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Vertical Bone Construction with Bone Marrow-Derived and Adipose Tissue-Derived Stem Cells

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Abstract: The purpose of this study was to conduct a histomorphometric analysis of bone marrow-derived and adipose tissue-derived stem cells, associated with a xenograft block, in vertical bone constructions in rabbit calvaria. Ten rabbits received two xenograft blocks on the calvaria, after decortication of the parietal bone. The blocks were fixed with titanium screws. The blocks were combined with the bone marrow-derived mesenchymal stem cells in the bone marrow stem cell (BMSC) group (right side of the calvaria) or with the adipose tissue-derived mesenchymal stem cells in the adipose tissue stem cell (ATSC) group (left side of the calvaria). After 8 weeks, the animals were sacrificed and their parietal bones were fixed in 10% formalin for the histomorphometric analysis. The following parameters were evaluated—newly formed bone (NFB), xenogeneic residual particles (XRP), and non-mineralized tissue (NMT). The histomorphometric analysis revealed 11.9 ± 7.5% and 7.6 ± 5.6% for NFB, 22.14 ± 8.5% and 21.6 ± 8.5% for XRP, and 65.8 ± 10.4% and 70.8 ± 7.4% for NMT in groups BMSC and ATSC, respectively, with statistically significant differences in the NFB and the NMT between the groups, but no differences in the XRP. Therefore, it can be concluded that the bone marrow-derived stem cells seem to have more potential for the bone formation than do the adipose tissue-derived stem cells when used in combination with the xenogenous blocks in the vertical bone construction.

Keywords: bone marrow cells; grafts; adipose tissue-derived stem cells

1. Introduction

Large bone constructions represent a challenge for the implant therapy team. In these situations, a bone graft is commonly considered the biological gold standard, once it has osteogenic, osteoinductive, and osteoconductive potentials [1]. However, autografts have a few disadvantages, such as prolonged surgical time and the need for an additional surgery to harvest tissue from the patient at the donor site, which can result in higher morbidity [2,3]. Thus, the study of different types of cell therapy is justified as they present minimal donor site morbidity and a lower risk of autoimmune rejection and disease transmission [4].

Concerning the usage of cell therapy in dentistry, there are many studies on the potential of stem cells for the regeneration of some tissues, such as periodontal tissues [5,6], bone [7,8], and the dentin–pulp complex [9,10]. Most of these studies focused on the use of stem cells from different sources (e.g., bone marrow, adipose tissue, periodontal ligament, and pulp). However, there are a limited number of studies comparing the results of tissue regeneration with stem cells from different sources.

Most bone substitute biomaterials have only osteoconductive potential due to the lack of proteins and of a cellular component [11]. However, the adjunctive use of stem cells with the possibility of osteoblastic differentiation could theoretically result in a composite graft (i.e., stem cell scaffold construct) with osteoconductive, osteoinductive, and osteogenic potential, as demonstrated by Victorelli et al. [12]. Autologous stem cells are adult stem cells that are considered undifferentiated cells found in specialized postnatal tissues and organs. Autologous mesenchymal stem cells have the capacity to differentiate into specialized cells of at least one mesenchymal lineage such as bone, cartilage, fat, or muscle [13], and they can be found in some types of postnatal tissues, such as bone marrow [14], adipose tissue [15], dental tissue [16], and gingival tissue [17].

Therefore, studies comparing the capacity of mesenchymal stem cells from different sources are of major importance in bone tissue engineering, especially in critical situations such as vertical bone constructions. In this study, as in a previous study by our group [7], we compared two of the most frequent types of tissue used in cell therapy—adipose tissue and bone marrow. However, as the delivery vehicle is important to the performance of mesenchymal stem cells [18,19], in the present study, we used a scaffold in block form, which is a common clinical strategy when treating bone defects that require appositional reconstructions.

