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***Basic Study***

**Dose of Alendronate directly increases trabeculae expansivity without altering bone volume in rat femurs**

Weiss SG *et al.* Aledronate increases trabeculae expansivity

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**Abstract**

***AIM***

to evaluate the effects of sodium alendronate on bone repair in fractures created in appendicular bones.

***METHODS***

Thirty-six *Wistar* rats were allocated into three distinct groups: group C (control), group B1 (received 1 mg/kg of alendronate) and group B2 (received 3 mg/kg of alendronate). The rats underwent femoral transversal linear fracture surgery using stable internal fixation with a 2.0-mm plate and screw system. Each animal randomly received intraperitoneal applications of sodium alendronate at a dose correspondent to group B1 or B2 three times a week, while the control group received a 0.9% saline solution. Drug administration was performed until euthanasia at 45 days. The femurs were removed and each surgical piece was sent for radiographic, tomographic and microtomographic analysis. Data were submitted to descriptive and inferential statistical analysis (95% CI).

***RESULTS***

Quantitative evaluations of bone neoformation did not show differences among the groups in the radiographic (*p =* 0.341), microtomographic (*p =* 0.581) and tomographic evaluations (*p =* 0.171). In the qualitative microtomographic analysis, a smaller distance was observed between the internal bone trabeculae in the groups that used alendronate (*p =* 0.05). On the other hand, group B2 had a higher amount of bone trabeculae per unit length when compared to the other groups (*p =* 0.04).

***CONCLUSION***

It can be suggested that the use of alendronate did not have a direct influence on the amount of bone neoformation, but it influenced, in a dose-dependent way, the bone quality, affecting the distance and quantity of trabeculae.

**Key words:** Alendronate; Bisphosphonates; Bone regeneration; Fracture; Femur

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**Core tip**: Several studies have been carried out to determine the influence of alendronate in bone repair or the appropriate dose of this drug to influence bone regeneration. In this research, thirty-six Wistar rats were allocated into three distinct groups that received either applications of alendronate at different doses or saline solution 3 times a week for 45 d. The rats underwent femoral fracture surgery with stable internal fixation. The imaginologic results suggested that the use of alendronate did not have a direct influence on the amount of bone neoformation, but it influenced, in a dose-dependent way, the bone quality.

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**INTRODUCTION**

Repair on transversal appendicular bones requires a complex process involving several biological stages that include cell recruitment, proliferation and differentiation[1]. The literature has described at least 2 types of bone repair for these fractures: direct or indirect. Direct bone healing is uncommon and is characterized by a healing area with a lack of a periosteal or endosteal callus formation. This effect occurs when the fracture area is rigidly fixed, causing direct remodeling of the lamellar bone with evident angiogenesis and formation of Haversian channels[2]. Indirect bone repair, the most common form of fracture healing, consists of endochondral and intramembranous bone healing and is characterized by the formation of a bone callus[3].

The establishment of a fracture pattern in an animal model requires surgical and technical abilities as well as accurate positioning and adequate bone fixation. The majority of the studies on rats are conducted with intramedullary pins, external fixators and pin-clips, among others[4]. It is known that the use of plates and screws for stable fixation in animal models favors and accelerates the repair process when compared to dispositive techniques that present few stable methods[5], even if the bone repair is not considered direct in this case. In the pilot study of our group, it was observed that the 2.0 system with 4-hole plates presented better results regarding bone repair of femur fractures when compared to other fixation systems.

Pharmacological agents that may modulate bone formation and bone remodeling are widely used and developed for the treatment of osteoporosis and other disorders of bone fragility[6]. Bisphosphonates (BFs) are a class of drugs that may act on bone remodeling by reducing bone resorption in a dose-dependent manner, mainly by inhibiting recruitment and promoting apoptosis of osteoclasts, while stimulating osteoblastic activity. BFs are available orally (alendronate, ibandronate and risedronate) and intravenously (ibandronate and zoledronate). Among these, sodium alendronate is part of the second generation of the BF class, presenting fewer side effects than the first generation and being the most widely used antiresorptive drug[7].

