between the two. The results of Mokbel et al.[14] and Pinto et al.[11] corroborated ours, indicating (1) low bone volume in the bone defects by using the bovine bone mineral free of cells and (2) high bone volume in the bone defects by using α-TCP free of cells. Thus, this osteoconductive capacity associated with the favorable chemical and physical properties makes them a potential matrix to transport and deliver multipotent adult stem cells in bone defects.

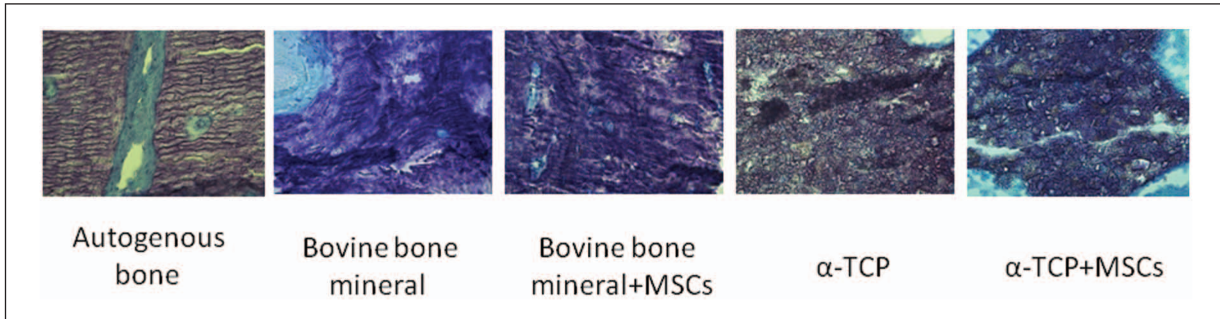

Figure 5. Toluidine blue staining of representative sagittal sections of alveolar osseous defects at 8 weeks after surgery in each group. Woven bone is displayed in all samples (from left to right; Groups 1, 2, 3, 4, and 5, respectively, under 400× magnification).
TCP: tricalcium phosphate; MSC: mesenchymal stem cell.

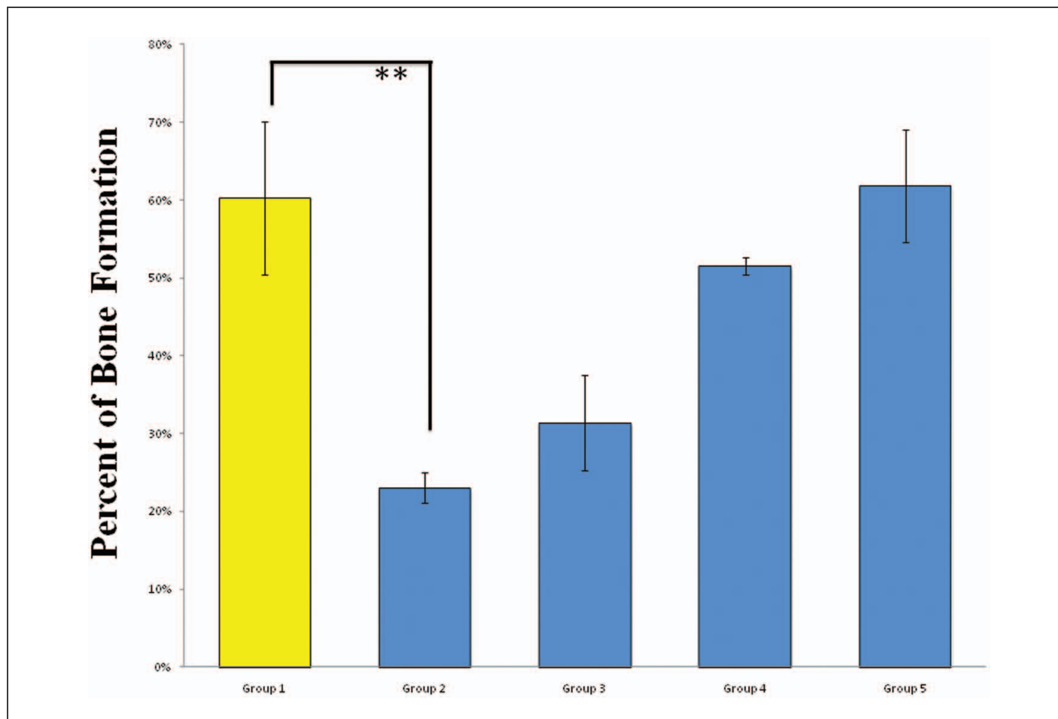

Figure 6. Histomorphometric quantification of bone formation within the alveolar osseous defect. *Asterisks* denote statistical significance ($p < 0.05$).

