Repair of Critical-Size Bone Defects Using Bone Marrow Stem Cells or Autogenous Bone With or Without Collagen Membrane: A Histomorphometric Study in Rabbit Calvaria

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Purpose: The aim of this study was to evaluate bone healing after the use of a xenograft scaffold enriched with bone marrow mesenchymal stem cells (BM-MSCs), an autogenous bone graft, or the scaffold without BM-MSCs.

Materials and Methods: Eighteen rabbits were used for this study; bilateral 12-mm-diameter defects were created in the animals’ parietal bones. The bilateral defects were filled with a xenograft enriched with BM-MSCs (test group [TG]), with autogenous bone graft (positive control group [PCG]), or with a xenograft alone (negative control group [NCG]). In all groups, randomly, one defect was covered with a collagen membrane. The rabbits were sacrificed 8 weeks after surgery, and their parietal bones were harvested and analyzed histomorphometrically.

Results: Within the PCG and the NCG, the defects covered with the barrier membrane showed better bone healing. In the TG, the defects covered with the barrier membrane did not show better bone healing (intragroup comparisons by Wilcoxon and Friedman tests for paired data). TG showed percentage of mineralized tissue (MT) of 56.03% ± 3.49% with membrane and 57.71% ± 5.31% without membrane. PCG showed MT of 55.13% ± 4.83% and 49.69% ± 3.81% with and without membrane, respectively, and NCG showed MT of 26.77% ± 7.29% and 19.67% ± 2.66% with and without membrane, respectively. Conclusion: Both autogenous bone graft and a xenograft enriched with BM-MSCs were equally effective for bone reconstruction and better than the xenograft alone. The use of a barrier membrane seemed to have a synergistic effect on bone healing in PCG and NCG but not in TG. INT J ORAL MAXILLOFAC IMPLANTS 2015;30:208–215. doi: 10.11607/jomi.4010

Key words: bone marrow, bone repair, cell transplantation, osteogenesis, stem cells

Bone repair of critical defects can be assisted with therapies that involve bone marrow, because this tissue possesses cell populations that play an important role in bone homeostasis, known as bone marrow stromal cells. However, there is no consensus about the best methodology for obtaining and using bone marrow stromal cells. The relevant literature has reported three main alternatives: (1) fresh bone marrow ("in natura")²⁻⁴; (2) bone marrow mononuclear cell concentrate, also called bone marrow mononuclear fraction (BMMF)⁵⁻⁶; and (3) cultivated bone marrow mesenchymal stem cells (BM-MSCs).⁷⁻⁸

Bone marrow stromal cells were discovered in the late 1970s by a group led by Alexander Friedenstein, who showed that bone marrow contains a population of plastic-adherent, highly proliferative cells that are able to form a colony of fibroblasts (hence the name colony-forming unit fibroblasts).⁹ Following implantation in diffusion chambers, colony-forming unit fibroblasts spontaneously formed bone, cartilage, and fibrous tissue in vivo.¹⁰ Whereas Friedenstein et al termed them “determined osteogenic progenitors,”¹² the subsequent findings of their multipotentiality toward other mesenchymal lineages led Caplan to coin the term mesenchymal stem cells,¹³ in analogy to hematopoietic stem cells, which were the best described adult stem cell type at that time.

Modern bone-engineering strategies based on osteogenic cells, osteoinductive stimulators, and osteoconductive scaffolds are recognized as potential ways to create biologic tissue substitutes for regenerating large bone defects.¹⁴ The choice of cell sources that can efficiently differentiate into bone tissue is the first important step of bone engineering. Several cell
types can potentially be used as cellular components in bone engineering. These include osteoblast, embryonic, and adult stem cells. Among these candidates, MSCs, as adult stem cells, possess some characteristics that make them more appropriate for use in promoting bone regeneration.\textsuperscript{15}

