Repair of critical-size bone defects using bone marrow stromal cells: a histomorphometric study in rabbit calvaria. Part I: Use of fresh bone marrow or bone marrow mononuclear fraction

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

Abstract
Objectives: The aim of this study was to compare the bone healing observed after the use of (1) a scaffold enriched with fresh bone marrow, (2) a scaffold enriched with bone marrow mononuclear fraction, and (3) a scaffold alone.

Material and methods: Twenty one rabbits were randomly divided into three groups of six animals and 1 group of 3 animals. Bilateral 12-mm diameter defects were created in the animals’ parietal bones. In Control Group, the defects were filled with a xenograft alone (n = 6); in Group 1, with a xenograft enriched with fresh bone marrow (n = 6); in Group 2, with a xenograft enriched with bone marrow mononuclear fraction (n = 6) and in Unfilled Group, nothing was grafted (n = 3). In Groups 1, 2, and Control, one of the calvarial defects was randomly covered with a barrier membrane. The rabbits were sacrificed 8 weeks after surgery, and their parietal bones were harvested and analyzed histomorphometrically.

Results: The histomorphometric analysis showed no difference between Group 1 and the Control Group regarding non-vital mineralized tissue area, but Group 2 showed a statistically significant higher percentage than the Control Group (P < 0.05) for both situations, with membrane (21.24 ± 3.68% and 13.52 ± 3.00%, respectively) and without membrane (20.91 ± 2.01% and 13.08 ± 1.72%, respectively). Group 2 showed the highest percentage of vital mineralized tissue area, followed by Group 1 and the Control Group (P < 0.05) for both situations, with membrane (28.17 ± 3.19%; 21.14 ± 7.38% and 13.06 ± 5.24%, respectively) and without membrane (21.13 ± 0.55%; 12.45 ± 6.34% and 6.56 ± 1.20%, respectively). Group 2 showed the lowest percentage of non-mineralized tissue area, followed by Group 1 and Control Group (P < 0.05) for both situations, with membrane (50.59 ± 6.64%; 58.75 ± 7.14% and 73.41 ± 6.87%, respectively) and without membrane (57.97 ± 1.91%; 71.74 ± 6.63% and 80.37 ± 2.67%, respectively). The sides in which the defects were covered with the barrier membrane showed better bone healing compared with the uncovered sides, in all groups (intragroup comparison, P < 0.05). The Unfilled Group specimens showed no bone formation.

Conclusions: Both methods using bone marrow stromal cells contributed to enhancing bone healing, especially that using the bone marrow mononuclear fraction. The use of a barrier membrane seemed to have a synergistic effect.

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procedures, but harvesting this tissue bears some risks (Jensen & Terheyden 2009). Other biomaterials have thus been studied to avoid harvesting autogenous bone (Grisdale 1999, Smiler et al. 2007; Araujo & Lindhe 2009, Macedo et al. 2009). However, autogenous bone substitutes have no osteogenic potential because they are acellular. Only an autogenous bone graft preserves some osteogenic potential after it is harvested, owing to some degree of cellular viability (Prolo & Rodrigo 1985).

Muschler & Midura (2002) stated that “the efficacy of all current clinical tools depends entirely on the cells in the grafted site, particularly the small subset of stem cells and progenitor cells that are capable of generating new tissue.” Therefore, the presence of these cells in an autogenous bone graft renders the reconstruction of large bone defects more predictable, even though the level of required cellularity is low. However, if autogenous bone cells were added to an appropriate scaffold, for example, commercially available lyophilized bone, the resulting biomaterial could display an adequate osteogenic potential, comparable with that of autogenous bone.

Pelegrine et al. (2010) stated that harvesting bone marrow, which contains mesenchymal stem cells, is relatively easy, painless, and does not entail significant risks of complications or postoperative morbidity. Therefore, if a safe protocol for using a concentrate of bone marrow stromal cells [BMSCs] could be established, it would be possible to obtain an osteogenic graft without harvesting large amounts of autogenous bone. As stated by Soltan et al. (2005), this could allow the use of a platinum standard bone graft material instead of the gold standard autogenous bone graft.

