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ORIGINAL ARTICLE

Morphological and immunohistochemical features of tooth extraction sites in rats treated with alendronate, raloxifene, or strontium ranelate

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Abstract

Objective The aim of this study was to evaluate morphological and immunohistochemical features of tooth extraction sites in rats subjected to different antiresorptive drugs.

Materials and methods Wistar rats were allocated into 4 groups according to the treatment: (1) alendronate, (2) raloxifene, (3) strontium ranelate, and (4) control. The animals underwent tooth extraction (60th day of treatment) and afterwards were euthanized (90th day of treatment). Tooth extraction sites were analyzed by means of scanning electron microscopy (SEM), hematoxylin-eosin staining (H&E), and immunohistochemical staining (RANKL and OPG).

Results On H&E analysis, the alendronate group showed greater amounts of non-vital bone, biofilm, inflammatory infiltrate and root fragment, and smaller amount of vital bone. The strontium ranelate group showed great amount of non-vital bone. This group also had lower levels of OPG, while the alendronate group showed lower OPG and RANKL than the other groups. On SEM analysis, the alendronate group showed a considerable number of microcracks on the alveolar bone surface and few Howship lacunae and lack of bone cells as well. The raloxifene, strontium ranelate, and control groups showed a large number of bone cells and Howship lacunae on the bone surface and few microcracks.

Conclusion Alendronate therapy is associated with macro- and microscopic features of medication-related osteonecrosis of the jaw at tooth extraction sites, whereas raloxifene therapy is not, and strontium ranelate therapy is associated with non-vital bone.

Clinical relevance Osteonecrosis of the jaws is a serious side effect of alendronate therapy, where tooth extraction is a major risk factor. Considering the significant number of patients undergoing antiresorptive therapies worldwide, the present study investigated whether raloxifene and strontium ranelate interfere with bone repair after tooth extraction in a similar way to bisphosphonates.

Keywords Bisphosphonate-associated osteonecrosis of the jaw · Osteoporosis · Tooth extraction · Alendronate · Electron scanning microscopy · Jaws

Introduction

Medication-related osteonecrosis of the jaw (MRONJ) is an important side effect of antiresorptive drugs, whose poor response to treatment can lead to lesion persistence for months or years [1]. When this condition is associated with oral bisphosphonates, the withdrawal of the medication, also called *drug holiday*, can improve it [2]. A drug holiday period has been pointed out as imperative for MRONJ healing [3, 4]. However, osteoporosis, which is the most common disease treated with antiresorptive drugs, still demands therapy and can even be aggravated by a drug holiday.

Oral bisphosphonates are indicated as first-line therapy for osteoporosis. By means of inhibiting osteoclastic activity, these drugs increase bone mineral density and decrease the risk of fractures [5]. Bisphosphonate accumulates in bone, with a half-life of around 10 years, and has a cumulative effect. During bone resorption, the drug is taken up by

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osteoclasts, causing inhibition of cholesterol synthesis by disrupting the mevalonate pathway. This process culminates in the loss of the ruffled border of osteoclasts and a decrease in their activity and cell number [6, 7].

Although bisphosphonates are by far the most prescribed drugs to treat osteoporosis [5], the pharmaceutical industry offers some alternatives, such as hormonal therapy, monoclonal antibody to receptor activator of nuclear factor-kappa B ligand (anti-RANKL, denosumab), selective estrogen receptor modulators (SERM: raloxifene, bazedoxifene), anabolic agents (teriparatide), and strontium ranelate [5, 8, 9]. Among drugs other than bisphosphonates used to treat osteoporosis, denosumab and raloxifene have been reported in some cases of MRONJ patients. While denosumab has been strongly associated with the disease [10, 11], for raloxifene the association has been implied in a few case reports and still lacks some evidence [12–14].

Strontium ranelate has been prescribed to MRONJ patients as an alternative antiresorptive medication [15–18]. However, the literature lacks studies showing whether strontium ranelate does or does not interfere with alveolar bone healing after tooth extraction. That is, the literature does not provide scientific evidence of the benefit of strontium ranelate over bisphosphonate among patients with MRONJ. The mechanism of action of strontium ranelate has not been completely elucidated, with reports that it can either stimulate bone formation or inhibit bone resorption. This mixed effect on bone turnover is partially mediated by calcium receptors, which induce osteoclast apoptosis and osteoblast proliferation by increasing osteoprotegerin (OPG) and decreasing RANKL levels [19–21].

