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Absence of medication-related jaw osteonecrosis after treatment with strontium ranelate in ovariectomized rats

Leomar Emanuel de Almeida MECCA[a]
Amanda Regina FISCHBORN[a]
Jessica Daniela ANDREIS[a]
Gabrielle DELFRATE[a]
Tayline MROCZEK[a]
Natália Mariane RIGO[a]
Fábio Brasil de OLIVEIRA[a]
Myrella Lessio CASTRO[b]
Mario TABA JUNIOR[c]
Marcela CLAUDINO[a]
Gilson Cesar Nobre FRANCO[a]

[a]Universidade Estadual de Ponta Grossa – UEPG, Department of Dentistry, Ponta Grossa, PR, Brazil.

[b]Faculdade de Ciências do Tocantins – Facit, Department of Pharmacology, Araguaína, TO, Brazil.

[c]Universidade de São Paulo – USP, Ribeirão Preto School of Dentistry, Department of Oral Surgery and Periodontology, Ribeirão Preto, SP, Brazil.

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Corresponding Author:
Gabrielle Delfrate
E-mail: g_delfrate@hotmail.com

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Abstract: This study aimed to evaluate the potential of strontium ranelate (SR) in medication-related jaw osteonecrosis (MRONJ) after tooth extraction in ovariectomized rats. Thirty ovariectomized rats were divided into three groups (n = 10): bisphophonate (BP) group (zoledronic acid; 0.4 mg/kg/week), SR group (625 mg/kg/day), and control group (saline solution). The lower first molars were extracted after 60 days of drug therapy. Drug administration was continued for another 30 days after tooth extraction. The mandibles were subjected to clinical, histological, radiographic, and microtomographic evaluations. Only the BP group showed clinical changes, characterized by the presence of 70% (n = 7) and 20% (n = 2) of ulcers and extraoral fistulas. Radiographic evaluation demonstrated bone sequestration only in the BP group (n = 7, 70%). Microtomographic analysis revealed increased bone porosity after ovariectomy, particularly in the the control group ($p < 0.05$). The BP group showed a higher bone surface density, bone volume, and trabecular number than SR and control groups, but with less trabecular separation ($p < 0.05$). All the animals in the BP group demonstrated histological osteonecrosis. There was no evidence of osteonecrosis in the control and SR groups, which was characterized by the absence of empty osteocyte gaps and associated with the gradual healing of the extraction area. Also, an increased number of blood vessels and a reduced number of osteoclasts were observed in the SR group ($p < 0.05$). Therefore, SR treatment increased angiogenesis and osteoclastogenesis in the healing socket and was not associated with MRONJ development after tooth extraction in ovariectomized rats.

Keywords: Zoledronic Acid; Bisphosphonate-Associated Osteonecrosis of The Jaw; Rats; Mandible; Strontium.

Introduction

Medication-related osteonecrosis of the jaw (MRONJ) is characterized by nonhealing exposed bone in the maxillofacial region for more than 8 weeks in patients treated with antiresorptive drugs such as bisphosphonates (BPs), without a history of radiotherapy in the head and neck region.[1,2] Although there are occasional reports of spontaneous development of MRONJ, clinical procedures such as tooth extraction and dental implant

placement are considered the main triggering factors for MRONJ.[3,4] This disease is multifactorial and involves inhibition of bone remodeling as a result of osteoclast dysfunction, unregulated inflammatory processes and/or infectious conditions, inhibition of angiogenesis, soft tissue toxicity, and innate or acquired immune dysfunction. However, the mechanisms involved in the pathophysiology of MRONJ remain unclear.[5,6]

Initially, in 2008, American Dental Association named this condition as "bisphosphonate-related osteonecrosis of the jaw," since the use of zoledronic acid the most potent medication in the BPs, was associated with a high risk for the development of osteonecrosis in the jaws. The FDA Adverse Event Reporting System recorded 17.119 cases of MRONJ from 2010 to 2014 in the United States and 67% were associated with the use of zoledronic acid.[7] However, in 2014, the American Association of Oral and Maxillofacial Surgeons recommended a nomenclature change to "medication-related osteonecrosis of the jaw (MRONJ)," considering an increasing number of osteonecrosis cases involving the maxilla and mandible were associated with other antiresorptive therapies (*e.g.*, denosumab).[2]