A number of in vitro assays can be used to assess the multipotentiality of these cell preparations.\textsuperscript{16} Osteogenic differentiation of human MSCs can be triggered by exposure to specific culture supplements, including dexamethasone, ascorbate-2-phosphate, and beta-glycerophosphate.\textsuperscript{17,18} Adipogenic differentiation can also be induced through the use of induction medium containing dexamethasone, indomethacin, isobutylmethylxanthine, and insulin, but these cultures require a higher seeding density than that used for osteogenic differentiation.\textsuperscript{16} Finally, human MSCs placed in aggregate or pellet cultures in a defined medium containing dexamethasone, ascorbate-2-phosphate, insulin, selenious acid, transferrin, sodium pyruvate, and transforming growth factor-beta will undergo chondrogenic differentiation.\textsuperscript{16,19,20} The ability of MSCs to differentiate along these lineages is strongly suggestive of their multipotency and stem cell nature. However, human MSCs do not maintain these characteristics indefinitely, and they present senescence with extensive subcultivation in vitro, whereby they lose their proliferation and differentiation potential.\textsuperscript{21–23}

In this study, the bone healing rate after the use of a tissue-engineering technique of bone xenograft scaffolds enriched with BM-MSCs was compared with the gold standard autogenous bone graft in a rabbit model.

**MATERIALS AND METHODS**

For this study, 18 adult, skeletally mature New Zealand white rabbits from the animal colony of Federal University of São Paulo, Brazil (UNIFESP), weighing 3.5 to 4 kg each were used. The animals were divided into three groups of six animals each: the test group (TG), in which BM-MSCs were associated with a xenograft; the positive control group (PCG), which were treated with autogenous bone graft; and a negative control group (NCG) treated with the xenograft alone. All animals were operated by the same researcher, and all evaluations were done by the same researchers.

The surgical protocol for this study was analyzed and approved by the UNIFESP Ethics Committee (no. 2139/2011).

The animals underwent a period of adaptation to environmental conditions prior to being housed on UNIFESP premises. Rooms with a controlled temperature ranging from 18°C to 20°C and individual cages specifically designed for rabbits were used. The animals were fed commercial pellets and water ad libitum.

Anesthesia was induced by ketamine (40 mg/kg), midazolam (2 mg/kg), and fentanyl citrate (0.8 µg/kg) and maintained using a mixture of isoflurane/nitrous oxide (1%:1.5%) and oxygen (2/3:1/3) using a pediatric-size laryngeal mask airway. The top of the head of each of the 18 animals was shaved and the site disinfected with a povidone-iodine solution. After administration of a local anesthetic injection (2% lidocaine with epinephrine 1:100,000), a sagittal incision was made and the skin and periosteum were retracted.

**Bone Marrow Harvest and Culture**

After general anesthesia was achieved, autologous bone marrow was obtained by an aspiration technique. Two milliliters of bone marrow aspirates were obtained from each tibia of the 18 rabbits using a disposable 40 × 10 needle (1.10 × 38 mm) with 20-mL disposable syringes, previously heparinized to prevent blood clotting. Only the initial volume aspirated of bone marrow was used, because a greater amount could result in peripheral blood aspiration.\textsuperscript{24}

The following procedure was performed to obtain the stem cells. After the bone marrow was aspirated, it was transferred into polypropylene tubes containing preservative-free heparin (1,000 units/mL). The bone marrow and heparin were mixed well. The BMMF was then separated from the bone marrow tissue by density-gradient centrifugation with Ficoll Histopaque-1077 (Sigma-Aldrich) according to the manufacturer’s instructions. In essence, 3.5 mL of the bone marrow tissue was diluted in a 1:1 ratio with phosphate-buffered saline (PBS), which was placed in a conical tube (15 mL) containing the same volume of Ficoll Histopaque-1077. The final solution was centrifuged at 400g for 30 minutes at 22°C. The mononuclear cell layer was then pipetted and washed twice with PBS. A volume of 4 mL was established, and another centrifugation was performed at 200g for 10 minutes at room temperature. The supernatant was discarded, and the pellet was resuspended in 5 mL of Dulbecco minimum essential medium (DMEM) (Sigma) containing 10% fetal bovine serum (Sigma), 1% penicillin, 100 U streptomycin, and 100 µg/mL solution (designated the standard culture medium) and transferred to a 75-cm\textsuperscript{2} culture flask (Corning) that already contained 5 mL of culture medium. The cells were incubated at a temperature of 37°C in an atmosphere composed of 95% air and 5% carbon dioxide. The culture medium was exchanged every 48 hours, and the culture was monitored daily using an inverted optical microscope.
Creation and Grafting of the Critical-Size Bone Defects