Raloxifene, in turn, is a SERM indicated to treat patients at high risk of vertebral fractures and also at high risk of breast cancer, when bisphosphonate therapy use is not possible for some reason [9]. This drug inhibits bone resorption through β-estrogen receptor activation, upregulating transforming growth factor β_3 (TGFβ_3), whereas interleukin 6 (IL-6) and tumor necrosis factor (TNF) are downregulated. As a result, osteoblast stimulation and osteoclast apoptosis occur [22, 23]. This drug has also been prescribed as a bisphosphonate alternative for MRONJ patients [13]. Based on the different mechanisms of action of these three drugs in the treatment of osteoporosis, this study aimed to compare the effects of alendronate, raloxifene, and strontium ranelate on the morphological and immunohistochemical features of tooth extraction sites in rats.

Materials and methods

All procedures used in this study were in accordance with the guidelines of the National Council for Animal Experimentation Control (CONCEA) and conformed to the guidelines of Animal Research: Reporting of In Vivo Experiments (ARRIVE) [24]. The study protocol was approved by the Ethics Committee on Animals Use of the Pontifical Catholic University of Rio Grande do Sul (PUCRS, protocol #7700). The sample was composed of 48 female rats (*Rattus norvegicus*, Wistar strain) from the Center for Experimental Biological Models of PUCRS (CeMBE-PUCRS), which were 100 days old with a mean weight of 250 g at the beginning of the experiment. The animals were identified on the tail and housed in standard microisolators (4 per cage) at 22 °C with a 12-h light/dark cycle (lights on at 7:00 am and off at 7:00 pm). During the experiments, a standard diet of rat chow (Nuvilab, Colombo, PR, Brazil) and filtered water were provided ad libitum [25]. After 21 days of acclimatization, the animals were randomly allocated into 4 groups of 12 rats each: (1) alendronate, (2) raloxifene, (3) strontium ranelate, and (4) control. All the groups were subjected to tooth extractions.

Drug administration and tooth extractions

Sodium alendronate (Dimed, Porto Alegre, RS, Brazil) was administered by the subcutaneous route, at a dose of 0.05 mg/kg every 7 days with a total of 13 doses [26]; raloxifene (Day Pharma Brasil, Itu, SP, Brazil) and strontium ranelate (Protos®, Servier, Gidy, France) were administered by oral gavage respectively at 1 mg/kg/day and 625 mg/kg/day [27, 28]. In the control group, 6 animals received 0.1 mL of saline by the subcutaneous route every 7 days with a total of 13 doses; and 6 animals received 1 mL/kg of filtered water daily by oral gavage. The dose regimen of the drugs was based on previous studies and corresponded to the human equivalent dose [26–28].

Tooth extractions were performed 60 days after the beginning of the experiment. Animals were anesthetized with a single intraperitoneal injection (IP) of a mixture of ketamine hydrochloride at 70 mg/kg (Syntec, Cotiá, SP, Brazil) and xylazine hydrochloride at 7 mg/kg (Syntec) [27]. The three upper right molars were extracted using an adapted 3s spatula (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) for luxation and pediatric forceps (Edlo, Canoas, RS, Brazil) adapted to the size of the teeth [26, 29, 30].

Euthanasia, macroscopic analysis, and specimen processing

After 90 days from the start of drug administration, the animals were euthanized by anesthetic overdose with 210 mg/kg ketamine hydrochloride and 21 mg/kg xylazine hydrochloride by the IP route [31]. The maxilla was dissected and examined with a #5 clinical probe (SS White, Duflex, Rio de Janeiro, RJ, Brazil) to determine the presence/absence of oral mucosal lesion, which was defined as loss of mucosal integrity [30].

Afterwards, the maxilla was cut into two fragments in the coronal direction, using an extra fine diamond disk (KG Sorensen, Cotia, SP, Brazil) [29, 30]. The maxilla fragments designated for histological analysis were fixed for 24 h in 10% buffered formalin, and those designated for electron microscopy analysis were fixed in 2.5% glutaraldehyde. These steps and macroscopic analysis are represented, respectively, in Fig. 1 and Fig. 2.

