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Alveolar bone dynamics in osteoporotic rats treated with raloxifene or alendronate: confocal microscopy analysis

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Abstract. In this study, the characteristics of the alveolar bone of rats with induced osteoporosis were examined. Thirty-two rats were divided into four groups according to the induction of osteoporosis and drugs administered: OG, osteoporotic rats without treatment (negative control); SG, rats which underwent sham surgery ovariectomy (SHAM); alendronate (AG), osteoporotic rats treated with alendronate; and RG, osteoporotic rats treated with raloxifene (RG). On the 8th day after ovariectomy and SHAM surgeries, drug therapy was started with AG or RG. On the 52nd day, 20 mg/kg calcein was administered to all of the rats, and on the 80th day, 20 mg/kg alizarin red was administered. Euthanasia was performed on the 98th day. The bone area marked by fluorochromes was calculated and data were subjected to two-way ANOVA test and Tukey's post-hoc test ($p < 0.05$). The comparison of the induced osteoporosis groups showed no statistically significant differences in bone turnover only between RG and SG ($p = 0.074$) and AG and OG ($p = 0.138$). All other comparisons showed significant differences ($p < 0.001$). The largest bone turnover was observed in RG and SG groups. RG was the medication that improved the dynamics of the alveolar bone of rats with induced osteoporosis, resembling that of healthy rats. © *2015 Society of Photo-Optical Instrumentation Engineers (SPIE)* [DOI: 10.1117/1.JBO.20.3.038003]

Keywords: osteoporosis; alendronate; raloxifene; microscopy.

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1 Introduction

Osteoporosis is a systemic disorder that affects both men and women but is very common in postmenopausal women.[1,2] A fall in estrogen (E2) production leads to an increase in the release of cytokines that induce osteoclastogenesis, a key characteristic of osteoporosis.[3,4] These changes in bone metabolism result in reduced bone mass and alterations in structural microarchitecture, increasing the risk of fractures.[5,6]

One feature of E2 deficiency-induced osteoporosis is the increased rate of bone remodeling, where bone resorption predominates bone formation, leading to an imbalance in bone microarchitecture, increased risk of fracture, and decreased bone density.[3–6] During osteoporosis, the rate of bone turnover is accelerated, resulting in increased bone resorption without compensation by the formation of new bone tissue.[7,8]

By conducting bone density assessments using computed tomography (CT) scans and radiography, clinical studies have shown that maxillary bones are also affected by osteoporosis, resulting in a loss of bone density.[9–11] However, data supporting this assertion and explaining how the systemic disorder affects alveolar bones are lacking in the literature.

Several therapeutic agents are used to treat osteoporosis, including estrogen replacement therapy,[12,13] calcitonin,[14,15] the bisphosphonates alendronate sodium and risedronate,[16] and the selective estrogen receptor modulator raloxifene (RG).[17–19] Randomized clinical trials have shown that these agents have significant efficacy in postmenopausal osteoporosis, increasing the density of mineralized bone and reducing the risk of vertebral fractures.[19–23]

One concern regarding the use of these drugs in osteoporosis patients, particularly alendronate sodium, is the potential risk of osteonecrosis of the jaw,[24] leading to postexodontia complications[25] and the failure of dental implants.[26,27] Moreover, alterations to alveolar bone turnover in these patients remain unknown. Thus, it is critical to determine whether osteoporosis treatments alter bone turnover in these tissues and promote a balance between bone formation and resorption.

In this study, we conducted histometric analysis of epifluorescence to evaluate alveolar bone turnover in rats with osteoporosis induced by bilateral ovariectomy and a calcium- and phosphate-deficient diet. Further, we evaluated the effect of alendronate sodium and RG treatment in these rats. We hypothesized that drug therapy would improve the rate of bone remodeling in the mature bones of osteoporotic animals.

2 Materials and Methods

2.1 *Experimental Design*

The study was approved by the Ethics Committee for the Use of Animals (2010/003045) of the Aracatuba Dental School,

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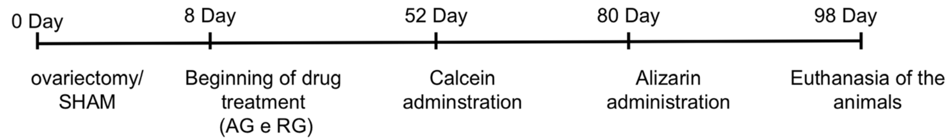

Fig. 1 Chronological flowchart of the events in this study.

