Ectopic Implantation of Hydroxyapatite Xenograft Scaffold Loaded with Bone Marrow Aspirate Concentrate or Osteodifferentiated Bone Marrow Mesenchymal Stem Cells: A Pilot Study in Mice

Gabriela Victorelli, DDS1/Antonio Carlos Aloise, DDS, MS, PhD2/Fabricio Passador-Santos, DDS, MS, PhD3/Rafael de Mello e Oliveira, DDS, MS2/André Antonio Pelegrine, DDS, MS, PhD4

Purpose: To evaluate the influence of bone marrow cells in the bone formation in an ectopic subcutaneous model in mice. Materials and Methods: Six BALB/c mice were divided into three groups of two each. In all groups, xenograft was implanted subcutaneously. In the negative control group, the xenograft was hydrated with saline solution. In the positive control group, the xenograft was embedded with osteodifferentiated adult mesenchymal stem cells derived from the bone marrow. In the experimental group, the xenograft was embedded with bone marrow aspirate concentrate. After 4 weeks, the animals were sacrificed and prepared for histologic, histomorphometric, and immunohistochemical analysis. The following tissues were evaluated: preosteoid tissue, loose connective tissue, and remaining xenograft particles. Results: There was a statistically significant difference (P = .008) in the preosteoid tissue area between the negative control group (0 ± 0%) and the other two groups, with 42 ± 11% for the experimental group and 56 ± 5% for the positive control group. Similarly, there was a statistically significant difference (P = .006) in the loose connective tissue area between the negative control group (49 ± 18%) and the other two groups, with 3 ± 9% for the experimental group and 0 ± 0% for the positive control group. Regarding the xenograft area, there was not a statistically significant difference between the three groups (P = .143). Conclusion: The use of a mineralized scaffold loaded with either concentrated bone marrow aspirate or with osteogenically induced bone marrow mesenchymal stem cells favored the formation of osteoid tissue as opposed to the scaffold alone. INT J ORAL MAXILLOFAC IMPLANTS 2016;31:e18–e23. doi: 10.11607/jomi.4509

Keywords: bone marrow, cell transplantation, osteogenesis, stem cells

Repair of critical-size bone defects is a major challenge in implant dentistry. There is a consensus that autologous bone grafting is the gold standard for bone reconstruction, due to its potential for osteoconduction, osteoinduction, and osteogenesis.1 Nevertheless, autologous bone harvesting implies the need for a second surgical site, thereby increasing morbidity.2 Depending on the amount of bone needed for reconstruction, harvesting extraoral sites—usually the skull, iliac, or tibia—may be necessary, which would require hospitalization and a medical team.3

Different cell therapy approaches have been used to enhance or induce bone formation in critical situations in an attempt to replace autologous grafting.14-9 In recent years, studies involving the isolation and expansion of stem cells for regeneration of bone tissue have reported a promising approach for repairing critical defects10,11; however, the techniques involving stem cell culture are complex and expensive.12

Previous studies by the present authors have demonstrated that less complex and less costly methods, such as gross bone marrow centrifugation, without aiming for a specific cell population, can also

1Postgraduate Student, Department of Implant Dentistry, São Leopoldo Mandic Institute and Research Center, Campinas, Brazil.
2Assistant Professor, Department of Implant Dentistry, São Leopoldo Mandic Institute and Research Center, Campinas, Brazil.
3Assistant Professor, Department of Oral Pathology, São Leopoldo Mandic Institute and Research Center, Campinas, Brazil.
4Professor and Head, Department of Implant Dentistry, São Leopoldo Mandic Institute and Research Center, Campinas, Brazil.

Correspondence to: Prof André Antonio Pelegrine, Rua das Areias 37, Campinas SP Brazil 13024-530. Email: pelegrineandre@gmail.com

©2016 by Quintessence Publishing Co Inc.
contribute to bone tissue regeneration. Therefore, the purpose of this study was to evaluate the formation of bone tissue at ectopic sites using cell therapy with minimal handling.

MATERIALS AND METHODS

Experimental Model
Six 3-month-old immunocompromised BALB/c male mice were used in this study. The animals were purchased from Anilab-Animal breeding and Trade LTD Laboratory. During the study period, the animals were kept in ventilated cages with light and dark cycles of 12 hours, pelleted feed, and water ad libitum. This study was approved by the Animal Research Center of the São Leopoldo Mandic Dental School and Research Center.