Strontium ranelate (SR) is a relatively new antiresorptive agent commonly used for postmenopausal osteoporosis treatment owing to its beneficial effects on bone metabolism, including increased bone formation, collagen synthesis, and reduced bone resorption through decreased osteoclast differentiation and resorption activity.[8,9]

However, considering the increasing number of patients under treatment with SR, the scientific literature is scarce in demonstrating its clinical safety for dental procedures, such as tooth extraction and implant placement. Seeking to evaluate a possible association between the use of this anti-resorptive drug and the development of osteonecrosis in the jaw, this study aims to evaluate the potential of SR therapy to induce MRONJ after tooth extraction in ovariectomized rats.

Methodology

The experimental procedures of this study were performed in accordance with NCNC3Rs ARRIVE Guidelines, Animal Research: Reporting of In Vivo Experiments[10] and approved by the the Ethics Committee for Animal Care and Use of State University of Ponta Grossa (study protocol 031/2016). The sample size was calculated using the G*Power 3.1 software. It was considered as type I (5%) and type II (20%) errors, and a significant difference of 1.5 standard deviations. Thirty adult female Wistar rats (*Rattus norvegicus*), 10–12 weeks old,[11-13] weighing approximately 250 g, were kept at room temperature (23 ± 2°C) and received full feed and water.

All rats received intraperitoneal administration of xylazine (0.125 mL/250 g body weight) and 10% ketamine hydrochloride (0.3 mL/250 g body weight) and were submitted to bilateral ovariectomies. Before surgery, the animals were subjected to water and food restriction for 12 h.[14] Ovariectomy was confirmed by the presence of uterine horn atrophy after autopsy. In addition, osteopenia resulting from ovariectomy was confirmed using a 3-point femur test[15,16] and microtomographical evaluation.

Thirty days after ovariectomy, rats were randomly divided into one of three groups: control (C, n = 10), SR (n = 10), and zoledronic acid (BP, n = 10) (Figure 1). The control group was administered with saline solution via gavage until euthanasia. In the BP group, zoledronic acid (Sigma-Aldrich, Charleston, United States) was applied once a week intraperitoneally, at a dose of 0.4 mg/kg for 90 days, maintaining a 2-h fast.[17] The SR group received 625 mg/kg/day for 90 days of SR (Protos®; Les Laboratoires Servier Industrie, Gidy, Loiret, France) under water and food restriction for 2 h before and 2 h after gavage. This dosage represents the 2 g/day used for osteoporosis treatment in human adults.[18-20]

Tooth extraction

After 60 days of treatment, the rats received intraperitoneal administrations of xylazine (0.125 mL/250 g body weight) and 10% ketamine hydrochloride (0.3 mL/250 g body weight) for tooth extractions. All animals underwent extraction of the lower first molars as a triggering factor for MRONJ.[21] Initially, a number 5 explorer probe was placed between the first and second molars for luxation. Clinical forceps adapted to the size of the teeth were used for the extrusion.

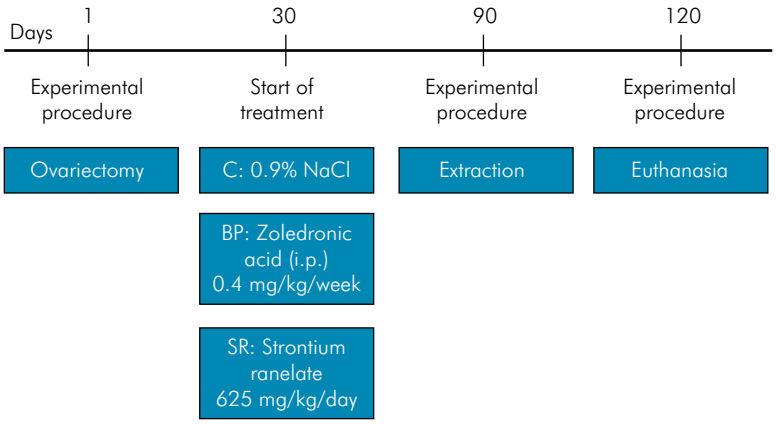


Figure 1. Schematic representation of the experimental design.