Universidade Estadual Paulista (UNESP). A total of thirty-two 50- to 60-day-old female adult rats (*Rattus norvegicus albinus*, Wistar) weighing approximately 200 g were used. The animals were maintained in cages and were fed with balanced chow (NUVILAB, Curitiba PR, Brazil) containing 1.4% calcium (Ca^{++}) and 0.8% phosphate (PO_{4-}). The animals received water *ad libitum* for 10 days during acclimatization and after the start of the experiment.

The animals were divided into four groups ($n = 8$) according to the induction of osteoporosis and the drug treatment received. Twenty-four rats underwent bilateral ovariectomy and were divided into the following groups: ovariectomized rats fed a low Ca^{++} (0.1%) and PO_{4-} (0.5%) diet (RHOSTER Ind. Com., Vargem Grande Paulista, SP, Brazil) without drug treatment (OG, negative control) and ovariectomized rats fed a low Ca^{++} and P diet and treated with raloxifene (RG, test 1) or alendronate sodium (AG, test 2). Eight rats underwent a sham surgery, exposing the ovaries such that the rats underwent the same surgical stress as the other groups. The animals in this group were fed a balanced diet containing 1.4% Ca^{++} and 0.8% PO_{4-} (SG; positive control; NUVILAB, Curitiba PR, Brazil).

The chronological sequence of events in this study is detailed in Fig. 1. Before ovariectomy, the rats were subjected to estrous cycle analysis for more than three regular cycles. After ovariectomy, animals in the RG and AG groups were fed with a low Ca^{++} and PO_{4-} diet. The remaining groups were fed a balanced standard diet. On the 8th day after ovariectomy, drug treatments were initiated by gavage with $1\ mg \cdot kg^{-1} \cdot day^{-1}$ RG[8,28] or with $0.1\ mg \cdot kg^{-1} \cdot day^{-1}$ alendronate sodium (AG).[29]

On the 52nd day after ovariectomy or sham surgery, 20 mg/kg calcein (Sigma Chemical Company, St. Louis, Missouri) was intramuscularly administered, and on the 80th day, 20 mg/kg alizarin red (Sigma Chemical Company) was administered. Fluorochromes were diluted to 1.5 mL in deionized water with a magnetic stirrer (Max Labor, Presidente Prudente, SP, Brazil). The animals were euthanized 18 days after the application of alizarin red by overdose of the inhaled anesthetic, halothane (Tanohalo, Cristália, Cristal Pharma, Contagem, MG, Brazil).

2.2 *Laboratory Procedures*

The right jaws of the rats were removed and fixed in 10% paraformaldehyde solution. After 48 h, the specimens were washed in running water for 24 h and dehydrated in increasing concentrations of alcohol. The bones were then embedded and infiltrated in a solution of acetone and methyl methacrylate slow (MMAL) (Clássico, Artigos Odontológicos Clássico, São Paulo, SP, Brazil) at a ratio of 1∶1. This was followed by three MMAL baths. Benzoyl peroxide catalyst (1%, Riedel—de Haën AG, Seelze—Hannover, Germany) was added to the last bath. The specimens were placed in glass jars covered with a lid and were maintained in an oven at 37°C for 5 days until the resin polymerized.

After polymerization, the blocks containing the specimens were reduced with a "Maxcut" drill mounted on a Kota countertop motor (Strong 210, São Paulo, SP, Brazil), parallel to the long axis of the jaw (sagittal plane). The specimens were then subjected to progressive manual wear with a polishing machine (ECOMET 250PRO/AUTOMET 250, Buehler, Lake Bluff, Illinois) with sandpaper (granulation of 120, 300, 400, 600, 800, and 1200; Carbimet 2, Buehler, Lake Bluff, Illinois) under fluorescent light, until a thickness of 80 μm was reached, as measured by a digital caliper (Mitutoyo, Pompeia, SP, Brazil).