2. Materials and Methods

This study was analyzed and approved by the Research Ethics Committee of the São Leopoldo Mandic Dental School, Campinas, SP, Brazil (process 0191/14).

2.1. Bone Marrow Harvest by Aspiration

Autologous bone marrow was obtained by aspiration after anesthesia in all 10 animals. Anesthesia was induced with ketamine (40 mg/kg), midazolam (2 mg/kg), and fentanyl citrate (0.8 μg/kg), and maintained with isoflurane/nitrous oxide (1:1.5%) and oxygen (2/3:1/3) with a pediatric facemask. In addition, a local anesthesia was provided via 1 mL of 2% lidocaine HCl and epinephrine 1:100,000 diluted in 1 mL of physiological saline solution.

Two-milliliters of bone marrow aspirates were obtained from each tibia of the ten rabbits using disposable 40 × 10 needles (1.10 mm × 38 mm) and 20-mL disposable syringes previously heparinized to prevent blood clotting.

2.2. Culture of Adult Bone Marrow-Derived Mesenchymal Stem Cells

The procedure to obtain the BMSCs followed the standard guidelines and was completely described in a previous publication of our group (Coelho de Faria et al.) [7].

The BMMSCs, after culture, were detached and resuspended in the culture medium and subsequently mixed with the xenograft in the BMSC group.

2.3. Lipectomy for Adipose Tissue Isolation

The adipose tissue was obtained from the back of all the 10 animals following the same general anesthesia protocol previously described by our group (Coelho de Faria et al.) [7].

The collected material of all the animals was immediately taken to the cell culture laboratory for tissue processing.

2.4. Culture of Adult Adipose Tissue-Derived Mesenchymal Stem Cells

The procedure to obtain the ATSCs followed the standard guidelines and was completely described in a previous publication of our group (Coelho de Faria et al.) [7].

The ATSCs, after culture, were detached and resuspended in the culture medium and subsequently mixed with the xenograft in the ATSC group.

2.5. Cell Adhesion Capability

Cell adhesion was analyzed using an inverted optical microscope 3 days after seeding the cells into the culture flask, and it was verified that the cells were plastic adherent when maintained under the standard culture conditions.

2.6. Differentiation Assays

Adipogenic, osteogenic, and chondrogenic differentiation assays were done, following the same methodology adopted previously by our group (Coelho de Faria et al.) [7].

2.7. Immunophenotypic Characterization

The bone marrow-derived and adipose tissue-derived stem cells from the second passage were used for immunophenotypic characterization, following the same methodology used previously by our group (Coelho de Faria et al.) [7]. The cells showed compatible immunophenotyping (CD16+, CD34−, CD45−, CD73+, CD90+, and CD105+).

2.8. Seeding Cells into the Scaffolds

In all the groups, 1 mL of the solution containing phosphate buffered saline (PBS) and 1×10^5 cells were seeded into the scaffolds. The solution was slowly pipetted onto the scaffold which, due to its characteristic, was fully absorbed. At the end of the procedure, the scaffolds containing the cells inside were ready to be inserted in the surgical site.

2.9. Experimental Design and Surgical Protocol

Ten adult male New Zealand rabbits aged between 10 and 12 months and weighing between 3.5 and 4 kg were selected. The animals were acclimatized in individual cages for 14 days, in a temperature-controlled room (18 °C to 20 °C), subjected to a 12-h light cycle. The animals were fed a commercial pelleted diet and allowed ad libitum access to water.

All animals were subjected to anesthesia following the same general anesthesia protocol previously described. The animals received two commercial xenogenous blocks of bovine origin (Baumer, Mogi Mirim, SP, Brazil). The blocks were placed on their parietal bones, bilaterally to the midline with the aid of one fixing screw, after performing five perforations into the cortical plate of the parietal bone to encourage bleeding and graft nutrition (Figure 1).