A 1.0-mm round bur at 15.000 rpm was used to remove possible root fragments and standardize trauma at the extraction socket. After the surgical procedures, the animals were maintained individually in a warm environment. The treatment protocols were maintained for another 30 days for a total of 90 days of pharmacological therapy. Euthanasia was performed using an anesthetic overdose of xylazine (Xilasin®, Syntec, Santana de Parnaíba, Brazil) and ketamine (Cetamin®, Syntec, Santana de Parnaíba, Brazil). In the sequence, mandibles were collected, separated into right and left hemimandibles, and fixed with 10% buffered formaldehyde for 24 h.

Clinical analysis

Prior to euthanasia procedures, with the anesthetized animals, the clinical signs of MRONJ were evaluated by a unique blinded examinator. All animals were placed in appropriate experimental beds, and the extraction area was evaluated for the presence of ulcers with exposure to necrotic bone and extraoral fistulas.

Radiographic and microtomographic analysis

The left hemimandibles were radiographed using a digital system with phosphorus plates. Initially, the hemimandibles were positioned parallel to the radiographic film, supported in wax for stabilization, with a focusing distance of 10 cm and exposure time established by the manufacturer (0.18 s). Qualitative radiographic analysis assessed the presence of bone sequestration and mottled appearance, which are both characteristics of MRONJ.[22] Hemimandibles were also scanned using microtomography equipment with a high-resolution imaging system (SkyScan® 1172, SKYSCAN N.V., Kontich, Belgium). The software CTAn® v 1.10.11.0, DATA Viewer® v 1.4.3, and CTVOX® v 2.0.0.0 were used for three-dimensional visualization. The samples were quantitatively evaluated for bone surface density (BS/TV), bone volume (BV), trabecular number (Tb.N), trabecular separation (Tb.S), and percent total porosity (Po). Regions of interest were delineated as described by Molon[23] et al.[23] All analyses were performed by a blinded examinator.

Histological analysis

After fixation procedures, right hemimandibles were decalcified for 60 days in 18% EDTA and washed, dehydrated, and embedded in paraffin. Serial sections of 5-µm thickness were obtained and stained with hematoxylin and eosin. From the mesial root of the second molar, four histological fields were photographed using a binocular microscope (Olympus, São Paulo, Brazil).[21,24] Morphometric evaluations were performed by a unique blinded examiner using ImageJ software (National Institute of Mental Health, Bethesda, United States) and evaluated based on the following criteria:

a. Presence of osteonecrosis, characterized by eight adjunct empty gaps in osteocytes;[24]

b. Rate between necrotic bone area (empty adjacent gaps without osteocytes) and vital bone area (viable osteocytes);
c. Bone vascularization is characterized by the number of blood vessels per bone area;[25]
d. The number of osteoclasts, characterized by multinucleated cells associated with bone resorption gaps.

Statistical analysis

Data were analyzed for normality using the Kolmogorov–Smirnov test. Since the data demonstrated normality, ANOVA followed by Tukey's post-hoc test was performed. For qualitative evaluations and data without normal data distribution, the Kruskal–Wallis test and Dunn's post-test were performed. Fisher's exact test was used to analyze dichotomous qualitative nominal data. The significance level established for all analyses was 5% ($p < 0.05$).

Results

Clinical analysis

Macroscopic evaluation showed an absence of evidence of MRONJ in the C and SR groups. Intraoral ulcers with bone exposure were more frequent in the BP group (n = 7). Extra-oral fistulas were detected in 20% (n = 2) of the BP group, as described in Table 1.

Table 1. Macroscopic evaluation of intraoral ulcer and extraoral fistula in C, BP, and SR groups.

Groups	Control	BP	SR
Intraoral ulcer	n = 0 / 0%^{A}	n = 7 /70%^{B}	n = 0 /0%^{A}
Extra-oral fistula	n = 0 /0% ^{A}	n = 2 /20%^{A}	n = 0 /0%^{A}

*Frequency of the presence/absence of intraoral ulcer and extra-oral fistula in the areas of extraction of the 10 lower molars in the C, BP, SR, and BP+SR groups. A ≠ B indicates a significant difference between groups (Fisher's exact test, $p < 0.05$).

Radiographic and microtomographic analysis

Radiographic and microtomographic evaluations revealed the presence of fractured roots in all groups related to the tooth extraction procedures. The presence of bone sequestration produced a mottled aspect consistent with MRONJ and was observed only in the BP group samples (Figure 2). Bone sequestration was not detected in the C or SR groups (Table 2).