Histological processing

The specimens were decalcified in 10% nitric acid for 8 h. Next, they were paraffin embedded, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E) and also cut into 3-μm sections for immunohistochemical processing.

Immunohistochemical technique

Tissue sections were deparaffinized, rehydrated, and processed. Antigen retrieval was performed with citrate buffer [EnVision FLEX TRS low (Dako Denmark A/S, Glostrup, Denmark)] at pH 6 for 40 min, and endogenous peroxidase was blocked with EnVision™ FLEX Peroxidase-blocking reagent (Dako). Sections were incubated overnight at 4 °C with the primary antibodies for RANKL [RANKL(G-1)HRP: sc-377079 HRP), Santa Cruz Biotechnology, Dallas, TX, USA] and OPG [OPG(E-10)HRP:sc-390518 HRP, Santa Cruz Biotechnology] both at a dilution of 1:50. Next, anti-mouse secondary antibody EnVision FLEX was added for 20 min. Bound antibodies were detected by EnVision FLEX/HRP (Dako) and visualized by diaminobenzidine (DAB) with chromogen [FLEX DAB+CHROMOGEN (Dako)]. Sections were counterstained with EnVision FLEX hematoxylin (Dako). Slides were dehydrated with ethanol and xylene and coverslipped in Entellan (Merck Millipore, Darmstadt, Hesse, Germany). Giant cell lesion tissue sections were used as positive controls, and samples of the study processed without the primary antibodies served as negative controls.

Histological evaluation

The histological images were captured with an Olympus BX-43 light microscope (Olympus, Tokyo, Japan), connected to a computer with an Olympus DP-73 digital camera (Olympus). Images were captured in a standardized manner using the ×10 objective for H&E and ×20 objective for immunohistochemical analysis. In H&E staining, five fields were captured in each slide aiming to cover the entire tooth extraction area; in immunohistochemical analysis, four fields were captured per slide, including bone and the adjacent connective tissue at the tooth extraction site. The captured images were stored in TIFF format (uncompressed) [29].

The H&E images were analyzed by using the manual counting technique in the Image Pro Plus software (Media Cybernetics, Bethesda, MD, USA). A quantitative analysis (proportion) was made for the variables epithelium, connective tissue, vital bone, non-vital bone, biofilm, inflammatory infiltrate, and root fragment (Fig. 3**a**-**d**). A point-grid of 768 points was superimposed on each image, and each point was counted corresponding to the matching morphological structure. In immunohistochemical images, positive-staining areas for RANKL and OPG (Fig. 3**e**, **f**) were quantified by using the semiautomated segmentation technique [29].

The images were analyzed by a calibrated blinded examiner, who did not know the group to which each image belonged. Intraobserver calibration was performed by evaluating 25 images in each technique twice at two different moments (7 days apart). The results of these two analyses were tested by the intraclass correlation coefficient, which showed $r > 0.8$.

Scanning electron microscopy

Specimens were dehydrated with gradually increasing concentrations of acetone, critical-point dried and subsequently mounted on a stub and coated with a gold layer to be analyzed with an XL 30 scanning electron microscope (Phillips, Eindhoven, Holland). We scanned the topography of the tooth extraction area of two samples of each group. The entire tooth