There is no consensus about the best methodology for using BMSCs. Relevant literature has reported three main alternatives: (1) fresh bone marrow (“in natura”) (Pelegrine et al. 2010, Costa et al. 2011), (2) bone marrow mononuclear cell [MNC] concentrate (Sakai et al. 2008; Yoshioka et al. 2011), and (3) cultivated bone marrow mesenchymal stem cells (Quarto et al. 2001; Cerutti Filho et al. 2007). Owing to the use of a cell culture methodology, the mesenchymal stem cell technique must be considered with caution because it normally requires a waiting period of some weeks between cell collection, culture, and transplantation, and this holds a risk of contamination (Lucarelli et al. 2007).

Because studies comparing the effectiveness of these methodologies in vivo are scarce in the related literature (Krzymanski et al. 1997, Petite et al. 2000, Clarke et al. 2007), the aim of the present study was to compare the effectiveness of two clinically acceptable methods of using BMSCs [fresh bone marrow or bone marrow mononuclear fraction] in an animal model.

Material and methods

Surgical protocol

The surgical protocol for this study was analyzed and approved by the UNIFESP Ethics Committee (number 2139/2011).

Twenty-one adult, skeletally mature New Zealand white rabbits from the animal colony of UNIFESP (Cedeme, São Paulo, SP, Brazil), weighing 3.5–4 kg, were used in the study.

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/N₂O [1 : 1.5%]) : oxygen [2/3 : 1/3] using a pediatric-size laryngeal mask airway. The top of the head of the 21 animals were shaved, and the site was disinfected with a povidone–iodine solution. After administration of a local anesthetic injection of 2% lidocaine with epinephrine 1 : 100,000, a sagittal incision was made and the skin and periosteum were retracted.

Two defects were then created, one on each side of the midline, with a 12-mm diameter trephine bur (Fig. 1a,b). The 21 rabbits were randomly divided into 3 groups of 6 animals (Group 1, Group 2, and Control Group) and 1 group of 3 animals (Unfilled Group). The eighteen rabbits of Groups 1, 2, and Control were divided into 3 groups of 6 animals each, and the bilateral defects were filled by restoring their original contour. The following materials were used: a xenograft enriched with fresh bone marrow (Group 1), a xenograft enriched with bone marrow mononuclear fraction (Group 2), and a xenograft alone (Control Group). In these three groups, one of the calvarial defects was randomly covered with a barrier membrane (Fig. 2). In the remaining three rabbits, the defects were left empty (Unfilled Group). In all groups, the incision was closed in layers.

Randomization was performed using specific software, available at http://www.randomization.com/

Aspiration of autologous bone marrow was conducted under general anesthesia. Two-milliliter bone marrow aspirates was obtained from each tibia of the 21 rabbits using a disposable 40 × 10 needle (1.10 mm × 38 mm) with 20-ml disposable syringes, previously heparinized to prevent blood clotting. In the 12 rabbits enrolled in Groups 1 (n = 6) and 2 (n = 6), the bone marrow stromal cells – whether concentrated [Group 2] or not [Group 1] – were mixed with the xenografts, in the 9 rabbits enrolled in the Control Group (n = 6) and in the Unfilled Group (n = 3), the autologous bone marrow aspirates were discarded.
The following procedure was performed to obtain the bone marrow mononuclear fraction (BMMF). After the bone marrow was aspirated, it was transferred into polypropylene tubes containing preservative-free heparin (1000 units/ml). The bone marrow and heparin were mixed well. The BMMF was separated from the bone marrow tissue by density-gradient centrifugation using Ficoll-Histopaque-1077 (Sigma-Aldrich, Saint Louis, MO, USA) according to the manufacturer’s instructions. In essence, 3.5 ml of the bone marrow tissue was diluted in a 1 : 1 ratio with PBS, which was placed in a conical tube [15 ml] containing the same volume of Ficoll-Histopaque-1077. The final solution was centrifuged at 200 g for 10 min at room temperature of 4 ml, and another centrifugation was performed at 200 g for 10 min at room temperature. The supernatant was discarded and the pellet was resuspended in 1 ml of PBS.

This final suspension containing the BMMF was then mixed to the xenograft (Fig. 3b).

The animals were given an intramuscular injection of benzylpenicillin [40,000 UI] and sodium dipyrone [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, Wolhusen, Switzerland), which is a particulate xenograft bone substitute. The form used in this study was small particles, with granules ranging in size from 0.25 mm to 1 mm. The barrier membrane used was Bio-Gide® (Geistlich Biomaterials, Wolhusen, Switzerland), which is a resorbable bilaminar collagen membrane of porcine origin used to prevent unwanted invasion of tissues adjacent to the bone defect, thus optimizing bone healing.