In the literature, some studies have reviewed the positive effect of sodium alendronate in bone repair[8-10]. It is hypothesized, by the mechanism of action of this drug, that alendronate, when applied at the appropriate dose after fixation of the fracture, accelerates the bone repair process, which makes prognosis more favorable.

**MATERIALS AND METHODS**

The animal protocol was designed to minimize pain or discomfort to the animals. The experiments were carried out in the Vivarium and in the Imaging Laboratory at Positivo University and in the Laboratory of Analysis of Minerals and Rocks at Federal University Paraná, after approval by the Ethics Committee on the Use of Animals (ECUA 320). The study followed the guidelines of ARRIVE (Animal Research: Reporting *in Vivo* Experiment). Throughout the experiment, the ambient light, temperature and humidity conditions of the rooms were controlled in a digital panel in order to maintain a photoperiod of 12 h, a temperature range varying from 18 to 22 ºC and 65% humidity. The animals were euthanized on the 45th day.

***Experimental design***

Thirty-six Wistar rats from 4 to 5 mo of age, weighing approximately 500 g were randomly divided equally into 3 groups: Group C (control), group B1 (received 1 mg/kg of alendronate) and group B2 (received 3 mg/kg). After the fracture, the intraperitoneal applications of sodium alendronate were started 3 times a week, and the control group received applications of 0.9% saline solution concomitantly until the time of euthanasia. Intraperitoneal applications were performed on the opposite side of the fracture.

***Fracture preparation and stabilization***

During all surgical procedures, asepsis criteria were maintained. The rats were sedated for 1 minute via inhalation with isoflurane (Cristália, Itapira, SP, Brazil) and anesthetized with 10% ketamine hydrochloride (Vetbrands, Paulínia, SP, Brazil) and 2% xylasin hydrochloride (Vetbrands, Paulínia, SP, Brazil) by intraperitoneal injection. After anesthesia, the rats were placed in the left lateral decubitus position, then a right femur trichotomy was performed with vigorous antisepsis using iodopovidone. A straight incision was made approximately 5 cm along the long axis of the femur with blade number 15C, and with the aid of blunt scissors, the tissue was divulsed into muscular planes. Thus, after incision and detachment of the periosteum with a scalpel, the surface of the femur could be accessed.

Before performing the osteotomy, it was necessary to perform the positioning, drilling and adaptation of the 2.0-mm 4-hole titanium plate system with four 4-mm long screws (Orthoface, Curitiba-PR, Brazil) in order to avoid poor positioning of the bone segments. Then, the fracture was made with a reciprocating saw (Figure 1). Abundant lavage of the wound was done with saline solution. The suture was performed in planes with isolated stitches using Vicryl-0® thread (Ethicon, Johnson & Johnson, São José dos Campos, SP, Brazil) for the muscular plane and nylon 4-0 (Ethicon, Johnson & Johnson , São José dos Campos, SP, Brazil) for the skin. Control of analgesia, inflammation and infection was held in the postoperative period.

***Applications***

Immediately after the surgeries, intraperitoneal applications were initiated and were able to last until euthanasia. Three weekly applications were carried out. The control group (C) received physiological saline solutions at 0.9%, while the animals in groups B1 and B2 received alendronate at a dose of 1 mg/kg and 3 mg/kg, respectively.

***Euthanasia***

At 45 d, the animals were euthanized and had the right femurs as well as the plates and screws removed. The specimens were stored separately in pots containing 10% formaldehyde. For euthanasia, the rats were exposed to overdoses of isoflurane for about 10 min. The specimens were sent to analysis. The statistical methods of this study were reviewed by Rafaela Scariot, Professor of Biostatistics at Master Program in Dentistry at Positivo University.