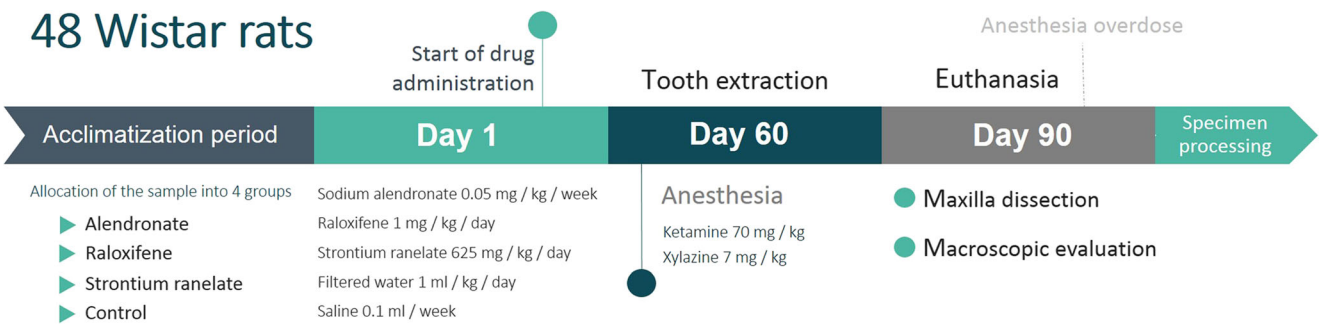

Fig. 1 The flowchart summarizes the study design and methodology

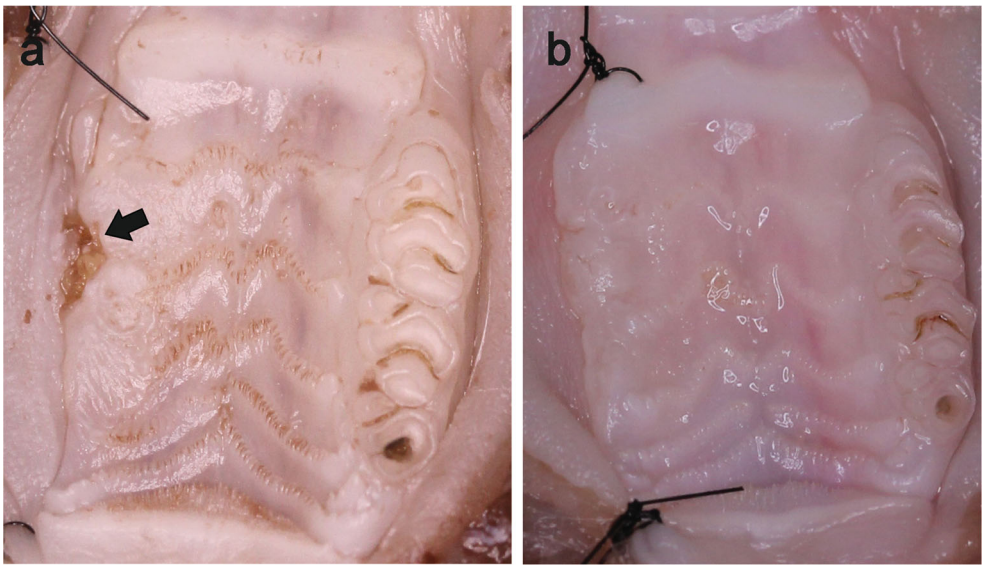

Fig. 2 Macroscopic analysis: presence of oral mucosal lesion (arrow, **a**) and absence of oral mucosal lesion (**b**)