The xenograft blocks from group BMSC were combined with the autologous bone marrow-derived mesenchymal stem cells (n = 10) and those from group ATSC were combined with the autologous adipose tissue-derived mesenchymal stem cells (n = 10); 1 mL of phosphate buffered saline (PBS) solution (Sigma Aldrich, Darmstadt, Germany) containing 1×10^5 cells was used in both groups, and the respective stem cells were added dropwise to each block. Each animal received one block of each group (i.e., BMSC group and ATSC group). The block from the BMSC group was fixed on the right side of the calvaria and the block from the ATSC Group was fixed on the left side of the calvaria. The surgical wounds were then subjected to primary closure.

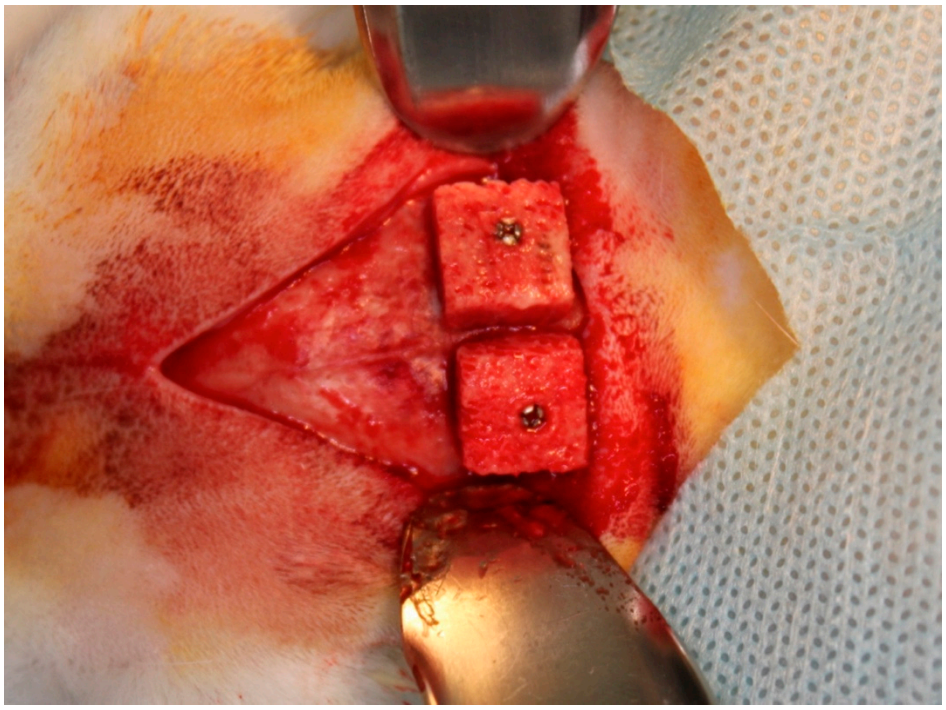


Figure 1. Xenograft blocks fixed on the rabbit's calvaria with titanium screws.

All animals were post-operatively medicated with benzylpenicillin (40,000 IU) and sodium dipyrone (0.25 mg/kg). The animals were sacrificed 8 weeks after surgery and their parietal bones were removed and processed for histological and histomorphometric evaluation.