All 21 animals were sacrificed 8 weeks after the initial surgery. Their parietal bones were then harvested and processed for histological and histomorphometric evaluation (Fig. 4).

Statistical analysis
All quantitative data were analyzed with SPSS-V17 software (SPSS Inc. 233, Chicago, IL, USA). The Wilcoxon test was used to compare the results obtained with and without membrane coverage in relation to variables NVMT, VMT, and NMT in the three groups (G1, G2, and Control Group). The Friedman test for paired data was used to compare the NVMT, VMT, and NMT results for the situations with and without membrane coverage in the three groups (G1, G2, and Control Group). The Kruskall–Wallis test was used to compare the groups in regard to each variable (NVMT, VMT, and NMT) and also in regard to the results obtained with and without membrane coverage. The Bartlett test was used to evaluate the homoscedasticity. A P-value less than 0.05 indicated statistical significance. Spearman’s correlation coefficient was used to assess interexaminer agreement.

Results
The coefficient of the correlation of Spearman showed a strong association between the examiners \( r = 0.998, \ P = 0.000 \).
All 3 specimens of the Unfilled Group showed no bone formation at the center of the defect and were therefore not included in the histomorphometric analysis (Fig. 5a), unlike the other groups (Fig. 5b).

In Group 1, Group 2 and Control Group, the sides in which the defects were covered with the barrier membrane showed better bone healing [\( P < 0.05 \)] (Intragroup comparison by Wilcoxon test and Friedman test for paired data).

Group 2 showed the lowest resorption level, with the highest percentage of NVMT area [represented by remaining Bio Oss\textsuperscript{®} particles], except for the Unfilled Group [21.23 ± 3.77\% and 20.9 ± 2.01\%, with and without membrane coverage, respectively]. In this regard, there was no statistically significant difference between Group 1 and the Control Group. Evidently, no NVMT was observed in the native bone.

Group 2 showed the highest percentage of vital mineralized tissue area, except for the Unfilled Group [28.17 ± 3.19\% and 21.12 ± 0.55\%, with and without membrane coverage, respectively], followed by Group 1 [21.14 ± 7.37\% and 12.45 ± 6.33\%, with and without membrane coverage, respectively] and the Control Group [13.05 ± 5.23\% and 6.55 ± 1.20\%, with and without membrane coverage, respectively]. The native bone showed a VMT level of 34.68 ± 4.43.

Tables 1 and 2 show the histomorphometric results for Group 1, Group 2, and Control Group.

**Discussion**

In this study, Bio-Oss\textsuperscript{®} osteoconductive xenograft was used in Groups 1 and 2 as a scaffold, allowing delivery of the BMSCs. This material is very similar to human bone, both from a chemical and physical point of view, and can be used as a substitute for an autologous bone graft in many cases (Berglundh & Lindhe 1997; Piattelli et al. 1999; Hallman et al. 2001; Tadjoeidin et al. 2003). Bio-Oss\textsuperscript{®} acts in the early differentiation stages of human mesenchymal cells, possibly contributing to bone formation (Sollazzo et al. 2010). Therefore, the use of this material as a scaffold in tissue engineering seems reasonable.

The best results were observed when the Bio-Gide\textsuperscript{®} barrier membrane was used, probably by preventing an unwanted invasion of adjacent tissues into the bone defect. Based on the histomorphometric results observed in Groups 1 and 2, and in the Control group on the side in which the defect received a membrane coverage, the use of barrier membrane prevented soft tissue growth in the early bone healing stages, increased the amount of VMT, and decreased the amount of NMT, corroborating the findings of Busenlechner et al. (2005). Bio-Gide\textsuperscript{®} was not used in the Unfilled Group, because, in rabbit calvaria, the soft tissue below the defect tends to occupy the space created. Therefore, a barrier merely placed over the defect, without any internal scaffold like the xenograft, does not act as a space maintainer, which is essential for guided bone regeneration (GBR).

In this study, a critical-size bone defect—a defect that cannot heal completely by itself—was effectively created in rabbit calvaria. This was demonstrated by the histological analysis performed in the unfilled specimens, where bone formation never bridged the defect. Thus, the 12-mm bone defects used in our study to create a bilateral experimental design proved effective in rabbit calvaria for studying reconstructive techniques. The 15-mm critical-size defects reported elsewhere in the literature present limitations, insofar as our experience has shown that these bilateral 15-mm defects tend to communicate with each other when created in 3.5–4-kg New Zealand rabbits, without preserving the sagittal suture [data not shown]. On the other hand, Borie et al. (2011) stated that an 8-mm diameter defect is critical in rabbit calvaria, because it cannot regenerate without the use of a bone graft. As 3.5–4-kg New Zealand rabbits allow the use of 12-mm bilateral defects, our choice seems reasonable.