Radiographic results were confirmed by computed microtomography, in which bone sequestrations were more frequent in the BP group (Figure 3). Regarding the quantitative values of computed microtomography, there was an increase in bone density only in the BP group (Figure 4a). However, the BV was higher in the BP and SR groups than in the C group (Figure 4b). A higher Tb.N and decreased Tb.S were observed in the BP group, whereas the SR group showed similar values compared to the C group (Figures 4c and 4d). A high percentage of bone porosity was observed in the control group (Figure 4e).

Histological analysis

No evidence of MRONJ was detected in the C or SR groups. All animals in the BP group (n = 10) presented histological characteristics of MRONJ (Figure 5). The proportion of necrotic bone was approximately 60% in the BP group (Figure 6).

An increased number ($p < 0.05$) of blood vessels was detected in the SR group compared to the C

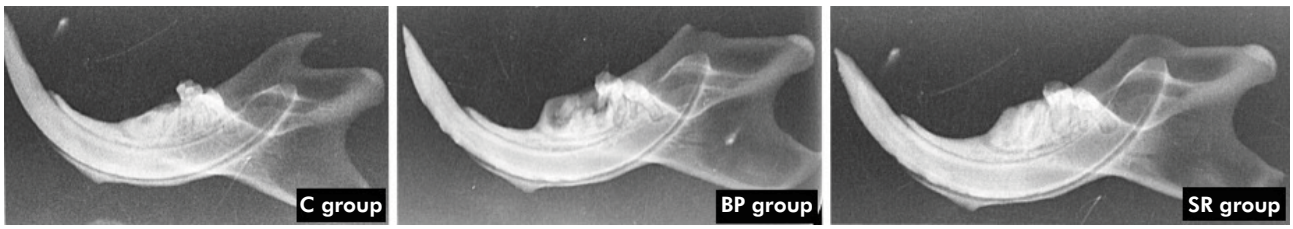


Figure 2. Radiographical aspect of control, strontium ranelate (SR), and zoledronic acid (BP) groups. Observe the presence of bone sequestration after extraction of the first molar in the BP group (white arrow).

Table 2. Radiographic evaluation of the hemimandibles in C, BP, and SR groups.

Groups	Control	BP	SR
Residual root	n = 3 / 30% ^{A}	n = 4 / 40%^{A}	n = 4 / 40%^{A}
Bone sequestration	n = 0 / 0%^{A}	n = 7 / 70%^{B}	n = 0 / 0%^{A}

*Frequency of the presence/absence of residual root and bone sequestration in the region of extraction of the 10 lower molars in groups C, BP, SR, and BP + SR. A ≠ B indicates a significant difference between groups (Fisher's exact test, $p < 0.05$).

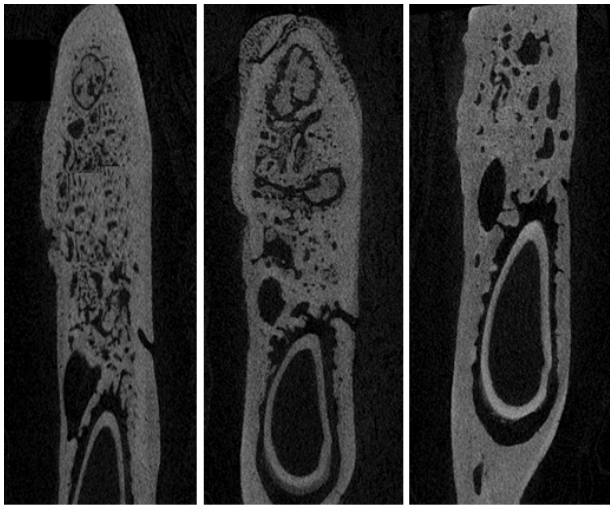


Figure 3. Cross-section from the occlusal direction to the base of the mandible in the control, BP, and SR groups. Observe the presence of bone sequestration in the BP group (white arrows).

and BP groups. Moreover, there was a significant reduction ($p < 0.05$) in the BP group compared to that in the C group (Figure 7).

The highest number of osteoclasts was observed in the BP group. The SR group showed a significant reduction ($p < 0.05$) in the number of osteoclasts compared with the control and BF groups (Figure 8).