Two defects were then created, one on each side of the midline, with a 12-mm-diameter trephine bur. In TG, a xenograft enriched with BM-MSCs was used to fill the bilateral defects, restoring their original contour. In PCG, autogenous bone graft was harvested from the created defect area and then particulated (Neodent) to fill the bilateral defects, restoring their original contour. In NCG, a xenograft was used to restore the contour of the defects bilaterally. In all groups, one of the calvarial defects (randomly chosen) was covered with a barrier membrane. Randomization was performed with specific software.25 In all rabbits, the incision was closed in layers.

The animals were given an intramuscular injection of benzylpenicillin (40,000 IU) and sodium dipyridone (0.25 mg/kg) postoperatively. The animals received food and water ad libitum for the remainder of the experimental period.

The xenograft used in this study was Bio-Oss (Geistlich Biomaterials), which is a particulate xenograft bone substitute. The form used in this study was small particles, with granules ranging from 0.25 to 1 mm. The barrier membrane used was Bio-Gide (Geistlich Biomaterials), which is a resorbable bilaminar collagen membrane of porcine origin that prevents unwanted invasion of tissues adjacent to the bone defect, thus optimizing bone healing.

All 18 animals were sacrificed 8 weeks after the initial surgery. The parietal bones were then harvested and processed for histologic and histomorphometric evaluation.

Assays and Characterization

Adhesion of MSCs. Cell adhesion was analyzed 3 days after seeding the cells in the culture flask using an inverted optical microscope.

Adipogenic Differentiation. To evaluate adipogenic differentiation, the cells were cultivated in six-well plates (TPP Techno Plastic Products) containing 2 mL of complete DMEM/F12 medium supplemented with 10 \( \mu \)mol/L insulin and 1 \( \mu \)mol/L dexamethasone (Sigma Aldrich) for 15 days (adipogenic medium). The plates were kept in a humidified incubator (37°C; 95% oxygen and 5% carbon dioxide), and the culture media was changed every 3 days. After this period, the medium was aspirated, and the cells were washed twice with PBS. Then, a solution of 2 mL paraformaldehyde (0.4% in PBS, Electron Microscopy Sciences) was added to the cells. The fixative solution was aspirated after 30 minutes, and the cells were washed three times with PBS as follows: once with PBS containing glycine 0.1 mol/L for 10 minutes and twice with PBS for 2 minutes.

The cells were then incubated in a solution of sodium alizarin (40 nM, pH 4.1; Sigma Aldrich) at room temperature for 30 minutes. The dye was carefully removed with a 2-mL disposable pipette, and the plate was washed five times with 2 mL of water. The fixed and dyed cells were observed with a Nikon Ti-U optical microscope and photographed using NIS-Elements version 3.2 software (Nikon Instruments).

Osteogenic Differentiation. To determine osteogenic differentiation, the cells were cultured in six-well plates (TPP Techno Plastic Products) containing 2 mL complete DMEM/F12 medium supplemented with 50 \( \mu \)mol/L ascorbic acid (Sigma Aldrich), 0.1 \( \mu \)mol/L dexamethasone, and 10 \( \times 10^{-2} \) mol/L beta-glycerophosphate (Mallinckrodt Baker) for 21 days (osteogenic medium). The plates were kept in a humidified incubator (37°C; 95% oxygen and 5% carbon dioxide), and the culture media was changed every 3 days. After this period, the medium was aspirated, and the cells were washed twice with PBS. Then, a solution of 2 mL paraformaldehyde (0.4% in PBS, Electron Microscopy Sciences) was added to the cells. The fixative solution was aspirated after 30 minutes, and the cells were washed three times with PBS as follows: once with PBS containing glycine 0.1 mol/L for 10 minutes and twice with PBS for 2 minutes.

