Alendronate Associated with Bovine Bone Graft in Bone Defect Repair: A Histomorphometric Study

Douglas Bertazo Musso¹, Conrado Dias do Nascimento Neto², Natália Marreco Weigert¹, Stela Maris Wanderley Rocha³, Robson Almeida de Rezende⁴, Elizabeth Pimentel Rosetti⁵, Rossiene Motta Bertollo⁶, Martha Chiabai Cupertino Castro⁷, Daniela Nascimento Silva⁸*

1. Dentistry Clinical, Federal University of Espírito Santo, Vitória, ES, Brazil.
2. Dentistry Science, Federal University of Espírito Santo, Vitória, ES, Brazil.
3. Dentistry Department, Federal University of Alagoas, Maceió-AL, Brazil.
4. Department of Dentistry Clinical, Federal University of Espírito Santo, Vitória-ES, Brazil.
5. Department of Dental Prosthodontics, Federal University of Espírito Santo, Vitória-ES, Brazil.
6. Department of Dentistry Clinical, Federal University of Espírito Santo, Vitória-ES, Brazil.
7. Department of Dental Prosthodontics, Federal University of Espírito Santo, Vitória-ES, Brazil.
8. Department of Dentistry Clinical, Federal University of Espírito Santo, Vitória-ES, Brazil.

Abstract

This study evaluated the effect of 0.5% ALN associated with bovine bone graft on new bone formation and graft resorption. Two defects were created in the calvarium of 18 rats and filled with: B = bovine bone graft; AB = 0.5% ALN + bovine bone graft; and C = control (blood clot). After 90 days, a histomorphometric analysis of the specimens provided total tissue volume and amount of newly formed bone and of remaining graft. Mean (SD) total tissue volume was 88.0% (28.8%) in group B, 66.9% (24.0%) in group AB, and 63.6% (10.7%) in group C. The amount of newly formed bone was group B = 46.0% (22.0%), group AB = 29.9% (17.8%) and group C = 49.9% (16.4%), and of remaining graft was group B = 9.2% (4.5%) and group AB = 12.2% (7.2%). After Student’s t-test (p ≤ 0.05), there was a statistical difference in total tissue volume between groups B and C (p = 0.03) and in amount of newly formed bone between groups AB and C (p = 0.025). ALN associated with bovine bone graft did not improve new bone formation and had no effect on bone graft resorption. The bovine bone graft alone showed better results in terms of total volume of tissue filling the bone defects.


Keywords: Heterologous transplantation, Alendronate, Calvaria.
Received date: 14 March 2021  Accept date: 24 April 2021

Introduction

Lyophilized bovine bone grafts have been widely used in dentistry to reconstruct alveolar ridges, to fill periodontal and peri-implant intrabony defects, and in procedures of maxillary sinus floor elevation. Due to its great similarity to human bone, bovine bone graft is added to the natural process of bone shaping and remodeling, and its presence provides permanent correction of alveolar defects. With its incorporation, bovine bone graft helps recreate the natural contour of alveolar ridges, enabling later implant rehabilitation.¹

Despite its several indications, one of the disadvantages of bovine bone graft is the time interval between grafting and its incorporation into the recipient bed, which may range from 6 to 10 months ², besides not being a suitable material in post-traumatic cases, in deformities or hypoplastic areas.³ This encourages its association with other substances that accelerate local new bone formation. Studies on bone regeneration have included the use of biological mediators to improve quantity and quality of regenerated bone.⁴

Bisphosphonates are a group of mediators of bone metabolism with a potent inhibitory effect on bone resorption that are able to control osteolysis or to minimize bone loss caused by various diseases.⁵ They have been used as antiresorptive agents for the treatment of diseases in which there is increased osteoclastic resorption, including post-menopausal osteoporosis, Paget’s disease, and tumor-
Nitrogen-containing bisphosphonates have shown greater potency in inhibiting bone resorption. Alendronate (ALN) is a nitrogen-containing bisphosphonate that has been available in the market since 1990 and whose effect on osteoclasts is approximately 70 times more potent compared to non-nitrogen-containing bisphosphonates.\(^6\)\(^7\)

A single local application of ALN may allow its proper deposition in bone tissue due to its high affinity for the mineral portion of bone tissue, resulting in reduction of bone resorption and induction of increased alveolar bone regeneration. Thus, ALN may be considered a therapeutic option in different cases of bone remodeling and resorption.\(^8\)\(^9\)

There have been few studies on the association of bisphosphonates with lyophilized bovine bone graft on bone repair, which have found, in general, positive and dose-dependent effects.\(^10\)\(^-\)\(^13\) No entanto, a concentração ideal do ALN não está bem estabelecida. Isso motiva pesquisas de novas associações, buscando reduzir o tempo de osteointegração do osso bovino liofilizado ao leito receptor e acelerar o reparo de defeitos ósseos.