Scaffold Implantation
The scaffold consisted of a fine-grained bovine hydroxyapatite block containing 10% porcine collagen (Bio-Oss Collagen) measuring $5 \times 5 \times 1.2$ mm ($W \times D \times H$). The procedure was performed under general anesthesia via intraperitoneal injection of 500 $\mu$L of 80% ketamine and 20% xylazine hydrochloride. Dorsal trichotomy was subsequently performed at the surgical site, the mouse was placed on the operating table, and the surgical area was decontaminated with povidone-iodine. The skin in the dorsal region was clamped, and a 7-mm incision was made using a pair of curved tip scissors. Subcutaneous tissue was then divulsed and the hydroxyapatite scaffold inserted. The surgical wound was sutured using a 5-0 nylon thread (Ethicon) and simple interrupted stitches.

Experimental Groups
Three experimental groups were designed: negative control group, which received the scaffold only; experimental group, which received the scaffold combined with the bone marrow aspirate concentrate; and the positive control group, which received the scaffold combined with adult mesenchymal stem cells derived from the bone marrow.

Bone Marrow Aspiration
The bone marrow was obtained via aspiration from a donor patient who was submitted to a bone marrow aspirate concentrate protocol, for bone augmentation purposes. The bone marrow aspiration procedure followed the guidelines described in Pelegrine et al. Briefly, the patient was laid flat on their side for access to the iliac crest; skin decontamination was carried out, and a 2% lidocaine hydrochloride (without vasoconstrictor) local anesthetic injection was administered. The puncture was performed at a preestablished point using a 40- $\times$ 12-mm needle with a mandrel. The needle was inserted until it touched the cortical bone and gently spun 1 cm deep into the marrow cavity. The mandrel was removed from the hollow needle, and a previously heparinized (5,000 IU) 20-mL syringe was used to aspirate 30 mL of bone marrow. The bone marrow was transferred to a 50-mL conical test tube and taken to a sterile environment, where the content was distributed equally into two 15-mL tubes. One tube was processed to produce the concentrated aspirate, and the remaining tube was processed to yield stem cells from the bone marrow.

Bone Marrow Concentrate
The tube containing 15 mL of bone marrow was centrifuged at 400 $g$ for 10 minutes (Centribio R, model 80-2B) at room temperature. The supernatant was discarded, and the cell pellet deposited at the bottom of the conical tube was used in the experiment, which averaged 2 mL.

Isolation, Expansion, and Osteodifferentiation of Adult Mesenchymal Stem Cells Derived from Bone Marrow
The second 15-mL tube containing the bone marrow was processed similarly to obtain the concentrate; however, after centrifugation, the cell layer located immediately above the pellet, also called the “buffy coat,” which was approximately 1 mL, was pipetted out and centrifuged at 200 $g$ for 10 minutes to form a cell pellet deposited at the bottom of the conical tube.

The solution containing the cells was then resuspended in 20 mL of osteogenic culture medium containing Dulbecco’s Modified Eagle Medium (DMEM; high glucose level [4.5 g/L] L-glutamine [584 mg/L], sodium pyruvate [110 mg/L]) with 10% fetal bovine serum (20% iron-supplemented) and penicillin/streptomycin (100 IU/mL=100 μg/mL), buffered with sodium bicarbonate (1 N), 0.5 μg/mL acid ascorbic, 2.16 mg/mL β-glycerophosphate, and 10$^{-7}$ M dexamethasone, and transferred to a 75-cm$^2$ culture flask (Corning). Subsequently, the flask was placed in an incubator featuring a controlled atmosphere, 5% $CO_2$, and a temperature of 37$^\circ$C. When the cells reached 80% confluence, they were removed from the flask using trypsin solution and counted. When the cell density reached 5.6 $\times$ 10$^5$, the cells were resuspended in 1 mL of phosphate-buffered saline (PBS; Sigma-Aldrich) and then used in the experiment.

Characterization of Adult Mesenchymal Stem Cells Derived from Bone Marrow
Bone marrow stem cells from the second passage were used for the immunophenotypic characterization.
The cells were trypsinized, and the cell suspension was centrifuged (300 g for 4 minutes). The cells were stained with antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin (APC). The sections were evaluated for alkaline phosphatase (ALP). The sections were evaluated for ALP activity. All bone marrow cells were counted and stained for CD73, CD90, and CD105. The sections were evaluated for the relative expression of CD73, CD90, and CD105.