The histological sections were mounted on slides with mineral oil (Petrolato Líquido, Mantecor, Taquara, RJ, Brazil) and fixed with coverslips and enamel to prevent oil leakage and section drying.

2.3 *Scanning Confocal Laser Microscopy*

Longitudinal scans of the right jaw of the alveolar bone adjacent to the apical third of the maxillary central incisor were obtained by a Leica CTR 4000 CS SPE microscope (Leica Microsystems, Heidelberg, Germany), using a ×10 objective (original amplification × 100) (Fig. 2). The area was chosen considering the reference of the bone close to the upper right incisor. Therefore, the tooth was taken as the reference, and then the area of the bone around this tooth was evaluated in each sample.

The images that were obtained from different sections were compressed in order to obtain the best fit. After the selection of the section thickness, all of the slices were obtained and the

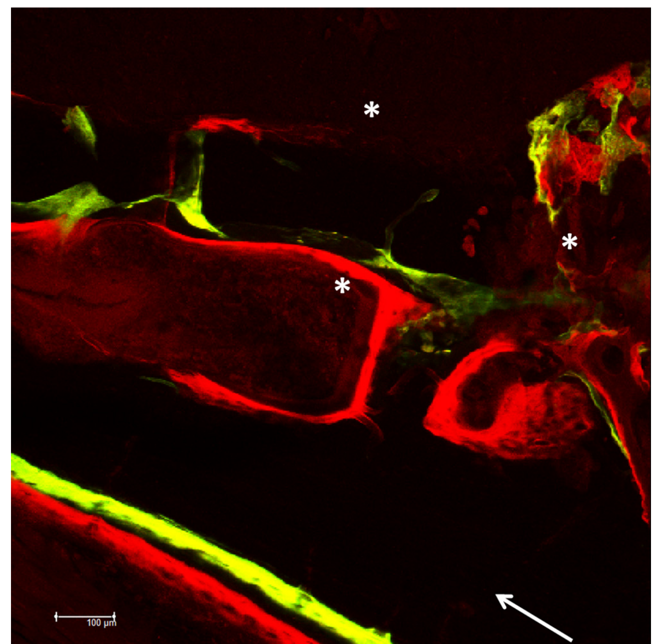

Fig. 2 Area corresponding to the alveolar bone selected for analysis by confocal microscopy (white asterisks). White arrow indicates the region of the right upper incisor.

software performed the z-stack that allowed achieving the best fit of images that represented the section from each animal of the experimental groups. The images started from the beginning of the fluorescence thus representing, in our methodological approach, the beginning of calcification or bone formation (calcium precipitation in the collagen organic matrix).

These images had a dimension of $1 \times 1\ \text{mm}^2$ and corresponded to optical sections of 512×512 pixels. Sections ($2\ \mu$m) were scanned over 2.5 min. Therefore, 28 sections were obtained for each $56\text{-}\mu$m slide. The barrier filters used were BP 530/30 nm and 590 LP, combined with activation of double dichroic 488/568 nm, and the photomultiplier was set to 534 for calcein and 357 for alizarin. The codes 534 and 357 nm represent the filters that allow the visualization of the fluorochromes. They are located in the microscope and when the light that comes from the mercury lamp passes through these filters and reaches the sample in the slice, it is possible to visualize the colors of the fluorochromes. One of the filters enables visualization of calcein (blue filter) and the other, alizarin (green filter).

The images obtained by confocal microscopy were reconstructed through the stack of the software that is installed to manipulate the confocal microscope (Leica CTR 4000 CS SPE, Leica Microsystems, Heidelberg, Germany); the alveolar bone showed two overlapping fluorochromes (calcein and alizarin; Fig. 3). The overlap represents the superposition of calcium that was precipitated in both periods, thereby showing the conversion of old bone to new bone. These images were saved in TIFF format and transported to the ImageJ software (Processing Software and Image Analysis, Ontario, Canada). Using the "color threshold" tool, each image was standardized according to hue, saturation, and brightness to reveal the fluorochromes. First, the calcein was highlighted, and the "measure" tool was used to provide the corresponding area in μm^2. The same procedure was performed for the alizarin, obtaining data related to the dynamics of the alveolar bone tissue.