The cells were then incubated in a solution of 0.5% at room temperature for 30 minutes. The dye was carefully removed with a 2-mL disposable pipette, and the plate was washed five times with 2 mL of water. The fixed and dyed cells were observed with a Nikon Ti-U optical microscope and photographed using NIS-Elements version 3.2 software (Nikon Instruments).

Chondrogenic Differentiation. To assess chondrogenic differentiation, the cells were cultured in six-well plates (TPP Techno Plastic Products) containing 2 mL complete DMEM/F12 medium supplemented with 10 \( \mu \)mol/L insulin, 0.1 \( \mu \)mol/L dexamethasone, 50 \( \mu \)mol/L ascorbic acid, and 10 ng/mL transforming growth factor-beta1 (Cell Signaling Technology) for 21 days (chondrogenic medium). The plates were kept in a humidified incubator (37°C; 95% oxygen and 5% carbon dioxide), and the culture media was changed every 3 days. After this period, the medium was aspirated and the cells were washed twice with PBS. Then, a solution of 2 mL paraformaldehyde (0.4% in PBS, Electron Microscopy Sciences) was added to the cells. The fixative solution was aspirated after 30 minutes, and the cells were washed three times with PBS as follows: once with PBS containing glycine 0.1 mol/L for 10 minutes and twice with PBS for 2 minutes.

The cells were then incubated in toluidine blue (0.1%; Sigma Aldrich) at room temperature for 30 minutes. The dye was carefully removed with a 2-mL disposable pipette, and the plate was washed five times with 2 mL of water. The fixed and dyed cells were observed with a Nikon Ti-U optical microscope and photographed using NIS-Elements version 3.2 software (Nikon Instruments).
Each specimen were selected and embedded in paraffin blocks. Next, 7-µm sections of each specimen were obtained and evaluated according to a semiquantitative histologic scoring system, in which an initial section was used and the following three were discarded, successively, until all sections had been evaluated. Thirty-six sections of each specimen were stained with Mallory trichrome and Stevenel blue and then qualitatively examined under light microscopy. Six areas of each section were analyzed (top left, lower left, top center, lower center, top right, and lower right) (Fig 2).

Digital images were captured with a digital camera (RT Color, Diagnostic Instruments) attached to a light microscope (×1.25 magnification). The digital images were merged to create a single image for each histologic section using Adobe Photoshop Elements 2.0 (Adobe Systems).

Two blinded examiners (AZ and RMO) were calibrated previously. In cases of disagreement, the specimen was reevaluated and a consensus was reached. The examiners traced all the images to measure new bone formation using Image Pro Plus 4.5 Software for Windows (Media Cybernetics). Mineralized tissue (MT) and nonmineralized tissue (NMT) were measured. In all groups, the nonvital mineralized tissue (NVMT) and vital mineralized tissue (VMT) were measured inside the MT area. All results were scored in square micrometers and then expressed as a percentage of the total area.
1.46 × 10^6 cells per sample. After 3 days, the culture medium was removed, and adherent cells were counted after trypsinization (0.25% trypsin + 5 mmol/L ethylenediaminetetraacetic acid) using trypan blue. A constant rate was observed of 15 adhered cells per flask of culture (0.001%). There were two subcultures until they reached confluence of 80% of the surface of a 75-cm² flask, which took an average of 35 days. Upon confluence, the BM-MSCs were removed and counted; an average of 2 × 10^6 cells per culture flask was obtained.

**Cell Adhesion and Differentiation Assays**

The cells were evaluated for their ability to adhere in the culture flask before the first exchange of culture medium. All flasks had a mean of 15 adherent cells (0.001%) (Fig 3). For the three differentiation tests (adipogenic, chondrogenic, and osteogenic) the BM-MSCs were cultured for 21 days and then photographed to evaluate the presence of the respective markers (oil red O, toluidine blue, and alizarin red). There was positive staining for all BM-MSCs in all cell cultures; the markers were uniformly distributed on the surfaces of the culture flasks (Fig 4).

**Immunophenotype of Cells**

Flow cytometry analysis showed that these cells expressed CD14, CD16, CD73, CD90, CD105. There was no expression of markers CD31, CD34, CD44, CD45, CD117 in any of the samples analyzed (Fig 5).