Discussion

BPs are routinely prescribed to patients for the treatment of several diseases related to bone metabolism and are the first-line treatment for osteoporosis.[26] However, this class of drugs is associated with MRONJ development. Despite this, BPs remain widely used, increasing the risk and incidence of MRONJ.[2,5,6] In addition to the BP class, other drugs that inhibit bone resorption have been related to MRONJ, such as denosumab and infliximab.[27] The treatment of MRONJ remains poorly described and has been highly prevalent due to the frequent use of bisphosphonates. Thus, it is of great relevance to elucidate the mechanisms by which antiresorptives are associated with the development of MRONJ.[28]

SR has been used in the treatment and prevention of postmenopausal osteoporosis because of its potential to increase bone mineral density and consequently reduce the risk of fractures. The mechanism of action is based on the activation of the calcium-sensitive receptor in osteoblastic and osteoclastic cells, which stimulates bone formation and inhibits resorption.[29,30] However, the relationship between the use of SR and the incidence of osteonecrosis remains unclear. Therefore, this study aimed to evaluate the potential of SR in MRONJ development after tooth extraction in ovariectomized rats.

Animal models are widely used to evaluate bone metabolism, and rats are the most common experimental model used in studies on MRONJ.[18,19,31,32] In our experimental design, ovariectomized rats were used to mimic postmenopausal osteoporosis models.[15,16,33,34] The results demonstrated an increase in the percentage of porous bone in the control group. In contrast, the other two groups that received treatment with antiresorptives had less porosity, mainly in the BP group.

Our experimental design was based on a pilot study in which the femurs of ovariectomized rats were evaluated for flexural strength using a three-point bending test. Moreover, atrophy of the uterine horns after autopsy was observed during euthanasia. Taken together, these results suggest that ovariectomy was effective in inducing osteopenic conditions.[15,16] However, a control group or sham would be interesting, which can be considered a limitation of this experimental design.

In addition, lower first molar extraction was chosen as a triggering factor for MRONJ, since these regions have less irrigation.[31,32] In fact, less favored blood flow in the cortical bone, such as mandibular bone tissue, favors the development of MRONJ because monocytes, precursor cells of osteoclasts, migrate