Bone turnover, in this methodological approach, is represented by the difference between old bone (green) and new bone (red). Fluorochromes are chemical compounds that have the property of binding to calcium in the moment of the precipitation on the organic bone matrix. Therefore, the extent of fluorochrome labeling represents the quantity of calcium precipitation, thereby enabling the measurement of the bone formation event. Another aspect to be considered is the period when the fluorochromes were injected; as the first one was calcein (green), the bone filled with the green fluorochrome represents the old bone. The last fluorochrome injected was alizarin; therefore, the bone filled with the red fluorochrome represents the new bone. Considering these points, it can be said that the different colors represent the different ages of the formed bone.[30,31] Bone tissue dynamics is represented by the bone turnover that is observed through the renewal bone represented by the red fluorochrome. The greater the prevalence of the red fluorescence the greater the formation of new bone while the green fluorescence represents the old bone. The dynamics make it possible to observe the two situations in the same slice, i.e., to observe what is new and what is old.[30,31]

2.4 *Statistical Analysis*

Sigma Plot 12.3 (San Jose, California) software was used for statistical analysis. The Shapiro–Wilk test ($p = 0.953$) was conducted to determine the homoscedasticity, i.e., whether the results were homogeneous. Data were analyzed with two-way analysis of variance (ANOVA; experimental groups: four levels; fluorochromes: two levels). Significant results were statistically evaluated with Tukey's post-hoc test. For all tests, a p-value less than 5% was considered significant.

3 Results

The administration of fluorochromes established fluorescent lines formed by the precipitation of calcium in the organic matrix next to calcein (injected 52 days after ovariectomy) and by precipitation of calcium next to alizarin (injected 80 days after ovariectomy). Importantly, the green line (calcein) marks the older bone, whereas the alizarin marked the new bone. Concomitant observation of the images showed the amount of old bone versus new bone (Fig. 4). The number of the bone represents the quantity of bone that was formed after the precipitation of calcium into the collagen bone matrix. The fluorochromes have the property of binding to calcium in the moment that it is going to precipitate into the matrix, and because of this direct relation, the quantity of fluorescence represents the quantity of the bone formed.[30,31]

Data were analyzed with two-way ANOVA to compare the differences between the experimental groups (OG, SG, AG, and

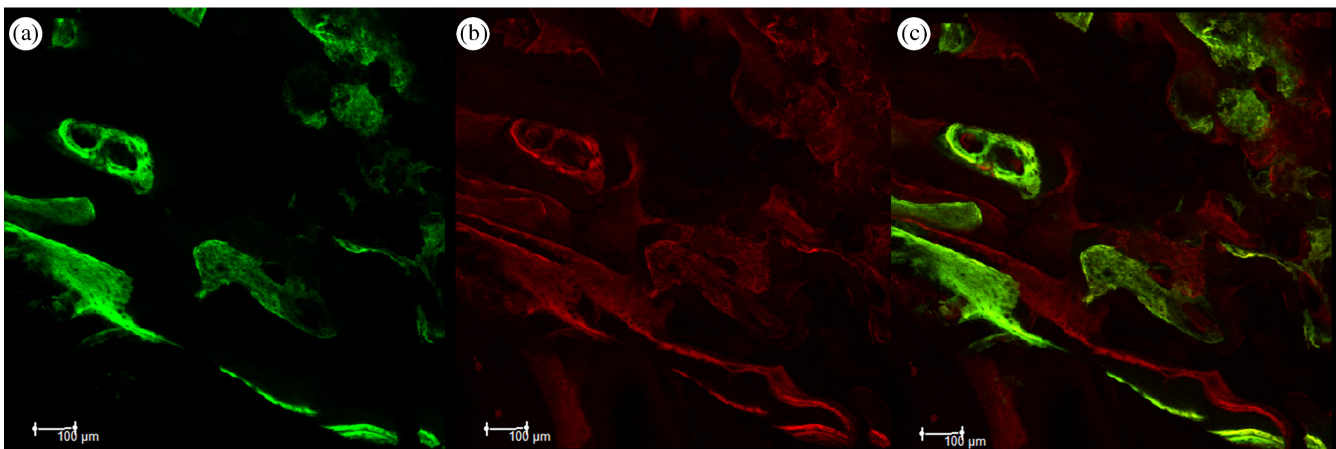

Fig. 3 Alveolar bone images obtained using confocal microscopy. (a) Bone area stained with calcein (green); (b) bone area stained with alizarin (red); and (c) merged images of both fluorochromes.