Histomorphometric analysis has been used for the evaluation of the bone repair process in several experimental studies.\(^8\)\(^,\)\(^9\)\(^,\)\(^14\)\(^-\)\(^16\) It has been widely used to investigate metabolic bone diseases, as it enables direct and accurate assessment of changes in bone remodeling, including tissue mechanisms involved in the process.\(^17\)

The aim of the present study was to evaluate the effect of 0.5% ALN associated with lyophilized bovine bone graft on the bone repair process of rat calvarial bone defects.

**Materials and methods**

This study was approved by the Institutional Animal Care and Use Committee of the Universidade Federal do Espírito Santo (Brazil) with protocol number 010/2014. The sample consisted of 18 adults male Wistar rats weighing an average of 300 g. Two bone defects were created in the calvarium of each animal, for a total of 36 bone defects.

The animals were anesthetized using a mixture of 10% ketamine (0.05 mL/100 g - Agener®, União Química Farmacêutica Nacional S/A, Brazil) and 2% xylazine (0.025 mL/100 g - Flotril®, Intervet Schering-Plough, Brazil) intraperitoneally, and received antibiotic prophylaxis with 2.5% enrofloxacin (10 mg/kg) subcutaneously. The region between the external ears of the rats was shaved and cleaned with 2% chlorhexidine, and 2% lidocaine with 1:100,000 norepinephrine (Alphacaine®, DFL Ind. e Com. S/A, Brazil) was infiltrated subcutaneously. Then, a 1.5-cm linear coronal dermo-periosteal incision was performed, followed by periosteal elevation. Two bone defects (right and left) measuring 5 mm in diameter were created in the parietal bone laterally to the sagittal suture (Figure 1) using a pear-shaped multi-laminated drill. External and internal cortical bones of the calvarium were ruptured without damaging the meninges.

Study substances were injected into the right defect of the parietal bone of each animal according to the following groups:

**Group B:** lyophilized bovine bone graft (Bio-Oss® - Geistlich Pharma AG, Wolhusen, Switzerland) in small granules (0.25 to 1 mm) and wetted with 0.9% saline solution (n = 9);

**Group AB:** bovine bone graft soaked in 0.5% ALN solution (Pharmácia Specífica Ltda, Bauru, Brazil) (n = 9);

**Group C (control):** the left defects of group B were filled with autologous blood clot from the surgical wound bed (n = 9). Left defects were also created in the calvaria of group AB to ensure that all animals underwent the same surgical procedure on both sides of the skull. However, these defects were not used as controls due to a possible systemic effect of ALN.\(^8\)

Tissues were repositioned and closed with simple interrupted 5-0 nylon sutures. After 90 days of observation, the rats were euthanized with a lethal dose of xylazine (15 mg/kg to 30 mg/kg body weight) and ketamine (150 mg/kg to 225 mg/kg body weight). The region of calvarial bone containing the defects was removed and fixed in 10% buffered formalin for histological processing. Seven-micrometer sections were cut from each specimen and stained with hematoxylin and eosin (HE).

Histological images were obtained with a light microscope (Primo Star®, Zeiss, Oberkochen, Germany) coupled to a microscope camera (Axioskop ERC 5s®, Zeiss, Oberkochen, Germany). Histomorphometric analysis was performed using an image analysis software...
(AutoCAD® 2010, Autodesk, San Rafael, CA, USA). The following data were obtained: total tissue volume (%) and amount (%) of newly formed bone, connective tissue (including fibrous tissue, blood vessels, and adipose tissue), and remaining graft (Figure 2). Student’s t-test for independent samples was used to compare these data between groups. Data were analyzed using a statistical software package (SPSS®, version 21.0; IBM, Armonk, NY, USA).

Results

There was no statistically significant difference in the mean amount of newly formed bone between groups B and C (control). However, connective tissue filled a mean volume of 32.8% of bone defects in group B and 13.7% in group C, and the difference was statistically significant ($P = 0.021$). Remaining bovine bone graft granules filled a mean volume of 9.2%, which contributed to greater tissue volume filling the bone defect in group B (mean volume of 88.0%) compared to group C (63.6%). This difference was also statistically significant ($P = 0.030$) (Table 1).