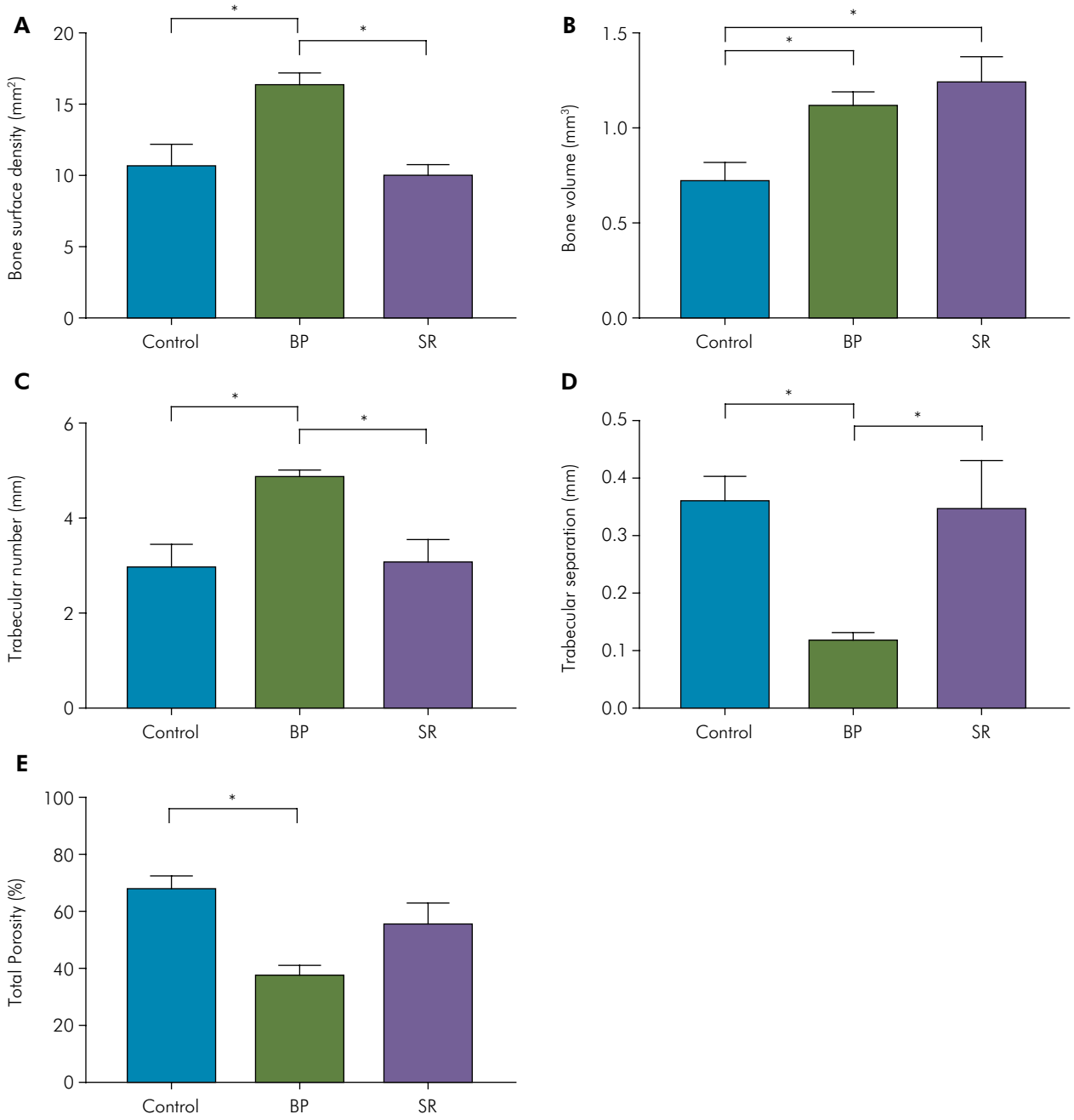


Figure 4. Microtomographical aspects of control, BP, and SR groups. a) Higher bone surface density in the BP group. Mean ± SEM *p<0.05 (ANOVA, followed by Tukey test). b) Higher bone volume in groups treated with anti-resorptives (BP and SR). Mean ± SEM *p<0.05 (ANOVA, followed by Tukey test). c) Expressive increase in the number of trabeculae in the BP group. Mean ± SEM *p<0.05 (ANOVA, followed by Tukey test). d) Decreased trabecular separation in the BP group. Mean ± SEM *p<0.05 (Kruskal–Wallis, followed by Dunn's test). e) High bone porosity percentage in the control group. Mean ± SEM *p<0.05 (Kruskal–Wallis, followed by Dunn's test).

from the interior of blood vessels to bone tissue. Thus, regions of less vascularized bone tissue are more susceptible to the development of MRONJ, as reported in several clinical studies.[2,32,35]

The use of zoledronic acid in the osteonecrosis model was effective for the development of MRONJ. Evidence of MRONJ was observed only in the BP group. This group showed large areas with empty osteocyte