**RESULTS**

**Isolation and Cell Culture**

The isolation and culture of bone marrow cells were performed uniformly for all six rabbits in the test group. There were no complications associated with harvesting bone marrow, and in all animals, it was possible to collect the same amount of bone marrow (a total of 4 mL, ie, 2 mL from each tibia). The BMMF was obtained from each animal for subsequent cell culturing procedures. The same amount of BMMF was obtained from all animals (2 mL) and contained 1.46 × 10^6 cells per sample. After 3 days, the culture medium was removed, and adherent cells were counted after trypsinization (0.25% trypsin + 5 mmol/L ethylenediaminetetraacetic acid) using trypan blue. A constant rate was observed of 15 adhered cells per flask of culture (0.001%). There were two subcultures until they reached confluence of 80% of the surface of a 75-cm² flask, which took an average of 35 days. Upon confluence, the BM-MSCs were removed and counted; an average of 2 × 10^6 cells per culture flask was obtained.

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Flow cytometry of BM-MSCs (21 days in culture).

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The PCG group showed no NVMT. The other groups showed varying amounts of NVMT (Table 2).

**Histomorphometric Observations**

In the TG, the sides on which the defects were covered with the barrier membrane did not show better bone healing ($P > .05$) (Table 1). In contrast, in the PCG and NCG, the sides on which the defects were covered with the barrier membrane showed better bone healing ($P < .05$) (intrigroup comparison by Wilcoxon and Friedman tests for paired data). The test group showed mean MT of $56.03\% \pm 3.49\%$ and $57.71\% \pm 5.31\%$ with and without membrane coverage, respectively. The PCG showed mean MT of $55.13\% \pm 4.83\%$ and $49.69\% \pm 3.81\%$ with and without membrane coverage, respectively. The NCG showed mean MT of $26.77\% \pm 7.29\%$ and $19.67\% \pm 2.66\%$ with and without membrane coverage, respectively (Table 1).

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<thead>
<tr>
<th>Tissue type</th>
<th>With membrane</th>
<th>Without membrane</th>
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<tbody>
<tr>
<td>MT</td>
<td>TG</td>
<td>56.03 ± 3.49</td>
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<tr>
<td></td>
<td>PCG</td>
<td>55.13 ± 4.83</td>
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<tr>
<td></td>
<td>NCG</td>
<td>26.77 ± 7.29</td>
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<td>P</td>
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<td>NMT</td>
<td>TG</td>
<td>44.97 ± 5.88</td>
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<td></td>
<td>PCG</td>
<td>44.87 ± 3.78</td>
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<td>NCG</td>
<td>73.23 ± 6.87</td>
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<td>P</td>
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$P \le .05$ indicates statistical significance (Kruskal-Wallis test).

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>With membrane</th>
<th>Without membrane</th>
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<tbody>
<tr>
<td>NVMT</td>
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<td>27.7 ± 2.72</td>
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<td></td>
<td>PCG</td>
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<td></td>
<td>NCG</td>
<td>13.52 ± 3.00</td>
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<tr>
<td>VMT</td>
<td>TG</td>
<td>28.24 ± 6.17</td>
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<td></td>
<td>PCG</td>
<td>55.13 ± 4.83</td>
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<td></td>
<td>NCG</td>
<td>13.06 ± 5.24</td>
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<tr>
<td>MT (NVMT + VMT)</td>
<td>TG</td>
<td>56.03 ± 3.49</td>
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<td></td>
<td>PCG</td>
<td>55.13 ± 4.83</td>
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<td>NCG</td>
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DISCUSSION

The definition of adult MSCs involves the observation of several factors, such as the capacity to adhere to the plastic of culture flasks; differentiation into three mesenchymal cell types (osteoblasts, adipocytes, and chondrocytes) through stimulation of extracellular matrix; and the expression of specific cellular markers. In this study, BMMF was used for the cell culture, rather than fresh bone marrow, because this procedure avoids undesirable cell types. The mean number of cells in this mononuclear cell fraction was $1.46 \times 10^6$ ($\pm 0.3$). On the third day after seeding these cells and immediately before the first culture medium change, there were 15 ($\pm 3$) cells attached (0.001%), which coincides with the findings of Minguell et al. Assays for differentiation of the adherent cells were performed with supplementation of the culture medium for 21 days and stains specific to each type of cell. It was observed that all cells evaluated differed, which proves the undifferentiated state of these cells before induction by culture medium.