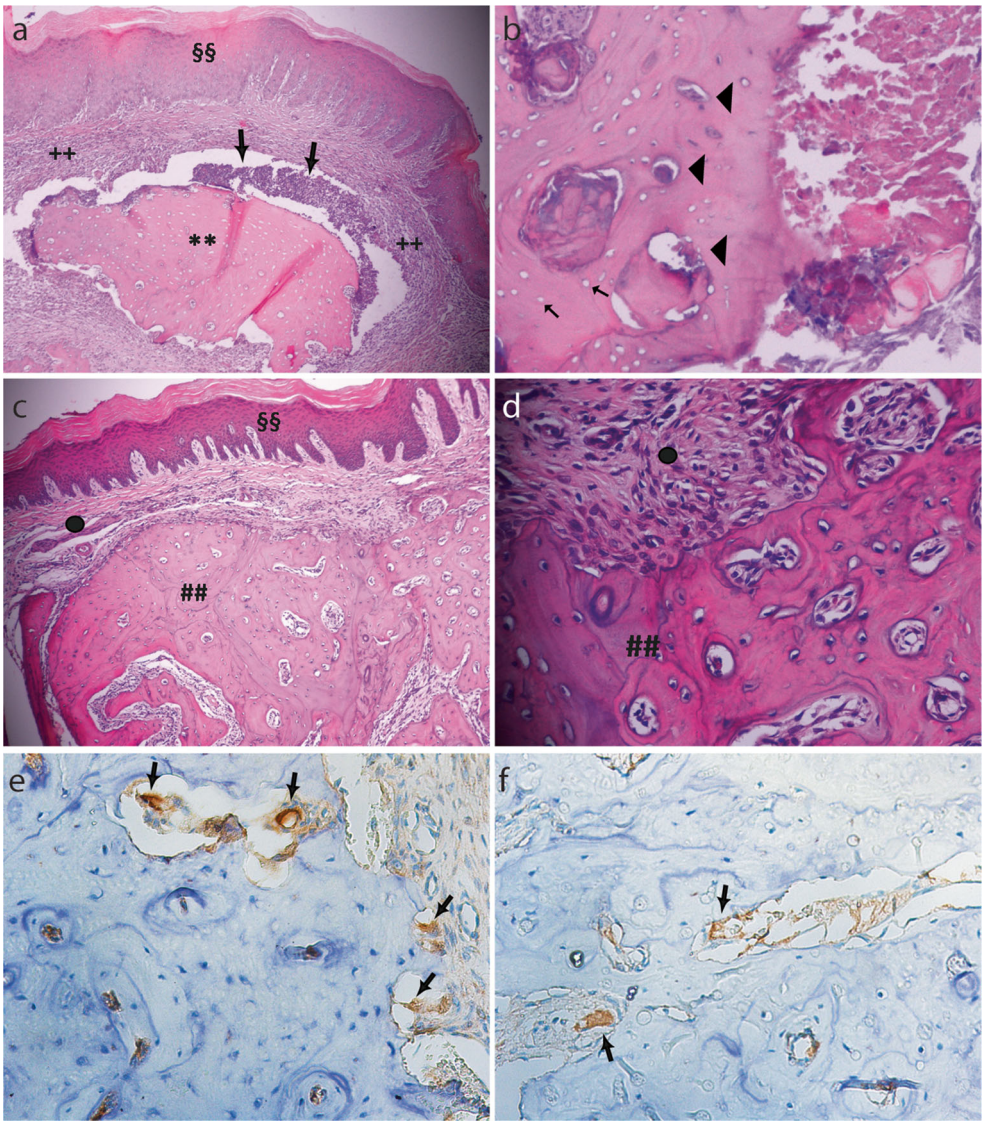

Fig. 3 Microscopic analysis: non-vital bone fragment (**) with biofilm (arrow head) circumscribed by connective tissue/inflammatory infiltrate (++) and epithelium coverage (§) (**a**, H&E, 100x); non-vital bone with empty osteocyte lacunae (arrows), bone marrow replaced by biofilm (arrowheads) (**b**, H&E, 400x); complete tissue repair show vital bone (##), connective tissue (•) and epithelium coverage (§) (**c**, H&E, 200x); vital bone (##) and connective tissue (**d**, H&E, 400x); immunohistochemical staining (brown) for RANKL (**e,** 400x) and OPG (**f**, 200x)

extraction area was scanned at 1000 x magnification, and areas of interest were subsequently scanned at higher magnifications up to 20,000 x. For chemical analysis, energy dispersive spectroscopy (EDS) of one sample from each group was performed.

Statistical analysis

Data were analyzed by means of descriptive (mean and standard deviation, median and 25th and 75th percentiles, and absolute and relative frequency) and inferential statistics. The Kolmogorov-Smirnov test was applied to analyze sample distribution. The Kruskal-Wallis test complemented by Dunn's multiple comparison test was used to compare quantitative variables between the groups, and Spearman's correlation coefficient was used to compare the variables to each other. Data were processed in SPSS 21.0 (IBM Corp., Armonk, NY, USA) at a significance level of 5%.

Results

During the experiment, 11 animals were lost: 2 from the raloxifene group, 2 from the alendronate group and 7 from the strontium ranelate group, making the new configuration of the groups as follows: alendronate (n = 10), raloxifene (n = 10), strontium ranelate (n = 5), and control (n = 12). In the alendronate and raloxifene groups, deaths occurred from anesthetic complication; in the strontium ranelate group, serious adverse events compatible with thromboembolism occurred.

Macroscopic analysis

All animals in the alendronate group showed oral mucosal lesion on macroscopic evaluation (P = 0.009, chi-square, adjusted residual analysis; Table 1). Also in this group, the area of the lesions was significantly larger compared with the other groups (P = 0.001, Kruskal-Wallis test, Dunn's multiple comparison test).