2.10. Histological Preparation and Histomorphometric Evaluation

A portion of the parietal bone with an approximate area of 18 mm^2 containing the block at the center was removed using an oscillating saw. Samples were fixed in 10% formalin for 7 days and then decalcified in 10% EDTA for 6 days at room temperature and fixed with 10% buffered formaldehyde. The histological slices were prepared using a microtome to cut 5 µm transverse sections of the entire set, including the graft, from the center of the block (where the screw was positioned) up to 2 mm from the center of the block. The sections were stained with Masson's trichrome and assessed by optical microscopy (Nikon Eclipse C1, New York, NY, USA). A digital CCD camera was used to acquire images for the subsequent analyses (Infinity-1, Lumenera®, Ottawa, ON, Canada). All slides were analyzed in four areas (upper left, lower left, upper right, and lower right), which allowed the determination of tissue status in the interface, near the recipient bed (by using the lower left and lower right measurements) and also far from the recipient bed, at the block's surface (by using the upper left and upper right measurements). Then, the overall average was calculated for each slide. Each evaluated site had a dimension (area) of 1,347,442 μm^2. Two previously calibrated examiners assessed the specimens blindly and, in case of disagreement, the sample was reviewed to reach a consensus. An average of the measurements for each area obtained by the two examiners was recorded. The examiners traced all images using Infinity Analyse® (Lumenera Corporation, Ottawa, ON, Canada), measuring the following parameters: (1) newly formed bone (NFB), (2) xenogeneic residual particles (XRP), and (3) non-mineralized tissue (NMT). All results were obtained in μm^2 and expressed as percentage of the total area.

2.11. Statistical Analysis

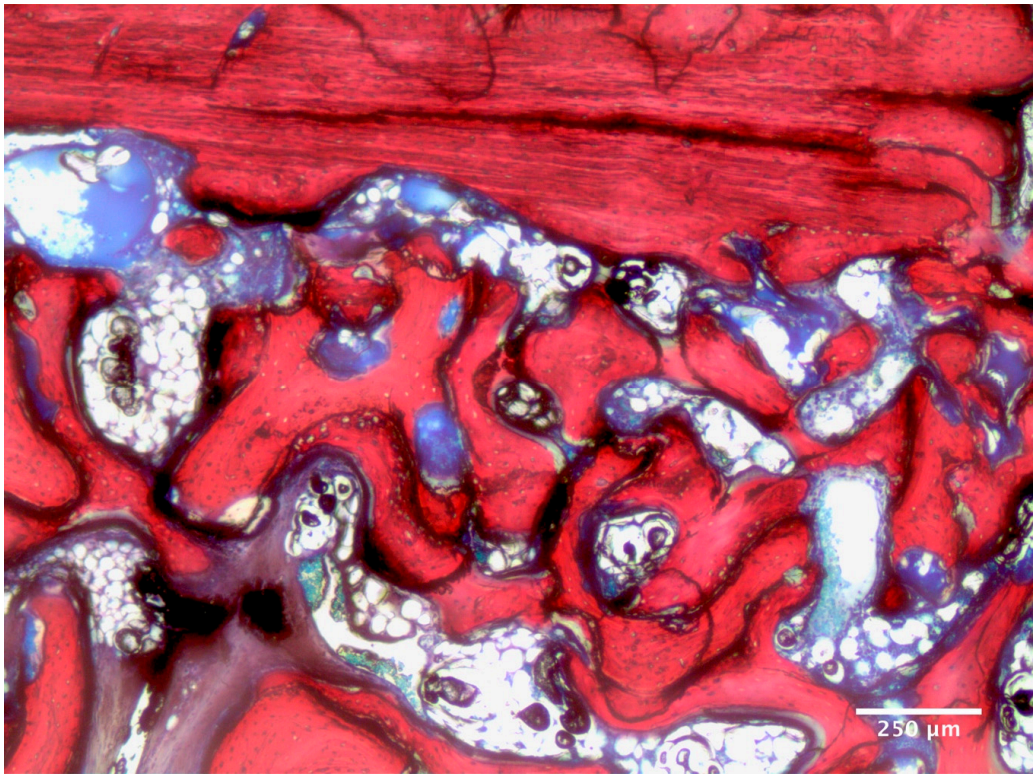
Prior to the analyses, data from the newly formed bone (NFB), the xenogeneic residual particle (XRP) and the non-mineralized tissue (NMT) were evaluated for normality (Shapiro-Wilk tests) and homogeneity of variance (Levene tests), and it was verified that both were attended.

Analysis of variance with two criteria for randomized blocks (two-way ANOVA) was applied to investigate whether percentages of NFB, XRP, and NMT were influenced by independent variables of stem cell type (bone marrow/adipose tissue) and calvaria distance (near/far) or by the interaction of both. If there was a significant interaction, Tukey's test was used for multiple comparisons.