Table 1. Total tissue volume and amount of newly formed bone, connective tissue, and remaining bovine bone graft in bone defects created in rat calvaria – Group B vs. Group C (control).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group B</th>
<th>Group C (control)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newly formed bone</td>
<td>46.0 ± 2.0</td>
<td>49.9 ± 16.4</td>
<td>0.675</td>
</tr>
<tr>
<td>Connective tissue</td>
<td>32.8 ± 20.6</td>
<td>13.7 ± 8.7</td>
<td>0.021*</td>
</tr>
<tr>
<td>Remaining bovine bone graft</td>
<td>9.2 ± 4.4</td>
<td>4.6 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>Total tissue volume</td>
<td>88.0 ± 20.6</td>
<td>63.6 ± 10.7</td>
<td>0.030*</td>
</tr>
</tbody>
</table>

*statistically significant difference for $P \leq 0.05$.

There was no statistically significant difference in the mean volume of connective tissue between the groups ($P = 0.175$). Total tissue volume was higher in group AB (66.9 ± 24.0%) compared to group C (63.6 ± 10.7%), but this difference was not statistically significant ($P = 0.078$) (Table 2).

The amount of newly formed bone was 46.0% in group B and 29.9% in group AB, with no statistically significant difference ($P = 0.107$). There was a greater amount of connective tissue in group B, contributing to a total tissue volume of 88.0%. In group AB, total tissue volume was 66.9%, but the difference was not statistically significant ($P > 0.05$) (Table 3).

Table 2. Total tissue volume and amount of newly formed bone, connective tissue, and remaining bovine bone graft in bone defects created in rat calvaria – Group AB vs. Group C (control).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group AB (Bovine bone graft + 0.5% ALN)</th>
<th>Group C (control)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newly formed bone</td>
<td>29.9 ± 17.8</td>
<td>40.9 ± 15.4</td>
<td>0.025*</td>
</tr>
<tr>
<td>Connective tissue</td>
<td>24.8 ± 21.8</td>
<td>13.7 ± 8.7</td>
<td>0.175</td>
</tr>
<tr>
<td>Remaining bovine bone graft</td>
<td>12.2 ± 7.2</td>
<td>4.6 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>Total tissue volume</td>
<td>68.9 ± 24.0</td>
<td>63.6 ± 10.7</td>
<td>0.708</td>
</tr>
</tbody>
</table>

*Statistically significant difference for $P \leq 0.05$. 

Table 3. Total tissue volume and amount of newly formed bone, connective tissue, and remaining bovine bone graft in bone defects created in rat calvaria – Group AB vs. Group C (control).
osteoclasts have suggested its involvement in the elucidation. Clinical and histological studies through various biochemical processes. Interaction with osteoclasts and osteoblasts incorporation into the bone, enabling a direct biological effects are primarily attributed to their when applied locally o

other bisphosphonates reduce bone resorption systemic circulation. Should be applied, as opposed to excessive and bioactive substances and exactly where they have provided information about optimal doses of bioactive substances and exactly where they should be applied, as opposed to excessive and unnecessary administration of drugs through the systemic circulation.

Some studies have shown that ALN and other bisphosphonates reduce bone resorption when applied locally or systemically. Their biological effects are primarily attributed to their incorporation into the bone, enabling a direct interaction with osteoclasts and osteoblasts through various biochemical processes.

The mechanism of action of ALN has yet to be elucidated. Clinical and histological studies have suggested its involvement in the recruitment, activation, and apoptosis of osteoclasts. After its incorporation into the bone matrix, ALN remains pharmacologically inactive until bone remodeling occurs, when its interaction with osteoclasts starts again. The suppression of osteoclast activity causes loss of cytoskeletal integrity with disappearance of its convoluted membrane structure, called ruffled border, which is a strong indication of osteoclast inactivation by bisphosphonates in vivo, followed by apoptosis.

Although the primary effect of ALN is inhibition of osteoclast-mediated bone resorption, some studies have demonstrated that these drugs also interact with osteoblasts. This may increase proliferation and maturation of osteoblasts and inhibit their apoptosis. These findings strongly suggest that ALN has an anabolic effect on osteoblasts, stimulating subsequent bone formation. There is evidence that the effect of ALN on osteoblast activity may be more important to an increase in bone formation during defect repair than its effect on osteoclast activity.