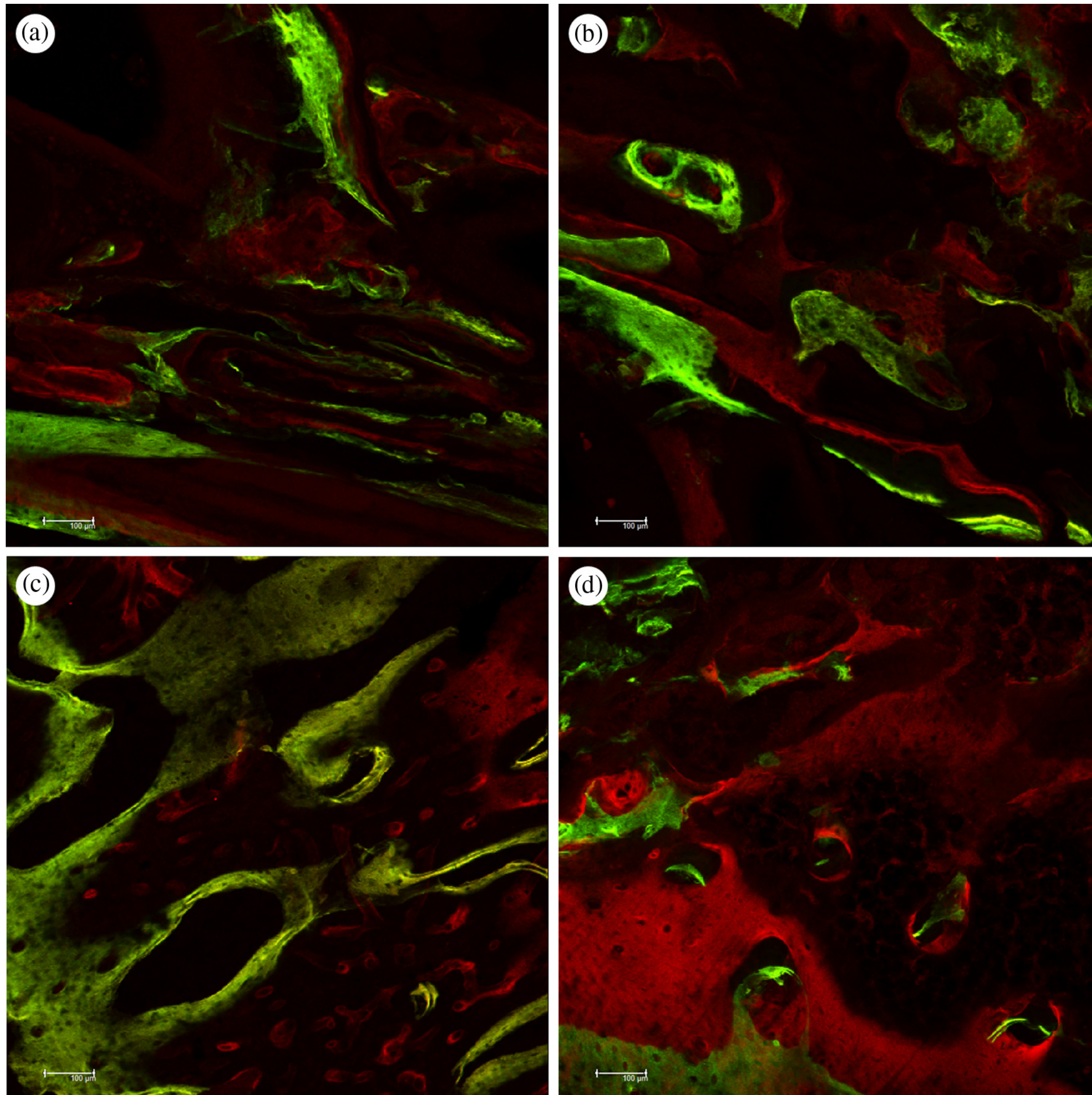

Fig. 4 Alveolar bone images obtained using confocal microscopy with overlapping of calcein (green) and alizarin (red) fluorochromes in the experimental groups. (A) OG: osteoporotic rats without drug treatment; (B) SG: rats subjected to sham surgery (SHAM) fed with a balanced diet; (C) AG: osteoporotic rats treated with alendronate (AG) sodium, and (D) RG: osteoporotic rats treated with raloxifene (RG).