***Radiographic analysis***

In order to evaluate the postoperative recovery and the positioning of the bone segments, the animals were submitted to digital radiography 7 and 45 d. The time of 7 d served only as a radiographic follow-up to observe the control and evaluation of the plaque adaptation, while the time of 45 d was used to evaluate bone repair. The animals were sedated within 7 d and had their femurs positioned on a digital sensor (Kodak RVG 5100, Carestream Dental, Rochester, NY, United States) for radiographic imaging with an exposure time of 1.0 s. The images were then processed and evaluated in Dental Imaging software (version 6.12.17.0 - A, Carestream Dental, Rochester, NY, United States). By the time radiographic evaluation was taken at 45 d, the animals were already euthanized

For evaluation of bone neoformation, in the Image J program (version 1.49t National Institute of Health-NIH Bethesda, MD, United States), the examiner was previously trained. The fracture’s region of interest was then established. To define the region of interest, a 10 mm line was drawn, 5 mm before the fracture line and 5 mm posterior. Then, from this line, a rectangle was created with the aid of the Selection Brush tool to delimit the edges surrounding the bone callus and obtain the total area value. Outside the fracture region, a rectangle was also constructed to obtain the total bone area in order to later compare the bone formation with the individual femur thickness. The area value in the fracture region was subtracted from the total bone area without the fracture; from this value, the excess bone value was acquired.

***Tomographic analysis***

The 36 specimens were sent to a dental tomography center, who used the same tomography device calibrated at 120 kVp and 36.12 mAs (i-CAT® CONE BEAM 3-D, Kavo Kerr, Joinville-SC, Brazil), to construct images with an exposure time of 40 s with 0.25 Voxel. From this scan, 216 tomographic sections with a 250-µm pixel size were obtained for each set of 5 samples. The femurs to be evaluated through the tomography were grouped into an acrylic base that accommodated up to 5 femurs in order to decrease the number of intakes. They were placed vertically and, with the help of utility wax, were attached to the base. After the acquisition of the tomographic sections, the images were analyzed using the I-CAT Vision software.

The longitudinal distance of the defect in which the amount of bone generated laterally in the sample was evaluated. The distance between the contact points of the femurs and the femoral diameter were also measured without considering the effects of formation and the formed bone callus. The I-CAT Vision software helps facilitate the analysis of distances by allowing the examiner to view samples in several ways, such as through the Implant Screen, an analysis of multiple tomographic sections of a selected region of interest, or the MPR Screen, which displays 3 views of the obtained femurs. In the MPR Screen it is possible to analyze the distances using the axial, coronal and sagittal views of the samples and using regions of interest that delineate the femurs individually. Therefore, this screen was used for the analysis. In addition to the advantage of easily performing the distance analysis, the brightness and contrast scale can be altered so that only the femur, which is more radiopaque, is noticed. This makes it possible to identify the diameter of the femur. Despite using the diameter for analysis, the femur is not exactly cylindrical, so the distance acquired corresponds to the greater distance of the femur and the longitudinal dimension of the defect. This sample movement was possible with the “Explore” function in the software. Once the region of interest, which corresponded to a single femur, was selected by means of the rulers shown on the sides of each image of the figure, the “Explore” function of the software was performed. Thus, in the coronal image, the formation of a circle via the presence of a diameter was observed. With the cursor, you can rotate the inscribed sample in any direction in the plane. The distance measure was done by selecting the “Distance” software tool and dragging the cursor.