Microscopic analysis

H&E

Table 2 displays the results for the quantitative histological analysis with H&E. The amount of vital bone was significantly greater in the control compared with the alendronate group (P = 0.018). Non-vital bone was significantly greater in the alendronate and in strontium group compared with control and the raloxifene-treated rats; there was no significant difference between alendronate and strontium ranelate group (P = 0.000). The alendronate group also showed more biofilm (P = 0.006) compared with the raloxifene and strontium ranelate groups, greater amount of root fragment compared with the raloxifene and control animals (P = 0.005), and more inflammatory infiltrate compared with other groups (P = 0.034). There was no significant difference for epithelium and connective tissue between the groups (Table 2, Kruskal-Wallis test complemented by Dunn's multiple comparison test, α = 0.05).

Correlation analysis

Table 3 displays the correlation analysis. Oral mucosal lesion and inflammatory infiltrate were correlated with each other (r = 0.664) and with non-vital bone (r = 0.406 and r = 0.664, respectively); inflammatory infiltrate was also correlated with biofilm (r = 0.370). Vital bone was negatively correlated with mucosal lesion (r = −0.550), epithelium (r = −0.347), connective tissue (r = −0.693), inflammatory infiltrate (r = −0.367), and root fragment (r = −0.400). There was also a negative correlation between non-vital bone and connective tissue (r = −0.422).

Immunohistochemical analysis

The alendronate group showed significantly lower expression of RANKL compared with the raloxifene and control groups (P = 0.000). OPG was significantly less expressed in the

Table 1 Macroscopic analysis: sample distribution according to presence/absence of oral mucosal lesion and size (mm^2) of the lesion

Group	Presence		Absence		P*	Area (mm^2)				P†
	n	%	n	%		MD	P25	P75	Mean rank	
Alendronate (n = 10)	10	100[a]	0	0	0.009	3	3	3.7	30.15[a]	0.001
Raloxifene (n = 10)	5	50[b]	5	50		0.5	0	2.2	15.65[b]	
Strontium ranelate (n = 5)	1	20[b]	4	80		0	0	2.5	15.00[b]	
Control (n = 12)	5	41.7[b]	7	58.3		0	0	1.7	14.17[b]	

n=number of animals; MD=median

*P value for chi-square test followed by adjusted residual analysis. †P value for Kruskal-Wallis test followed by Dunn's multiple comparison test. Values followed by different letters in the column showed significant difference

Table 2 Quantification of histological features (H&E stain) at the tooth extraction site in the alendronate, raloxifene, strontium ranelate, and control groups

Group	Alendronate				Raloxifene				Strontium ranelate				Control				P*
	Mean	SD	MD	Mean rank	Mean	SD	MD	Mean rank	Mean	SD	MD	Mean rank	Mean	SD	MD	Mean rank	
Epithelium	12.13	15.15	1.13	82.2[a]	10.37	9.86	7.11	85.2[a]	10.28	9.79	6.91	86.1[a]	13.28	15.12	12.32	90.0[a]	0.882
Conective tissue	40.61	13.62	40.74	92.6[a]	40.24	15.88	39.34	90.5[a]	39.34	13.32	37.7	88.3[a]	35.39	14.28	35.11	75.0[a]	0.271
Vital bone	31.7	25.65	27.89	68.2[a]	41.42	24.48	41.25	88.5[ab]	39.58	16.40	44.2	87.7[ab]	48.83	27.15	46.69	99.1[b]	0.018
Non-vital bone	2.15	5.76	0	94.0[a]	0	0	0	80.0[b]	4.26	9.86	0	95.4[a]	0.00	0	0	80.0[b]	0.000
Biofilm	0.67	1,18	0	100.1[a]	0.3	1.09	0	80.2[b]	0.15	0.736	0	71.8[b]	0.24	0.58	0	84.5[ab]	0.006
Inflammatory infiltrate	4.75	6.59	1.77	101.7[a]	2.02	4.69	0	78.1[b]	1.35	2.56	0	75.4[b]	1.99	3.14	0	83.4[b]	0.034
Root fragment	7.83	9.77	3.55	107.1[a]	5.56	10.73	0	82.2[b]	4.43	8.33	0	79.1[ab]	2.19	5.03	0	73.3[b]	0.005

SD standard deviation; *MD* median. **P* value for Kruskal-Wallis test, complemented by Dunn's multiple comparison test, $\alpha = 0.05$. Different letters in the same row indicate a significant difference.

alendronate group compared with the raloxifene-treated rats ($P = 0.000$), while the strontium ranelate group showed significantly lower OPG expression than the raloxifene and control groups ($P = 0.000$). No significant difference was observed between the alendronate and strontium ranelate groups regarding these variables (Fig. 4, *P* value for Kruskal-Wallis test complemented by Dunn's multiple comparison test).