**Immunohistochemical Analysis**

Three-micrometer sections were deparaffinized, hydrated, and immersed in 20% hydrogen peroxide for 30 minutes (Dinâmica, Diadema). The sections were subsequently incubated at 4°C with bovine alkaline phosphatase primary antibody overnight and then biotinylated secondary antibody peroxidase-conjugated streptavidin system (LSAB, Dako) for 1 hour at 37°C. The sections were stained for 10 minutes at 37°C with 3,3′-diaminobenzidine tetrahydrochloride (DAB, Dako) and counterstained with Mayer’s hematoxylin (Dinâmica, Diadema). Using a double-headed microscope (Olympus BX51), two examiners interpreted the immunohistochemical reactions for alkaline phosphatase (ALP). The sections were evaluated qualitatively according to the presence or absence of staining in the bone matrix, osteocytes, osteoblasts, and the xenograft cortical bone. Digital photomicrographs were obtained on a Zeiss Axioskop 2 plus microscope equipped with an Axiocam digital camera and AxioVision application software (Carl Zeiss).

**Statistics**

The intergroup analysis was performed using the Kruskal-Wallis test at a significance level of 5% on SPSS 20 (SPSS) and BioEstat 5.0 (Mamirauá Foundation).

**RESULTS**

**Differentiation Assays and Flow Cytometric Analysis**

The cells were initially evaluated on their ability to adhere to the culture flask before the first change of culture medium. All flasks had a mean of 15 adhered cells (0.001%). For the three differentiation tests, adipogenic, osteogenic, and chondrogenic, the AMSC-BM were cultured for 21 days. They were then photographed to evaluate the presence of their respective marker, oil red, toluidine blue, and alizarin red. All bone marrow stem cells in culture were positive, with a uniform distribution of the marker throughout the culture flask surface. The flow cytometry analysis showed that these cells were positive to CD14, CD16, CD73, CD90, and CD105 while negative to CD31, CD34, CD44, CD45, and CD117 in all samples analyzed.

**Histomorphometric Analysis**

The area was measured in square micrometers, and a percentage mean was calculated per specimen for the following tissues: (1) loose connective tissue; (2) preosteoid tissue, and (3) xenografts.

**Removal of Specimens**

The animals were sacrificed after 4 weeks using the anesthetic overdose method. The tissue samples were removed, which included the skin, connective tissue, and the scaffold from the dorsal region of the animals, measuring 2 × 2 cm, and fixed in 4% paraformaldehyde.

**Histomorphometric and Immunohistochemical Analysis**

The experimental and positive control groups had higher rates of preosteoid tissue and lower loose connective tissue rates than the negative control group (P < .05). Regarding the xenograft rates, the three groups showed similar levels (P > .05; Table 1). The immunohistochemical analysis showed strong and homogenous positive staining around the remaining particles of the xenogeneic graft, as well as the extracellular matrix (Fig 1). The osteocytes only showed cytoplasmic staining in focal areas of the section (Figs 2a to 2c).
Table 1  

<table>
<thead>
<tr>
<th>Tissue types</th>
<th>NCG</th>
<th>EG</th>
<th>PCG</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>POT</td>
<td>0</td>
<td>42 ± 11A</td>
<td>56 ± 5A</td>
<td>.008</td>
</tr>
<tr>
<td>LCT</td>
<td>49 ± 18A</td>
<td>3 ± 9B</td>
<td>0B</td>
<td>.006</td>
</tr>
<tr>
<td>XG</td>
<td>51 ± 18A</td>
<td>56 ± 3A</td>
<td>44 ± 5A</td>
<td>.143</td>
</tr>
</tbody>
</table>

NCG = negative control group; EG = experimental group; PCG = positive control group; POT = preosteoid tissue; LCT = loose connective tissue; XG = xenograft.

Kruskal-Wallis test with statistical significance considered when $P \leq .05$.

Mean followed by different superscripts (A and B) indicates significant difference between groups for each parameter (POT, LCT, and XG).

Fig 1  
Photomicrographs of the same specimens for each group showing the immunohistochemical reaction for ALP. Negative control group: (a) tissue section stained with hematoxylin and eosin (HE); (b) tissue section immunohistochemically tested for ALP ($\times$10 magnification). Experimental group: (c) HE-stained section; (d) tissue section immunohistochemically tested for ALP ($\times$10 magnification). Positive control group: (e) HE-stained section; (f) immunohistochemical reaction to ALP ($\times$10 magnification). Note the strong and homogenous positive staining around the remaining particles (*) of the xenogeneic graft.

Fig 2  
Photomicrographs of the histologic sections showing preosteoid tissue (#), loose connective tissue (^), and xenograft (*) in the three groups: (a) negative control group; (b) experimental group, and (c) positive control group (HE-stained $\times$100).
DISCUSSION

The use of animal models in cell therapy studies on bone tissue regeneration has been described by several authors. Studies in rabbit calvaria, where critical defects were created, revealed that such defects require a combination of a reinforced scaffold and a guided bone regeneration technique to predictably recover affected areas.