***Micro-CT analysis***

The computerized microtomography analysis was performed using a Skyscan computed micro-CT model 1172 (Bruker Skyscan, Luxembourg, Belgium), whose power and current was adjusted to 90 kV and 112 µa respectively. The pixel size was 12.8 µm, and a multichannel acquisition camera with a resolution of 2000 x 1336 pixels was used to make a signal detection. No filters were used to correct the energy of the X-ray beam. The portion was rotated 180º with a rotation step of 0.4º. A projection image of the part was obtained for each rotation step. The exposure time of the sample to the X-ray beam was 1.1 seconds per rotation step, and the total acquisition time of the projection images was 30 minutes. After the acquisition of the projection images, they were processed in the NRecon software (Bruker, Luxembourg, Belgium). The software reconstructed the projection images in tomographic sections using the Feldkamp algorithm. After obtaining the tomographic sections, the measurements of the three-dimensional space, trabecular formation and bone volume were evaluated by the software, CT Analyzer (v.1.16.1.0 +; Bruker Skyscan, Kontich, Belgium) and Dataviewer (v.1.5.2.4; Bruker Skyscan, Kontich, Belgium). In this software (CT Analyser), it is possible to separate different mineral densities from the contrast difference shown in the slices of acquired tomographic sections. The contrast of the tomographic section image comes from the different radiopacities of the materials in the sample, according to the interaction of these phases with the X-ray beam. Through this, it is possible to separate the neoformed bone from the autogenous bone and also from the cartilaginous material, all of which have different radiopacities.

***Quantitative analysis***

The quantitative analysis starts with the binarization process of the different phases of the sample. This binarization process corresponds to a range of gray tones to which each radiopaque material fits. The range of gray tones used to determine neoformed bone volume corresponds to 50 to 105 on a total dimensionless scale from 0 to 255. The determination of this volume occurred through the delimitation of a region of interest in a prismatic format (12 mm x 11 mm x 6.3 mm) that involves the whole region of the defect and bone callus, which is in the center of the defect (Figure 2). Thus, bone formation was investigated both in the defect region and in the volume of bone callus formed. This created region was used for all the pieces. From this procedure, the analysis of initial bone, neoformed bone and total surface area of bone formation, that is, the bone callus (lateral available area of the femoral bone), were obtained. The measurements of the relationship between the new bone formation and the femoral surface area were obtained using the difference in thickness between the samples.

***Qualitative analysis***

To analyze the trabeculae generated internally in the bone and in the external region, a region of interest was made inside and outside the femur bone (Figure 3) by considering a region that had the maximum density of trabecular bone within the bone callus. The calculations of the internal and external regions of the mean trabecular thickness (Tb.Th); trabecular linear density (Tb.N), which measures the average number of trabeculae per unit length, and average distance between the internal and external trabeculae (Tb.Sp) were possible through CTAn software[11,12].

***Statistical analysis***

The results were submitted to a descriptive and statistical analysis. Statistical evaluation was performed using a frequency data specific test, the Statistical Package for Social Science program (SPSS, version 24.0; SPSS Inc., Chicago, IL – United States), with a 95% confidence interval. The values obtained were submitted to a normality test (Shapiro-Wilk), and parametric variables were described by mean and standard deviation. Nonparametric variables were described as minimum, median and maximum. For comparison between groups, the ANOVA and Kruskal-Wallis tests were used, according to the normality of the variable. When there was a statistical difference between the groups, a Tukey test was performed for parametric samples. For nonparametric samples, comparisons were performed in two groups using the Mann-Whitney test.

**RESULTS**

***Radiographic analysis***

The bone formation, which was evaluated by radiographic analysis, was higher in group B2 (91.683 ± 35.657 mm²), followed by the control group (65.57 ± 32.642 mm²) and group B1 (62.670 ± 45.578 mm²). The measurements obtained (bone surplus) showed no difference between the groups (*p =* 0.341).

***Tomographic analysis***

Tomographic evaluation of the samples did not show quantitative differences in bone neoformation among the groups (One-way ANOVA test; *p =* 0.171). In Group C, the measure between the defect and femur ratio (mm³/mm²) was 1.76 ± 0.56. In group B1 and B2 were 1.59± 0.31 and 1.44 ± 0.21, respectively.