Scanning electron microscopy analysis

Scanning electron microscopy (SEM) showed microcracks on the bone surface in all groups, but with higher frequency and size in the alendronate group. The bone surface of the control and raloxifene groups showed similar appearance regarding the presence of Howship lacunae; in the strontium ranelate group, the lacunae were larger and deeper, and in the alendronate group, they were fewer and shallower. Bone cells compatible with osteocytes and osteoclasts could be seen in several areas of the control, raloxifene and strontium ranelate groups. However, only a small area in the alendronate group showed osteocytes, and no structures resembling osteoclasts were seen in this group. Other features observed in the alendronate group were shrunken cells with surface blebs, which suggested apoptotic bone cells (Fig. 5).

In the strontium ranelate group, bone structure showed fibrillar components with round superimposed particles, suggesting collagen fibrils with mineral deposition during the process of bone mineralization. The raloxifene and control groups showed apparently less collagen fibrils and more mineral content. In the alendronate group, there were mineral particles without fibrillar components, suggesting a greater mineral content (Fig. 6).

Table 3 Spearman's "r" for correlation between variables

Variables	Mucosal lesion	Epithelium	Connective tissue	Vital bone	Non-vital bone	Biofilm	Inflammatory infiltrate	Root fragment
Mucosal lesion	1							
Epithelium	−0.048	1						
Connective tissue	0.180	0.342*	1					
Vital bone	-0.550**	−0.347*	−0.693**	1				
Non-vital bone	0.406*	−0.169	−0.422**	−0.050	1			
Biofilm	0.243	0.218	−0.260	−0.094	0.437**	1		
Inflammatory infiltrate	0.664**	−0.110	0.106	−0.367*	0.430**	0.370*	1	
Root fragment	0.331*	−0.239	0.117	−0.400*	−0.087	0.072	0.074	1

Bold values show correlation

*$\alpha = 0.05$; **$\alpha = 0.01$

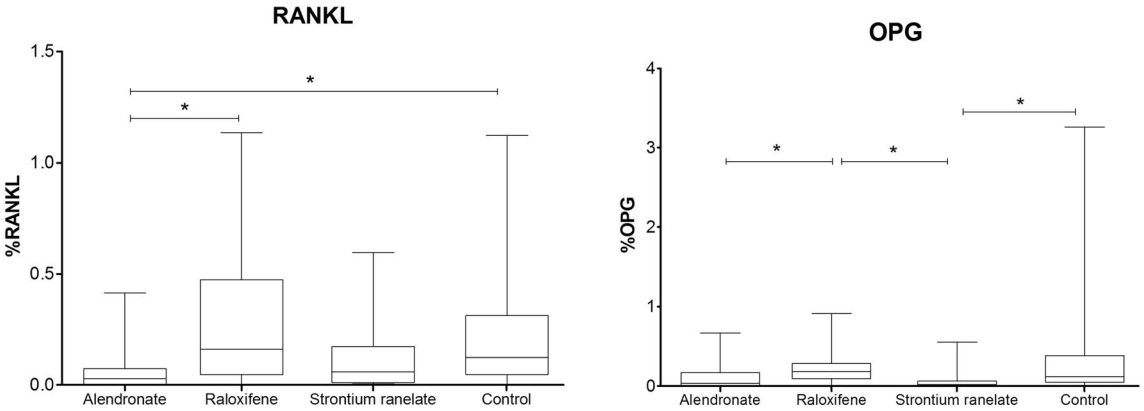

Fig. 4 Immunohistochemical quantification of RANKL and OPG in the alendronate, raloxifene, strontium ranelate and control groups. *$P = 0.000$, Kruskal-Wallis test complemented by Dunn's multiple comparison test