In this study, the bone marrow was harvested from the tibia because of the difficulty involved in obtaining more than 1 ml of bone marrow from the iliac crest [Eça et al. 2009]. Even though harvesting bone marrow from the femur epiphysis area could allow an adequate amount of material, we had noticed that the tibial epiphysis access facilitates the procedure [data not shown]. Although bone marrow was used only in Groups 1 and 2, it was harvested in all groups regardless, to standardize the rabbits’ stress level.

When transplanted, the mesenchymal stem cells from bone marrow follow differentiation pathways controlled by site-specific physiological conditions (Gurevitch et al. 2003). Therefore, the better histomorphometric results obtained in Groups 1 and 2, compared with those observed in the Control and Unfilled Groups, suggest that these cells differentiated into osteoblasts because of the physiological condition of the bone defect created. Nevertheless, it is important to state that there are other factors in bone marrow that could contribute to bone healing, such as the presence of other cells and growth factors (Soltan et al. 2009). The stem cell concentrates obtained by centrifugation [in Group 2] demonstrated even better results than the fresh bone marrow. However, because other cells and growth factors were also present in the concentrates, the benefits observed cannot be credited solely to the mesenchymal stem cells.

To date, there is no consensus about the best methodology for using BMSCs. Although cell cultures can increase the numbers of osteogenic cells, Krzymanski et al. (1997) and Clarke et al. (2007) failed to observe any increased osteogenic potential when using the cell culture technique, as compared to fresh bone marrow. Nevertheless, these findings were not corroborated by Petite et al. (2000). Different methods by which stem cells are isolated and cultured around the
world may account for the different results obtained. The routine clinical use of cell cultures remains unacceptable as yet, because definitive markers for mesenchymal stem cells have not been established, and this lack of standardization may be dangerous [Kotobuki et al. 2005]. The stem cell culture has many more disadvantages, compared with the mononuclear fraction or fresh bone marrow. These shortcomings include cost, time required between harvest and transplantation, contamination risk, and lack of agreement in regard to the number of cells required [Lucarelli et al. 2004]. Hence, the stem cell culture therapy was not evaluated in this preliminary study, owing to these clinical applicability difficulties. Nonetheless, our group will soon publish the results of a study using the cell culture method in the same animal model.

The histomorphometric analysis showed more VMT and less NMT per unit of area in Groups 1 and 2, suggesting that both the use of bone marrow and especially the bone marrow mononuclear fraction lead to a better quality of bone marrow and especially the use of the mononuclear fraction in this animal model led to a higher level of mineralized tissue after 8 weeks, as demonstrated by the higher level of vital mineralized tissue formation and by the preservation of Bio-Oss® particles for a longer period of time, as observed in this group. One hypothesis is that the microenvironment produced by the mononuclear fraction minimizes resorption. However, even though the sum of NVMT (28.17% and 21.23%, respectively) after the expected healing time for rabbits has also been observed elsewhere in the literature in humans after 6 months (Wang et al. 2012). In the cited study, these particles (called NVMT in our study) accounted for a higher level of non-vital mineralized tissue of the native bone was still not evident that the incorporation of the mononuclear fraction into the Bio-Oss® accelerated the healing process.

Bone metabolism in rabbits is approximately three times quicker than in humans [Roberts et al. 1987]. This fact justifies sacrificing the animals after eight weeks, as was performed in the present study, because bone augmentation procedures are usually performed six months (or 24 weeks) prior to implant placement in humans. The presence of residual Bio-Oss® particles after the expected healing time for rabbits has also been observed elsewhere in the literature in humans after 6 months (Wang et al. 2012). The present study, because bone augmentation procedures are usually performed six months (or 24 weeks) prior to implant placement in humans.

Conclusions

Associating fresh autologous bone marrow, and especially the bone marrow mononuclear fraction, to a scaffold, preferably with a barrier membrane, contributed to the enhancement of bone healing. However, the level of mineralized tissue of the native bone was still not reached after 8 weeks of healing in rabbits.

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Disclaimer

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Pelegrine et al. - Bone marrow stromal cells in bone repair

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