RG) and fluorochromes (calcein and alizarin); the interaction between groups and fluorochromes was statistically significant ($p < 0.001$).

In the comparison of bone turnover in the induced osteoporosis groups, no differences were observed between the RG and SG groups (Tukey, $p = 0.074$) and the AG and OG groups (Tukey, $p = 0.138$). All other comparisons showed significant results (Tukey, $p < 0.001$; Fig. 5).

The intragroup analysis of bone tissue dynamics using the enhanced color ratio showed significant changes between the fluorochromes injected 52 days (calcein) and 80 days (alizarin) after ovariectomy (Tukey test $p < 0.001$). Higher bone turnover was observed in the SG and RG groups, showing a larger alizarin red area (in μm^2) than calcein area. In the other groups (OG and AG), these proportions were reversed, with a larger calcein-stained area than alizarin red-stained area (Fig. 6).

4 Discussion

In this study, the hypothesis that drug treatments would improve the rate of bone remodeling in the mature bones of osteoporotic rats as opposed to in the mature bones of the untreated animals was partially supported. Bone turnover in the osteoporotic rats treated with RG was statistically higher than that in the untreated osteoporotic rats ($p < 0.001$). No difference was observed between the RG-treated rats and healthy rats (GS; $p = 0.074$). However, considering the statistical similarity to the osteoporotic rat group, treatment with AG did not improve the rate of bone remodeling (OG; $p = 0.138$).

AG can inhibit the antiresorptive activity of osteoclasts given the significant increase in calcein labeling. However, turnover is low, as evidenced by the weak alizarin staining (Figs. 5 and 6). In view of the marked use of this drug in the treatment and prevention of postmenopausal osteoporosis,[32–34] future studies should investigate the action of this drug in peri-implant

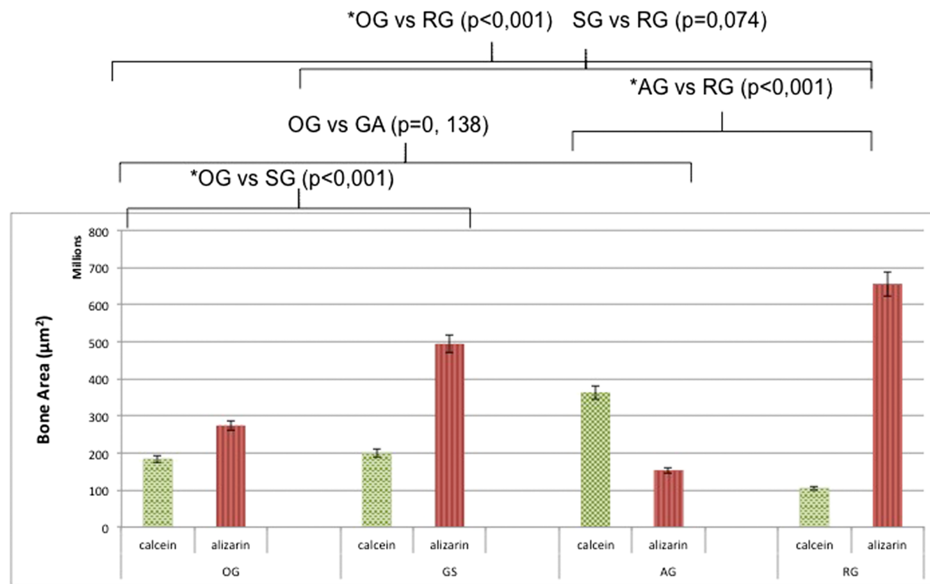

Fig. 5 Bone area (μm^2) for the experimental groups (OG, SG, AG, and RG) as determined by calcein and alizarin staining. *$p < 0.05$ (intergroup comparisons of statistical significance).

bone healing. The effect of AG on bone quality should be evaluated for use in oral rehabilitation of patients with osseointegrated implants.