One of the limitations of xenograft scaffolds is the risk of such materials not being osteogenic, due to the lack of a cellular component. The use of bone marrow to remedy this situation has been extensively investigated using experimental models. Sununliganon et al studied the use of concentrated bone marrow aspirate, while de Mello e Oliveira et al and Pelegrine et al investigated the bone marrow mononuclear cell fraction and Aloise et al used adult mesenchymal stem cells derived from the bone marrow. In all these studies, the use of bone marrow cells increased the percentage of bone neoformation. Nonetheless, when evaluating the results obtained via these methods, the studies that applied less complex methods, such as a concentrated bone marrow aspirate or the mononuclear cell fraction, produced comparable outcomes to those obtained from more complex approaches, such as the use of isolated stem cells.

Another common factor in these studies was the fact that despite the critical size of the bony defect for spontaneous regeneration, the graft recipient site was actually bone tissue; ie, both local and systemic cellular signaling focused on the production of young or immature bone. By contrast, ectopic implantation of grafts using animal models grants an overall evaluation of bone neoformation without the influence of specific signaling from direct contact with bone tissue. Following this philosophy, many authors have demonstrated higher levels of ectopic bone formation when using cell-loaded scaffolds (bone grafts) than with scaffolds alone.

In the present study, a positive control group consisting of osteogenically induced AMSC-BM as a comparative parameter to the experimental group (bone marrow aspirate concentrate) and the scaffold alone as a negative control were used. This decision was based on a previous study, which showed that the use of osteogenically induced AMSC-BM was more effective in ectopic bone formation than noninduced AMSC-BM. Ye et al reported that a beta-tricalcium-phosphate structure loaded with osteogenically induced AMSC-BM showed higher levels of bone formation than the scaffold loaded with a bone marrow cell concentrate. In the present study, a hydroxyapatite scaffold also loaded with either osteogenically induced AMSC-BM or bone marrow cells obtained from centrifugation were used on an ectopic implantation animal model. Unlike the results by Ye et al, the present study revealed no significant difference in bone formation between induced AMSC-BM and bone marrow aspirate concentrate. Indeed, these studies are not really comparable due to the different methodologies used for bone marrow cell culture and concentration as well as the evaluation criteria. In the study by Ye et al, no histomorphometric analysis was performed, but there was a qualitative histologic report. This study quantified the area of tissue neoformation involved in the process as opposed to solely indicating the presence or absence of evidence of bone formation.

In histologic analysis, three distinct types of tissues present in the area defined by the block after 4 weeks of implantation (preosteoid tissue, loose connective tissue, and xenograft tissue) were identified. Histomorphometric analysis revealed that the experimental group and the positive control group had higher rates of preosteoid tissue (42 ± 11% and 56 ± 5%, respectively) and lower rates of loose connective tissue (3 ± 9% and 0%, respectively) than the negative control group (P ≤ .05). Regarding the xenograft, all three groups showed similar rates (P ≤ .05). In a recent study, Wang et al used histomorphometric analysis following ectopic implantation of noninduced AMSC-BM and obtained 45 ± 5.3% of preosteoid tissue, a rate close to those found in the present study.

Histologic analysis was used in the present study to define the preosteoblastic tissue, which included parameters such as the presence of cells, orientation of fibers, and morphologic features, as well as immunohistochemical positivity for alkaline phosphatase, which is considered a marker of osteoblastic activity. In the present study, ALP positivity was confirmed immunohistochemically in both the experimental group and the positive control group. ALP was extensively expressed around the xenogeneic grafts as well as in the bone marrow in both groups. Since ALP is involved in the onset of mineralization, it is, therefore, an early marker of osteoblastic differentiation.

It is important, nevertheless, to highlight that further studies with a larger sample size are necessary to validate these findings.

CONCLUSIONS

The use of a mineralized scaffold loaded with either concentrated bone marrow aspirate or with osteogenically induced bone marrow mesenchymal stem cells favored the formation of osteoid tissue as opposed to the scaffold alone.
ACKNOWLEDGMENTS

The authors report no conflicts of interest related to this study.

REFERENCES


The International Journal of Oral & Maxillofacial Implants e23

© 2016 BY QUINTESSENCE PUBLISHING CO, INC. PRINTING OF THIS DOCUMENT IS RESTRICTED TO PERSONAL USE ONLY. NO PART MAY BE REPRODUCED OR TRANSMITTED IN ANY FORM WITHOUT WRITTEN PERMISSION FROM THE PUBLISHER.