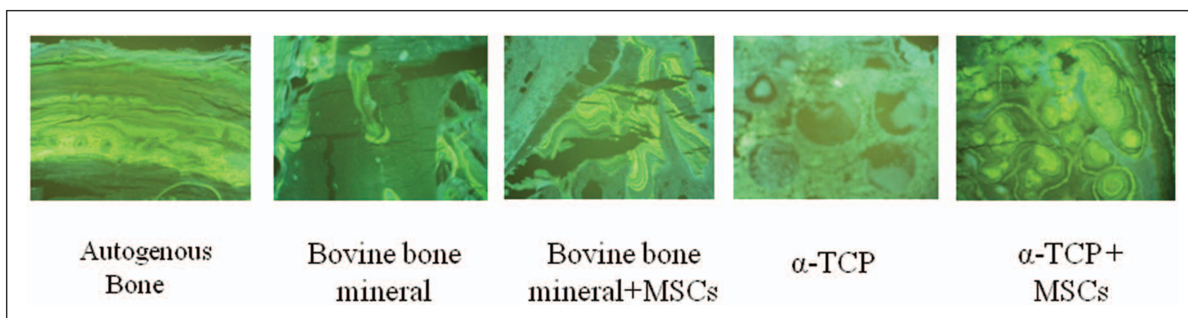

Figure 7. Tetracycline and calcein staining of maxillary alveolar defects showed a uniform platform of bone formation in Group 1, whereas, Groups 2 and 3 showed an island healing pattern of bone formation in the entire defect. Group 2 did not grossly differ from corresponding sections with mesenchymal stem cells. Groups 4 and 5 showed a bone island adjacent of α-TCP. Group 4 did not grossly differ from corresponding sections with mesenchymal stem cells (magnification 125×).
TCP: tricalcium phosphate; MSC: mesenchymal stem cell.

Langer and Vacanti[4] described the priorities of tissue engineering and the three most important characteristics of stem cells: (1) a reliable cell source of stem cells, (2) a high proliferative capacity, and (3) the ability to differentiate into a variety of cell types to restore lost tissue. The MSCs from the *orbicularis oris* of patients born with cleft lip and palate fulfill each of these criteria.[20] These cells can be easily isolated from small fragments of *orbicularis oris* muscle, which is usually discarded during the lip repair, and which offers an abundant source of cells. It offers satisfactory in vitro expansion and immunophenotype characterization in MSCs with positive reaction to key markers of MSCs.[20]

Finally, the density of MSCs also seems to play an important role in the effectiveness of a BTE strategy.[38]

Choi et al.[38] tested different volumes of human adipose tissue-derived MSCs to heal osseous defects in femoral regions of an animal model. They concluded that new bone formation is directly increased by the volume of cells loaded into the matrix. We showed a similar volume of bone in the defect using a different source of MSCs and approximately the same density of cells used by Choi et al.[38] Jafarian et al.[39] used bone marrow–derived stem cells to regenerate bone defects of the mandibles of a canine model using a similar BTE strategy. Jafarian et al.[39] also used the Bio-Oss and TCP as a carrier of adult MSCs (5×10^5 cells/mL) and found 51.31% ± 6.67% (for Bio-Oss + MSCs) and 65.78% ± 4.94% (for TCP + MSCs) of bone in the defect at 6 weeks after surgery. The relatively small difference between our results and those of Jafarian et al.[39] further confirms the osteogenic potential of muscular-derived MSCs. It is possible that an increasing volume of cells seeded in Bio-Oss could lead to a better result.

Taub et al.[40] also demonstrated the capacity of undifferentiated muscle-derived MSCs to regenerate bone in the calvarial region of an animal model. Although previous studies[41,42] showed the requirement of MSCs to undergo differentiation using bone morphogenetic protein (BMP-2) and other osteogenic mediums before being considered as a BTE strategy to regenerate bone in a calvarial bone defect, Taub et al.[40] showed calvarial bone healing using undifferentiated muscular-derived stem cells. We agreed with Taub et al.[40] and used undifferentiated cells, thereby decreasing the morbidity of the procedure by avoiding the necessary passages to obtain in vitro osteogenic differentiation before its inclusion into a matrix. Although we could not categorically affirm if the presence of MSCs determined the additional bone formation, we believe that local factors mediate MSC differentiation and induce bone formation by recruiting murine host osteogenic cells, especially in the presence of the Bio-Oss matrix, whose data showed a trend to generate statistically more bone in Group 3 (in which defects were repaired with bovine bone mineral loaded with MSCs) compared to Group 2 (in which defects were repaired with bovine bone mineral free of cells). Future studies to evaluate the osteoinductive role of MSCs will require MSC markers to confirm this hypothesis and also to identify the role of these matrices in cell differentiation. Another controversy is using human MSCs in the murine model. Previous studies[20,26–29,43] demonstrated that BTE strategies with human MSCs used to repair osseous defects in rats do not provoke immunological response or suffer rejection. It has been suggested that MSCs do not express MSC Class-II molecules or co-stimulatory molecules (B7 and CD 40), as they also may secrete factors that inhibit an immune response.[44] We also hypothesized that the primitive immune system of the murine model may contribute to this phenomenon.

A limitation of our study was to use CT (Shimadzu, SCT-7800TC), which was the only available equipment to analyze the bone formation in two different time periods without killing the animals. Since animals were imaged just after surgery to verify the consistence of osseous dimensions and location, the CT imaging used in this study aimed to complement the data found by the computerized histomorphometric analysis in the same specimens. Thus, the CT imaging score grading was a semi-quantitative method of analysis based on the blind and random assessment of two evaluators that assess the remaining osseous defects and the amount of bone-like tissue in the osseous defect. Interestingly, the data obtained from the radiological analysis corroborate the computerized histomorphometric analysis.

Given the aforementioned caveats, it is clear that MSCs combined with α-TCP to build a BTE strategy support satisfactory volume of bone formation, and it is not statistically different than autogenous bone transfer.

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Author contributions

C.E.R.-A., D.F.B., A.B.A., C.C.C., R.D.F., M.R.P.-B., and N.A. conceived and designed the experiments. C.E.R.-A., D.F.B., A.B.A., C.C.C., and R.D.F. performed the experiments. C.E.R.-A., V.J., C.H.G., M.R.P.-B., and N.A. analyzed the data. D.F.B., R.D.F., L.C.V., and M.R.P.-B. contributed reagents/materials/analysis tools. C.E.R.-A., M.R.P.-B., and N.A. wrote the article.

Declaration of conflicting interests

The authors have declared that no competing interests exist.

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