All quantitative data were analyzed by SPSS-V17® (SSPS Inc. 233, Chicago, IL, USA).

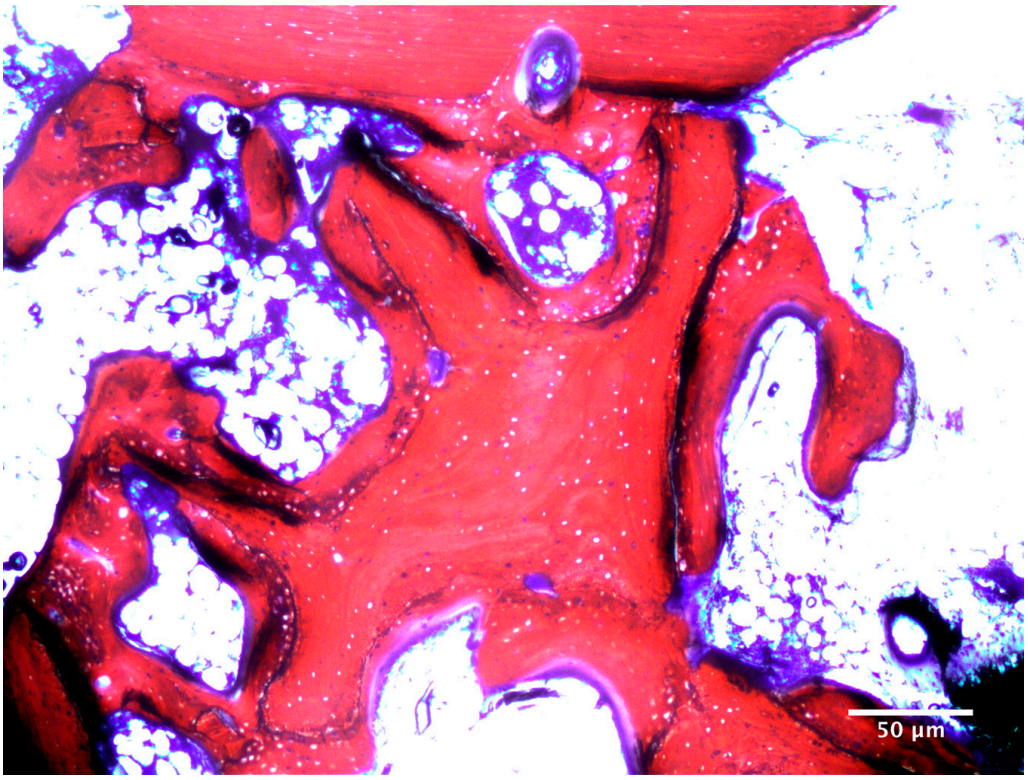
3. Results

Pearson's correlation test, which yielded a value of 0.99, was used to evaluate the measurements obtained from the two examiners. Therefore, we chose to use the mean measurements obtained by the two examiners. In low magnification (40×), the histological characteristics of the analyzed samples showed mineralized and non-mineralized tissues (Figure 2A). In higher magnification, both groups (BMSC and ATSC) showed areas of the newly formed bone, with a layer of osteoblasts adjacent to the reminiscent osseous trabeculae from xenograft blocks (Figure 2B,C, respectively). Variable quantities of the fibrovascular connective tissue were seen interspersed with the bone trabeculae (Figure 2B,C).