***Micro-CT analysis***

Regarding the relationship between the new bone formation and the femur surface area, the B2 group [5.87 (2.10 – 14.60)] mm³/mm² presented greater bone formation when compared with B1 group [4.88 (2.30–16.02)] mm³/mm² and C group [5.65 (3.64 – 12.40)] mm³/mm²; however, there was no difference among the groups (Kruskall-wallis test/*p =* 0.581).

***Qualitative analysis***

In the qualitative microtomographic analysis, it was possible to observe that, regarding Tb.N there was a difference in the number of trabeculae per unit length between groups (*p =* 0.05). Group B2, when compared with both groups, obtained higher linear density.

There was also a significant difference between the groups in the spacing of the internal bone trabeculae, showing that the Tb.Sp is lower when going from the control group to the B2 group (*p =* 0.04, Figures 4 and 5). The data of Tb.N, Tb.Th and Tb.Sp can be visualized in Table 1.

**DISCUSSION**

The aim of this study was to evaluate the evolution of appendicular repair of femurs fixed with plates in specimens that received alendronate in different concentrations through image analysis. It is known that during the early stages of bone healing, a less rigid mechanical environment results in a prolonged phase of chondral bone regeneration, whereas the intramembranous ossification process appears to be independent of mechanical stability[13]. In direct osseous repair, there is no formation of a bone callus. Therefore, it is possible to predict that when using fixation with plates and screws, a direct healing occurs because there is no movement of the bone preserves. However, in animal models (rats in this case), this is not true; it is instead indirect healing because the animal is not immobilized and the plates are not designed for animals, generating micromovement in the region. In the early healing of the fracture, mechanical stimulation seems to increase callus formation, but the amount of callus formation does not correspond to rigidity[14].

Sodium alendronate is a drug that prevents bone resorption and may induce osteogenesis by inhibiting osteoclast activity. As a result, it is able to maintain or promote callus formation in bone repair of fractures as well as increase bone mineral density in the fracture region[15]. In the present study, it was demonstrated that alendronate at concentrations of 1 mg/kg and 3 mg/kg, when evaluated by imaging, did not alter the amount of bone neoformation; it was equal for all groups.

Under a qualitative microtomographic analysis, it was observed that bone repair was more effective in the groups that received sodium alendronate applications, especially in the group with the highest dosage. This was visualized through the greater number of trabeculae and smaller spacing between the trabeculae in the 3 mg/kg group. Because alendronate promotes osteoblasts and mesenchymal cell osteoblastogenesis and inhibits osteoclastic activity[6,16], it may be suggested that the amount and arrangement of bone trabeculae is directly linked to the dosage and administration of alendronate. This study suggests that the higher the dose, the larger the expansion of mineral-like matrices, while spaces among these areas, such as in the chondroid or osteoid matrix, remain lower.

A hypothesis that should be considered and may explain all results found here is the likely action of TGF-β1, which was previously known to increase when alendronate is administrated[17]. It is noteworthy that this cytokine is an important growth factor that may contribute to mineral expansion. In the endosteum area, the bone matrix deposition occurs in a common situation independent of the chondroid area. Thus, alendronate could be responsible for the increase of this cytokine that in turn would increase the expression of BMP-2. This peculiar situation was observed in a recent study that demonstrated that specimens that received alendronate possessed a significant increase of this protein, improving the bone deposition in rabbit calvarias[18].

On the other hand, the same situation may be extrapolated for the periosteal area. Regarding this peculiar topography, it was described that the expansion of chondrocytes seems to be an effect strictly associated to functional endogenous TGF-β signaling. Besides that, TGF-β1 induces previous differentiation to hypertrophic cartilage, which is required for calcium deposition and ossification in this topography. For this conclusion, the authors of the study induced an inhibition of specific TGF-β receptors and verified that the suppression of the TGF-β would be an important condition that would culminate in the inhibition of cartilaginous growth and chondroid differentiation while inducing inhibition of the medullary area and hematopoiesis[19].