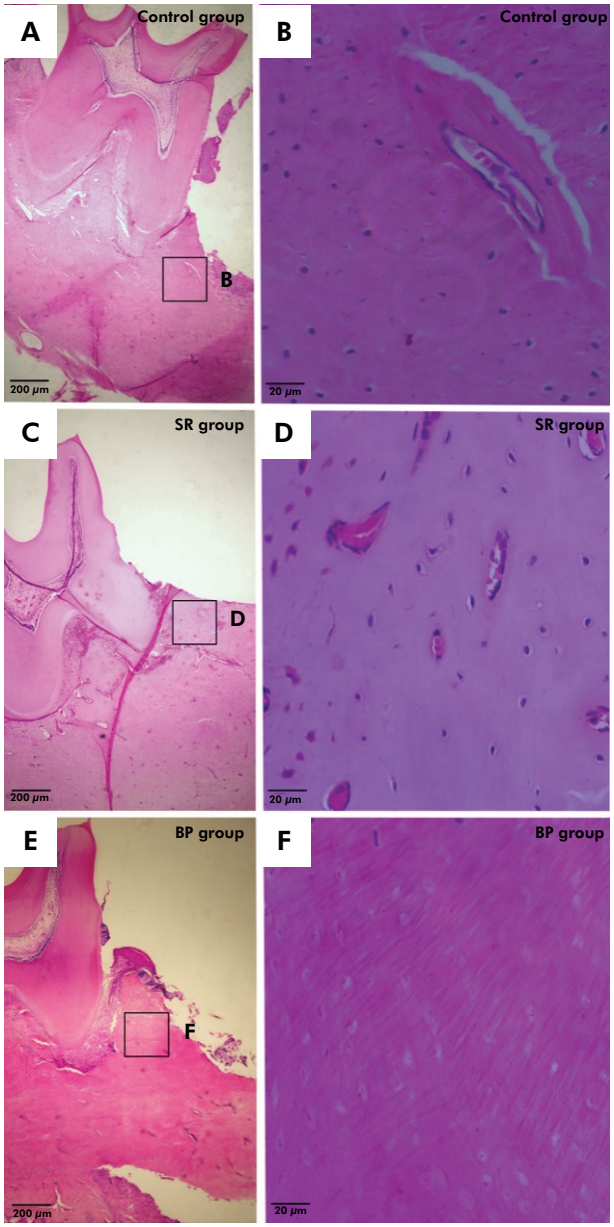
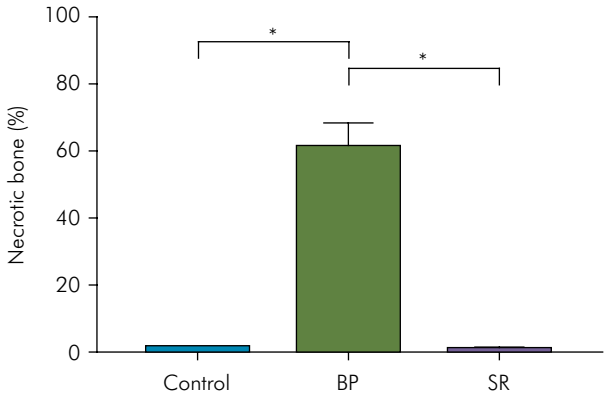


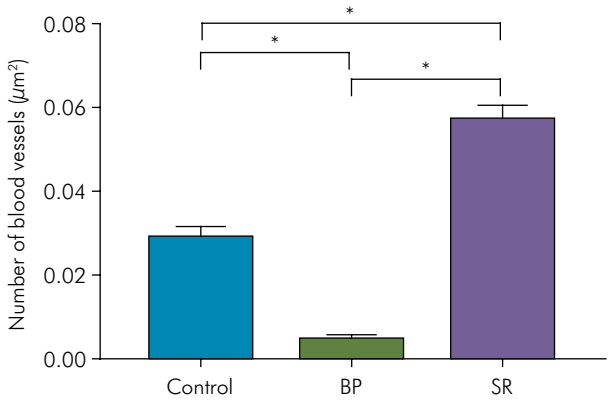
Figure 5. Histological features of control (A, B), SR (C, D), and BP (E, F) groups. Observe the presence of adjunct empty gaps of osteocytes in the BP group, characteristic of osteonecrosis.

gaps, bone sequestration, signs of fenestration and dehiscence, and histological, imagological, and macroscopic features of osteonecrosis. In contrast, both the control and SR groups did not present with MRONJ, suggesting that SR is a safe drug for MRONJ development. This result corroborates the study by Miranda et al.,[18] in which SR was associated with bone healing in alveolar sockets after tooth extraction in ovariectomized rats. However, different results were



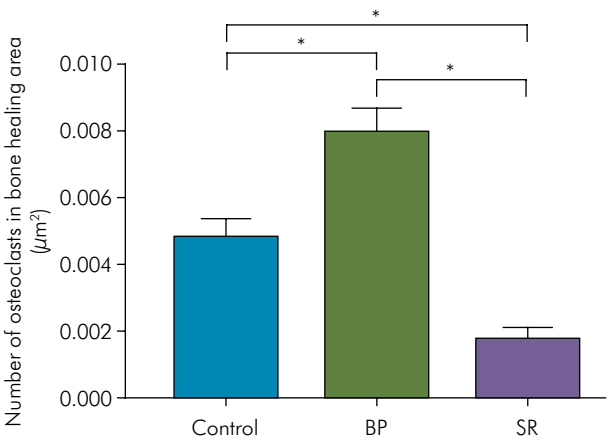
(Mean ± SEM) *p < 0.05 (Kruskal–Wallis, followed by Dunn's test).