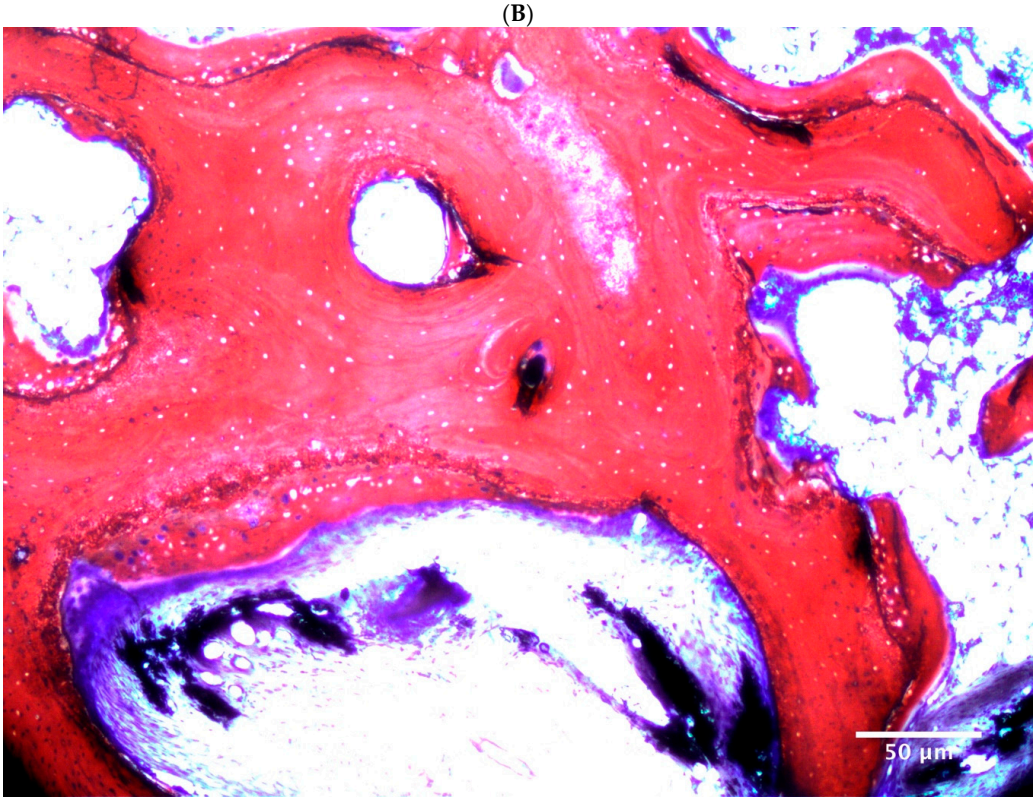


(**A**)

Figure 2. *Cont.*



(**B**)



(**C**)

Figure 2. (**A**) Histologic characteristics of xenograft blocks combined with BMMSC (G1) and ATSC (adipose tissue stem cells) (G2). Histologic characteristics of a sample, in low magnification, showing native bone (above) and the grafted area, 40×. Stain, Masson's trichrome. (**B**) Higher magnification showing reminiscent bone trabeculae from G1 xenograft block surrounded by newly formed bone, 200×. Masson's trichrome. (**C**) Higher magnification showing reminiscent bone trabeculae from G2 xenograft block surrounded by the newly formed bone, 200×. Stain, Masson's trichrome.

For the newly formed bone (NFB) data, the two-way ANOVA showed no significant interaction between the concentrate type variables and the distance in the rabbit calvaria ($p = 0.356$). The results showed that the type of stem cell used significantly affected the percentage of the newly formed bone (NFB) ($p = 0.036$), a fact that occurred near (interface between the graft and the recipient bed) or far (graft's surface) from the recipient bed. Specifically, by Tukey's test, it was found that the impregnation of the xenograft block with the adipose tissue stem cells resulted in a percentage of newly formed bone (NFB) statistically lower than that observed for the group in which the bone marrow stem cells were used. Analysis of variance with two criteria for randomized blocks also indicated that in groups in which the xenograft block was impregnated with the adipose tissue stem cells or the bone marrow stem cells, the highest percentage of newly formed bone (NFB) was observed at a location near the recipient bed ($p = 0.003$).