The optimal concentration of topical ALN for inhibiting bone resorption and increasing new bone formation has yet to be established in the literature, since different results have been found. In a study with rats, Yaffe et al. performed mucoperiosteal flap elevation, locally applied collagen sponges soaked with different doses of ALN (0.15, 0.75, and 1.5 mg/mL), and found no inhibitory effect on bone resorption. In 1997, Yaffe et al. repeated the experiment increasing the dose of ALN to 20 mg/mL and observed a significant reduction in alveolar bone resorption. Jaime et al., however, evaluated the effect of local delivery of ALN (20 mg/mL) soaked in collagen sponge on bone defects and found no difference between the treated and control groups. Binderman and Yaffe investigated the effect of lower doses of ALN (10, 50, 200, and 400 μg) soaked in collagen sponge and found that its topical application had a positive, dose-dependent effect with significantly reduced alveolar bone loss. Komatsu et al. also obtained favorable results in terms of bone repair with topical application of 1 mM ALN, which significantly reduced root and bone resorption and stimulated bone formation around the replanted teeth.

Regarding concentration levels, Sharma and Pradeep found that the local delivery of 10 μL of 1% ALN (equivalent to 0.1 mg/mL) resulted in increased new bone formation in patients with
periodontal disease. Rocha\textsuperscript{11} evaluated the effect of two concentrations of ALN (0.5 and 1%) associated with bovine bone graft on the repair of bone defects in rabbits and found, using microradiographic analysis, a statistically significant increase in new bone formation compared to bovine bone graft alone. The concentration of 0.5% ALN led to greater bone repair. Ming et al.\textsuperscript{40} reported lower bone mineral density in the group treated with 250 µg of ALN, demonstrating the inhibitory effect of this concentration on bone formation.

The results of the present study showed no difference in the amount of newly formed bone between groups B and C at 90 postoperative days. However, group B had greater total tissue volume (88.0%), as a result of a mean volume of 9.2% of graft remaining granules and a higher amount of connective tissue (with statistically significant difference between groups). This finding suggests that bovine bone graft is able to maintain connective tissue around its granules. A long-term assessment of the bone repair process could clarify whether this surrounding connective tissue would be partially or totally replaced by newly formed bone together with bovine bone graft.

The resorption of bovine bone matrix and its replacement by bone tissue have been widely discussed in the literature. Different degrees of resorption have been reported, especially when results of experimental studies with animals and humans are compared.\textsuperscript{41} Some studies have shown that bovine bone graft is quickly replaced by newly formed bone compared to other hydroxyapatites.\textsuperscript{42} Other studies have demonstrated a slow process of bone graft resorption.\textsuperscript{10,43}

In the present study, when 0.5% ALN was mixed with bovine bone graft and injected into the defect (group AB), new bone formation was significantly lower compared to controls (group C). However, the two groups showed a very similar total tissue volume due to the presence of graft granules and the amount of surrounding connective tissue.

Most studies on the association of bovine bone graft with a bisphosphonate have found an improvement in bone formation due to increased amount of trabecular bone.\textsuperscript{10,11,13} Houshmand et al.\textsuperscript{10} evaluated the effect of the bisphosphonate pamidronate disodium mixed with bovine bone graft on the repair process of bone defects created in the mandible of sheep. A histomorphometric analysis showed that this association improved new bone formation and significantly decreased the number of osteoclasts in the regenerated bone. Kim et al.\textsuperscript{13} added 1 mM and 10 mM ALN to bovine bone graft and found, using histomorphometric analysis, a significant increase in new bone formation in the group receiving a lower dose (1 mM ALN) at weeks 2 and 4. However, after week 8, this difference was not significant comparing the two dose levels with bovine bone graft alone. Results indicated that 1 mM ALN associated with bovine bone graft has a synergistic effect on bone regeneration due to decreased activity of the receptor activator of nuclear factor kappa-B ligand (RANKL) of osteoblasts.

In the present study, adding 0.5% ALN to bovine bone graft resulted in lower graft resorption, but with no statistical significance ($P = 0.310$). Möller et al.\textsuperscript{12} found significantly reduced graft resorption using a two-fold higher dose (1 mg/mL ALN) associated with bovine bone graft in the mandible of pigs. However, their study found signs of osteonecrosis on the bottom of the autologous bone grafts, suggesting that the dose of ALN was too high and needs to be adjusted.

There have been reports on the effect of ALN on decreased new bone formation, but this topic has yet to be elucidated. This influence is believed to be caused by the cytotoxic effect of high dosage, so lower concentrations need to be tested.\textsuperscript{11,12} Therefore, dosage is still uncertain and results are contradictory, requiring further studies to consolidate findings and provide more safety and predictability for the use of ALN in clinical practice.

Conclusions

ALN (0.5%) associated with bovine bone graft did not improve new bone formation and had no effect on bone graft resorption compared to bovine bone graft alone, which showed better results in terms of total volume of tissue filling the bone defects.

Acknowledgements

The authors have no financial relationships relevant to this article to disclose.
Declaration of Interest

The authors report no conflict of interest.

References