Thus, all of these hypotheses are possible explanations. We observed an important growth in minerals, whether the matrix was chondroid or osteoid, through accurate image analysis.

Sodium alendronate, at concentrations of 1 mg/kg and 3 mg/kg, when assessed by imaging tests, did not alter the amount of bone neoformation, which was equal for all groups.

Sodium alendronate interferes with the quality of bone neoformation regarding the quantity and disposition of bone trabeculae. The higher the dose of alendronate, the greater the number of trabeculae and the smaller the spaces among them.

**ARTICLE HIGHLIGHTS**

***Research background***

Bisphosphonates are potent inhibitors of bone resorption. Sodium alendronate is the most used drug of this class and may act on bone remodeling by reducing bone resorption in a dose-dependent manner, mainly by inhibiting recruitment and promoting apoptosis of osteoclasts, while stimulating osteoblastic activity.

***Research motivation***

Despite the knowledge of bone repair alteration by alendronate, it is not yet fully elucidated in the literature the appropriate dose to achieve better bone regeneration, neither the effects of this drugs when we use fixation methods.

***Research objectives***

To evaluate the influence of sodium alendronate, in different doses, on the bone repair of treated femur fractures with stable internal fixation by means of imaging tests (radiography, tomography and microtomography).

***Research methods***

It was allocated Wistar rats into three distinct groups to receive applications of alendronate at different doses or saline solution. Then, the rats underwent femoral transversal linear fracture surgery using stable internal fixation. Drug administration lasted 45 days. The femurs were sent for radiographic, tomographic and microtomographic analysis in order to evaluate bone quantity and quality.

***Research results***

The results did not show difference in bone quantity by radiographic, tomographic and microtomography analysis. However, when analyzing bone quality, it was observed that alendronate affected the distance and quantity of trabeculae, providing a better bone regeneration, in a dose-dependent way.

***Research conclusions***

The results of our research have stablished that sodium alendronate, at concentrations of 1 mg/kg and 3 mg/kg, when assessed by imaging tests, does not alter the amount of bone neoformation, which was equal for all groups. Although, it interferes with the quality of bone neoformation regarding the quantity and disposition of bone trabeculae. The higher the dose of alendronate, the greater the number of trabeculae and the smaller the spaces among them.

***Research perspectives***

More researches with this method of fixation and with the sodium alendronate are required, for example, related to mechanical force of the specimens. Also, it is important to compare the effects of alendronate with different markers. We suggest that the next studies use the dose of 1 mg/kg of alendronate, once we have demonstrated it provides bone regeneration.

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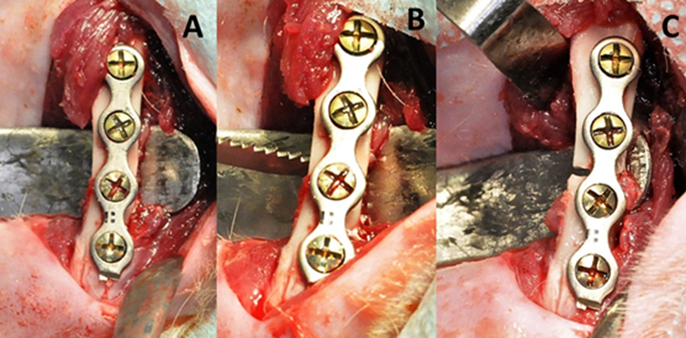
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Grade B (Very good): B, B, B

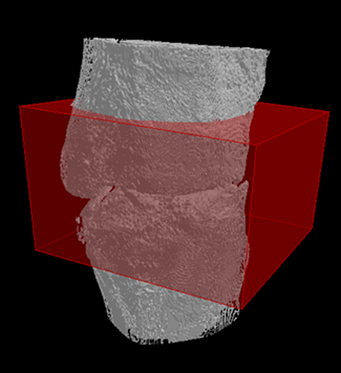
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Grade D (Fair): 0