For the percentage of xenogeneic residual particle (XRP), there was a significant interaction between both variables, type of cell and distance in rabbit calvaria ($p = 0.063$), as indicated by the analysis of variance with two criteria for randomized blocks. This test also identified no statistically significant difference ($p = 0.750$) between the groups (i.e., bone marrow and adipose tissue stem cells) in the percentage of xenogeneic residual particle (XRP). When the distances were compared, it was found that the percentage of xenogeneic residual particle (XRP) was significantly higher in the more distant location (i.e., far from the recipient bed) ($p = 0.002$).

Evaluating non-mineralized tissue (NMT), the two-way analysis of randomized blocks indicated a significant interaction between the stem cell type (i.e., BMSC and ATSC) and rabbit calvaria distance (i.e., near and far) ($p = 0.038$). Applying the Tukey's test, in close proximity to the calvaria, it was verified that the impregnation of the xenogeneic bone with the adipose tissue stem cells (ATSC) resulted in a greater percentage of non-mineralized tissue (NMT) in comparison to the group in which the impregnation occurred with the bone marrow stem cells (BMSC). In the more distant location, both groups did not differ significantly in relation to the percentage of non-mineralized tissue (NMT). The Tukey test also showed that the percentage of non-mineralized tissue (NMT) was higher in the distant location for the bone marrow stem cell group, whereas the adipose tissue stem cell group showed the highest percentage of non-mineralized tissue (NMT) at the nearest location. Table 1 shows the histomorphometric results and statistical comparisons.

Table 1. Histomorphometric results (mean and standard deviation) and statistical comparison between groups (in %)—BMSC, bone marrow stem cells group; ATSC, adipose tissue stem cells group; XRP, xenograft residual particles; NFB, newly formed bone; and NMT, non-mineralized tissue. Numbers inside the brackets are standard deviation and outside the brackets are mean. The mean values followed by different capital letters indicate statistically significant difference between the groups, within each column, considering separately each type of tissue. The mean values followed by different small letters indicate statistically significant difference between the sites, within each line, considering separately each type of tissue.

TISSUE	CELL SOURCE	DISTANCE-NEAR	DISTANCE-FAR	MEAN
NFB	BMSC	16.0 ± 6.1%	7.8 ± 6.7%	11.9 ± 7.5% A
	ATSC	9.9 ± 5.4%	5.3 ± 5.2%	7.6 ± 5.6% B
	MEAN	12.9 ± 6.4% a	6.6 ± 5.9% b	-
XRP	BMSC	20.7 ± 9.9%	24.1 ± 7.0%	22.14 ± 8.5% A
	ATSC	15.2 ± 4.9%	28.0 ± 6.3%	21.6 ± 8.5% A
	MEAN	17.9 ± 8.1% a	26.1 ± 6.8% b	-
NMT	BMSC	63.4 ± 10.5% Bb	68.3 ± 10.3% Aa	-
	ATSC	74.8 ± 7.4% Aa	66.7 ± 7.4% Ab	-

4. Discussion

There is consensus in the literature about the potential use of bone substitute materials to replace autogenous bone grafts in some clinical situations [20–22]. Nevertheless, in critical defects

(e.g., vertical bone reconstruction), the lack of a cellular osteogenic component may limit the use of bone substitute biomaterials [11]. Therefore, the study of cell therapy is extremely important in order to remedy this situation.

As the adult mesenchymal stem cells have the capacity to differentiate into specialized cells of at least one mesenchymal lineage such as bone, cartilage, fat, or muscle [13], the use of these undifferentiated cells harvested from the adipose tissue and the bone marrow seems to have clinical applicability in regenerative medicine and, as far as the implant therapy is concerned, in bone reconstruction as well. The mesenchymal stem cells used in the present study fulfilled the minimal criteria proposed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT) position statement, which defined multipotent mesenchymal stromal cells [23]. These criteria were tested by cell adhesion, differentiation assays, and immunophenotypic analysis carried out as described in the Methods section. However, despite the fact the authors of the present study have used this standardization, SEM analysis was not performed to visualize the mesenchymal stem cells inside the scaffold.