The cells expressed markers CD14, CD16, CD73, CD90, and CD105 and did not express markers CD31, CD45, and CD107, showing that the cells were BM-MSCs. These results are similar to those found by Horwitz et al and Hashimoto et al. A previous study that used the same critical-size defect in rabbits showed that the use of the scaffold without bone marrow cells provided poorer results for all histomorphometric parameters assessed, which suggests that cellular therapy increases bone regeneration, especially when BMMF or bone marrow stem cells are used.

For all groups evaluated by Pelegrine et al and for the PCG and NCG of the present study, the use of a collagen membrane contributed to superior results, with significantly higher levels of VMT. In guided bone regeneration, the membrane provides mechanical isolation of graft material from the overlying soft tissues. However, in the present study, in the TG, no benefit was conferred by the membrane ($P > .05$). The TG, in which BM-MSCs were used, was the only group in which the membrane did not provide any benefit. It may be that an interaction between the autologous mesenchymal stem cells and the bone marrow microenvironment inhibits cell migration of the overlying soft tissues. It is also possible that there is a greater induction of angiogenesis and vascular endothelial permeability (diapedesis) at the recipient site, as suggested by Blau et al. The most probable hypothesis to explain the similarity of action of stem cells with the technique of guided tissue regeneration might be the barrier action of the cells themselves; however, the MSCs do not act as a physical barrier, as does a traditional membrane, but rather as some sort of physiologic barrier.

Further research is warranted to confirm or reject this hypothesis.

The amount of VMT seen after the use of BM-MSCs (TG) was similar to the BMMF in the study of Pelegrine et al and higher than the PCG and NCG. This phenomenon can be explained by the presence of other populations in the bone marrow, in addition to the MSCs, that play a role in bone regeneration.

In the TG and the NCG, the level of mineralized tissue was the sum of NVMT and VMT; on the other hand, in the PCG, all the MT was VMT. This can be explained by the lack of NVMT in autogenous bone. In the test group, the sum of NVMT (27.79% ± 2.72% and 27.23% ± 6.80% with and without membrane coverage, respectively) and VMT (28.24% ± 6.17% and 30.38% ± 4.65%), with and without membrane coverage, respectively) after 8 weeks was similar to the MT observed in the PCG ($P > .05$). In the group in which only the xenograft was used (NCG), this sum (NVMT plus VMT) was lower. In the NCG, most of the MT was NVMT. This can be explained by the great distance between the remaining bone walls, ie, the critical-size bone defect. These results may also point to the incorporation of the MSCs into the Bio-Oss in the TG of the present study to accelerate the healing process. Furthermore, the use of MSCs inhibited resorption of the xenograft particles. However, regarding the presence of a VMT specifically, the PCG showed a higher level, and this might contribute to better osseointegration of titanium implants.

The clinical use of BM-MSCs is associated with variations in the method by which stem cells are isolated and expanded; methods are not the same around the world, resulting in different outcomes. At present, the routine clinical use of cell culture is not acceptable because of the absence of definitive established markers for MSCs, which reflects a dangerous lack of standardization. Although Yamada et al demonstrated excellent results when using a culture of MSCs from bone marrow associated with platelet-rich plasma for sinus floor augmentation, it must be recognized that stem cell culture has disadvantages versus other methodologies, such as the use of BMMF or fresh bone marrow, the cost, the time between removal and transplantation of the tissue, the risk of contamination, and the lack of agreement regarding the number of cells required.

CONCLUSIONS

The association of bone marrow mesenchymal stem cells with a scaffold and the use of autogenous bone graft, with the adjunct use of a barrier membrane, resulted in similar patterns of mineralized tissue, which were better than the xenograft alone. However, the
use of autogenous bone graft resulted in higher levels of vital mineralized tissue. The use of a membrane made no difference when bone marrow mesenchymal stem cells were used.

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