Figure 6. Incidence of MRONJ based on rate of necrotic bone. Necrotic bone was present only in the BP group. No evidence of osteonecrosis was detected in control and SR groups.



Mean ± SEM *p < 0.05 (ANOVA, followed by Tukey test).

Figure 7. Increased number of blood vessels in the SR group. Observe the decrease in the bisphosphonate-treated group.



Mean ± SEM. *p<0.05 (ANOVA, followed by Tukey test).

Figure 8. Increased levels of osteoclasts in the BP group.

reported by Koth et al.,[36] in which the presence of root fragments was related to the incidence of mucosal injury (r = 0.331) but was not related to the incidence of non-vital bone (r = -0.087). In the present study, the BP and SR groups had equal root fragment frequencies (n = 4/40%), even so, intraoral ulcers were observed in 70% of the animals in the BP group and in 0% of the animals in the SR group; therefore, the presence of residual roots did not seem to be related to the incidence of intraoral ulcers. However, the presence of residual roots may be considered as a limitation of our experimental model.

In our study, the SR group also showed an improvement in bone vascularization, characterized by an the increased number of blood vessels. Similarly, other authors described that SR treatment increased the expression of vascular endothelial growth factor in femoral head tissues in a model of steroid-induced osteonecrosis of the femoral head in rabbits.[30] The BP group showed a reduction of blood vessels, supporting the antiangiogenic effect of zoledronic acid.[37] Moreover, several drugs have been recently associated with MRONJ based on antiangiogenic effects.[27] Therefore, the angiogenic mechanism of SR can be associated with the non-development of MRONJ.

Interestingly, BP treatment also resulted in an increased number of osteoclasts, despite the BP's mechanism of action involving inhibition of these cells, as observed by Córdova[38] et al.[38] However, these cells present evident morphological changes and, consequently, may not be able to perform bone resorption properly. The SR group of our experimental design showed a significant reduction in the number of osteoclasts, consistent with its mechanism of action. According to Bakker et al.,[39] osteocytes strongly inhibit osteoclastogenesis. Treatment with SR was related to the maintenance of osteocyte viability, which was associated with reduced osteoclast numbers. In this context, treatment with BP reduced the number of osteocytes, which may have affected the number of osteoclasts in bone tissue. Therefore, SR also prevents ligature-induced bone loss in estrogen-deficient rats. Moreover, SR affected the expression of bone markers and was demonstrated to be an anti-resorptive agent.[18,19]

Despite the high number of osteoclasts, an increase in BS/TV and Tb.N, associated with a decrease in Tb.S, was detected in the BP group compared to the SR and C groups. Moreover, BP showed a lower porosity bone percentage than SR. Regarding the BV, higher values were observed in both the BP and SR groups, with a greater trend in the SR group. These results are compatible with the osteogenic mechanism of SR.[18,19,29] However, our experiments evaluated bone characteristics only in the mandible. In fact, interesting results could be obtained with histological and microtomographic evaluation of other regions, such as the femur.

Based on these findings, SR seems to be effective in controlling the resorptive process of bone tissue in osteoporosis and, consequently, in increasing bone density. However, this drug has been subject to some controversies regarding pharmacy surveillance over the years, leading to the question of its use for some period in some countries of Europe and the USA. These contraindications are related to side effects such as acute myocardial infarction and stroke and are not recommended for patients with a serious risk of thromboembolism events.[40,41] Thus, these systemic conditions should be evaluated, although some authors did not find evidence for a higher risk of cardiac events associated with the use of SR in postmenopausal osteoporosis.[42]

Finally, the presence of fractured roots during extraction is a limitation of the experimental design of this study, which was previously observed by Bolette et al.[32] However, in our experiments, all groups had residual roots, and only the BP group had deleterious effects on the alveolar repair process. In fact, the C and SR groups did not present significant complications in tissue repair, even in the presence of residual roots.

Conclusion

Taken together, treatment with SR was not associated with MRONJ development after extraction in ovariectomized rats. Moreover, SR has been associated with increased angiogenesis and osteoclastogenesis. However, other studies must be performed to improve our knowledge regarding the putative effect of SR

on the treatment and prevention of MRONJ, which may serve as a basis for the the development of more effective strategies for the management of this disease and conducting surgical procedures in the jaw.

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