In the present study, the percentage of newly formed bone was lower than in a previous study by our group [7], where we used a xenograft as a scaffold for the vertical bone construction on rabbit calvaria associated with the bone marrow-derived mesenchymal stem cells or the adipose tissue-derived stem cells. These differences in the levels of bone formation between the studies may have been caused by differences in (1) the characteristics of the scaffold and/or (2) the titanium device used to stabilize the scaffold. The presence of a particulate xenogenous graft in the first study could have contributed to a more adequate level of revascularization and, therefore, bone formation, when compared to the structured block graft of the present study. Moreover, the use of a titanium cylinder instead of a titanium screw certainly resulted in a larger titanium area in contact with the graft and bone in the previous study, which might have stimulated bone formation as titanium has a higher affinity for bone [24].

However, the XRP levels were similar between these two studies, with rates of 22.14 $\pm$ 8.5% and 21.6 $\pm$ 8.5% for the bone marrow-derived and the adipose tissue-derived mesenchymal stem cells, respectively, in the present study, and 23.31 $\pm$ 3.11% and 27.58 $\pm$ 3.98% for the bone marrow-derived and the adipose tissue-derived mesenchymal stem cells, respectively, in the previous study. Therefore, the use of a particulate or structured bone scaffold and the differences between the titanium areas probably did not have any influence. On the other hand, the NMT levels appear to be influenced by the scaffold characteristic and/or titanium area, since the percentage of this tissue in the group where the bone marrow-derived and the adipose tissue-derived stem cells were used was 65.8 $\pm$ 10.4% and 70.8 $\pm$ 7.4%, respectively, in the present study, and 50.23 $\pm$ 8.72% and 49.90 $\pm$ 8.76%, respectively, in the previous study. Therefore, the use of a particulate mineralized scaffold instead of a structured mineralized scaffold and the use of a larger area of titanium device to stabilize the scaffold may result in more newly formed bone and less soft tissue, which could contribute to an improved osseointegration when an implant is placed. However, it is important to state that the xenografts used in these two studies were not processed by the same company and, therefore, it might also contribute to different results.

Previous studies undertaken by our research group using the bone marrow-derived and the adipose tissue-derived mesenchymal stem cells in bone defects on rabbit calvaria showed the same tendency of higher levels of bone formation when using bone marrow stem cells (Pelegrine et al., 2014; Aloise et al., 2015; Zimmermann et al., 2015) [11,25,26]. Therefore, in both situations, onlay and inlay bone constructions, bone marrow may be considered a better choice for the tissue source of mesenchymal stem cells when compared to fat. The best results, in all studies, at the sites where the bone marrow-derived mesenchymal stem cells were used might be explained by the fact that cells from the bone marrow have a greater affinity for osteogenic differentiation, as stated before by our group [7]. Moreover, as mesenchymal stem cells are multipotent cells that are capable of multiple lineage differentiation due to the presence of inductive signals from the microenvironment [27,28],

we hypothesize that the microenvironment of bone marrow present in the recipient bed had a more pronounced effect on the stem cells isolated from the bone marrow than on those obtained from the adipose tissue. This might also have repercussion in the higher levels of non-mineralized tissue at the interface between the recipient bed and the graft, as observed in the ATSC group. This finding represents a worse integration of the graft combined with the adipose tissue stem cells and, if proven by future clinical studies, could reflect in the implants' survival rates, as the dental implants are commonly installed in this interface area.

5. Conclusions

The bone marrow-derived mesenchymal stem cells seem to have a higher potential for bone formation compared with the adipose tissue-derived mesenchymal stem cells when used in combination with the xenogenous blocks in the vertical bone construction.

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Conflicts of Interest: The authors declare no conflict of interest.

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