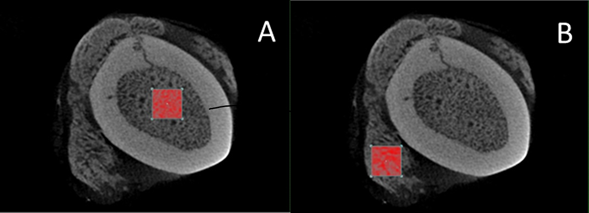
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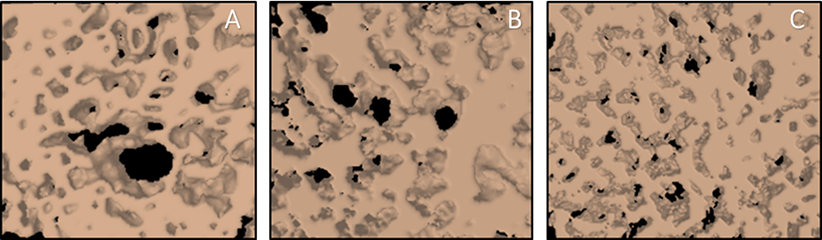
**Figure 1 fracture was made with a reciprocating saw.** Fixation in bone with plate and screws (A), followed by reciprocating saw used to induce fracture (B) and final fractured femur fixed (C).



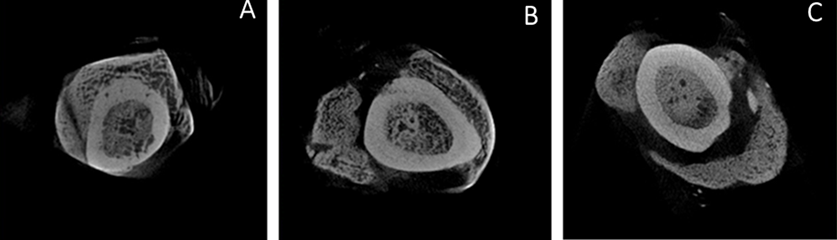
**Figure 2** **Delimitation of the defect for analysis.**



**Figure 3** **Region of internal interest (A), followed by region of external interest (B).**



**Figure 4** **Region of internal interest of the femur showing different patterns of trabeculae among groups.** Group C: control (A); group B1: bisphosphonate 1 mg/kg (B) and group B2: bisphosphonate 3 mg/kg (C).



**Figure 5** **Region of internal interest of the femur showing different patterns of trabeculae among groups.** Group C: control (A); group B1: bisphosphonate 1 mg/kg (B); and group B2: bisphosphonate 3 mg/kg (C).

**Table 1 Results of qualitative analysis by micro-CT [median (min-max)]**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | Group C | Group B1 | Group B2 | *P* |
| Tb.Th | 0.11 (0.09-0.14) | 0.09 (0.07-0.16) | 0.14 (0.09-0.16) | 0.07 |
| Tb.N | 5.26 (3.39-6.24)1 | 5.45 (1.42-5.87)2 | 5.94 (2.49-6.40)1,2 | **0.05** |
| Tb.Sp | 0.10 (0.06-0.16) | 0.09 (0.05-0.35) | 0.06 (0.05-0.21) | 0.07 |
| Tb.Th | 0.09 (0.07-0.13) | 0.10 (0.06-0,23) | 0.12 (0.06-0.15) | 0.06 |
| Tb.N | 4.60 (0.13-6.15) | 4.78 (0.15-6.90) | 6.15 (0.22-7.22) | 0.13 |
| Tb.Sp | 0.20 (0.08-0.50)3,4 | 0.09 (0.05-0.44)4 | 0.07 (0.05-0.42)4 | 0.04 |

1*p =* 0.049; 2*p* = 0.028; 3*p =* 0.032; 4*p =* 0.03. Kruskal-Wallis/Mann-Whitney. Data from external and internal areas, respectively.