One promising result of this study was that RG could treat osteoporosis. The antiresorptive action of RG, through its selective binding to estrogen receptors, reduced the expression of calcein and increased the expression of alizarin (Figs. 5 and 6). Therefore, we observed higher alveolar bone dynamics, evidenced by the balance between lower resorption and higher bone formation.

Previous studies[8,28,35] revealed that postexodontia alveolar repair in ovariectomized rats treated with hormone replacement (E2) or RG showed greater bone formation and intense immunostaining with osteocalcin, confirming their inhibitory effect on osteoclastogenesis. Our results, although representing the dynamics of the incisors of ovariectomized rats fed a low-calcium and phosphate diet, corroborate the findings of these previous studies. With regard to osseointegrated implants, we hypothesize that RG will improve peri-implant repair. Therefore, clinical studies should be conducted to determine whether this statement is true in order to establish a well-defined protocol for treating patients with osteoporosis.

The bone mass reduction caused by osteoporosis decreases the quality of the bone tissue, and the ability to reverse this situation is potentially mediated by bone turnover, represented by resorption and renewal.[3,6] In this study, superior bone dynamics were observed in the RG group, as compared to the AG and OG groups, similar to the rats with no change in bone metabolism (SG). This may be due to the Wnt/β-catenin signaling pathway,[36–38] which triggers cell turnover in bone and cartilage.[36]

In a clinical study with women treated with antiresorptive drugs (E2 or RG), Pineda et al.[37] observed a strong inhibition of bone resorption in both groups. The administration of RG inhibited the action of Dickkopf Homolog 1 (DKK1), an inhibitor of Wnt/β-catenin, and this increased the levels of

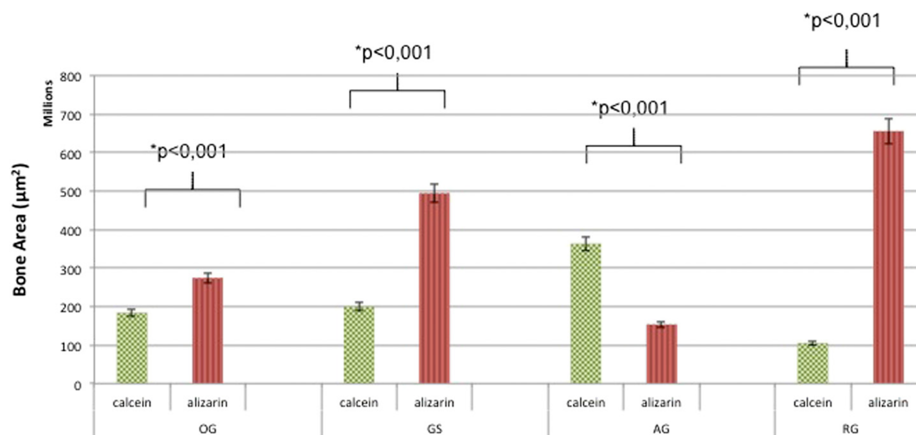

Fig. 6 Bone area (μm^2) for the experimental groups (OG, SG, AG, and RG) as assessed by calcein and alizarin staining. *$p < 0.05$ (intragroup comparisons of statistical significance).

osteoprotegerin, an inhibitor of osteoclastogenesis. This mechanism confirms the theory that RG is a superior drug for the treatment of osteoporosis. Moreover, it reinforces the role of osteoblasts as the main regulators of bone tissue turnover, releasing factors that activate and inhibit bone resorption.

Further analysis should be performed to characterize the bone tissue tested under these conditions, with regard to the quality of the bone tissue as well as the expression of proteins involved in bone dynamics, such as osteocalcin, RANK, and RANKL by means of immunohistochemistry and real-time polymerase chain reaction.

5 Conclusions

Therefore, considering the scope of this study, we can conclude that RG improved the dynamics of alveolar bone tissue in rats with induced osteoporosis, which consequently resembled healthy rats (SG).

Acknowledgments

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