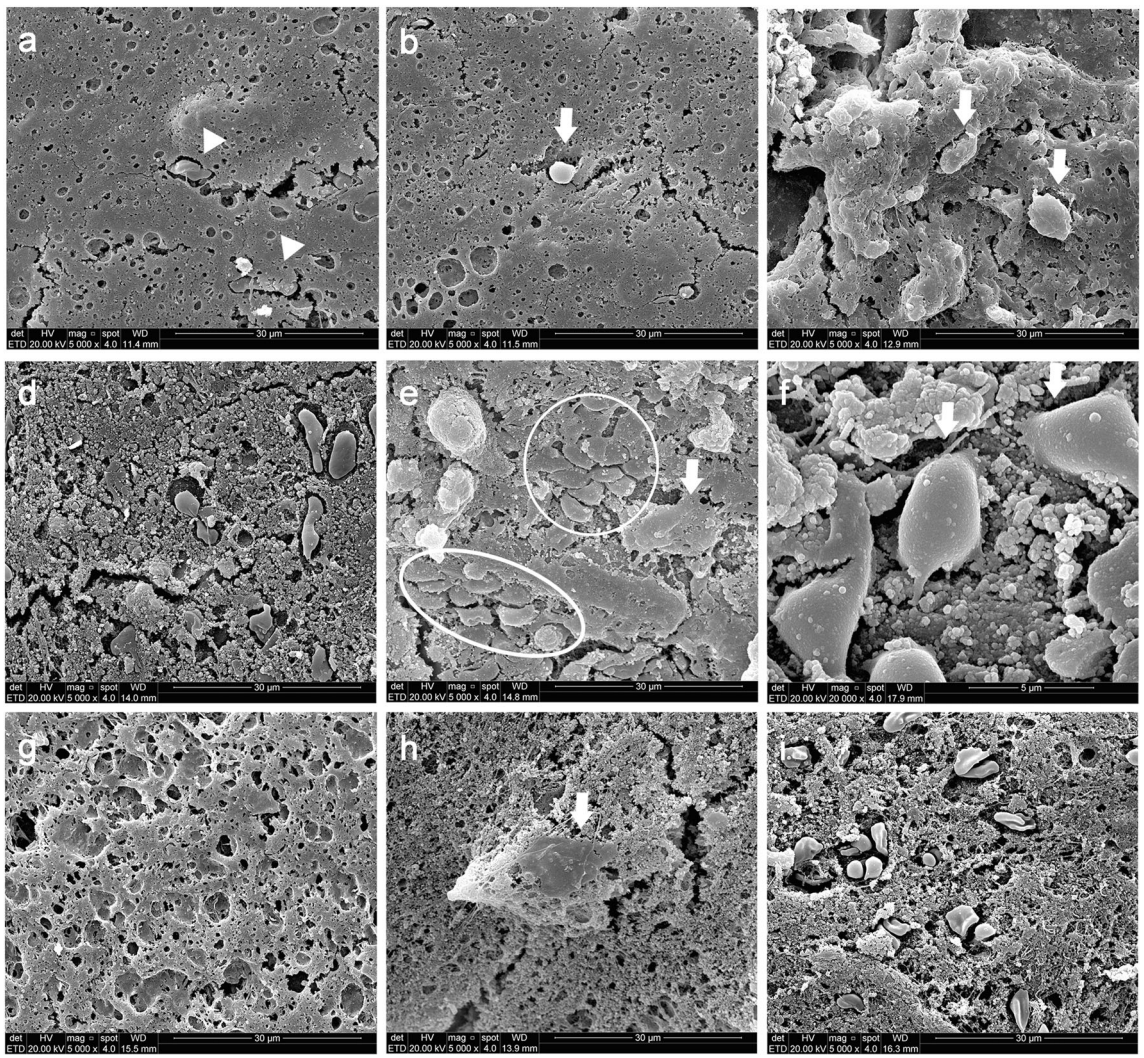

Fig. 5 Scanning electron microscopy (SEM): osteocytes (arrow, **a**) and a shrunken cell (**b**), microcracks and few and shallow Howship lacunae (**a**, **b**) in the alendronate group. Structure compatible with osteoclasts (arrows, **c**) and undifferentiated cells in medullary bone (**d**) in the raloxifene group. Strontium ranelate group shows structure compatible with osteoclast (inside circle and arrow, **e**; arrowhead, **f**) and large and deep Howship lacunae (**g**). Structure compatible with osteoclast over a bone spicule (arrow, **h**) and undifferentiated cells in medullary bone (**i**) in the control group. SEM magnification: 5,000x (**a,b,c,d,e,g,h**) and 20,000x (**f**)

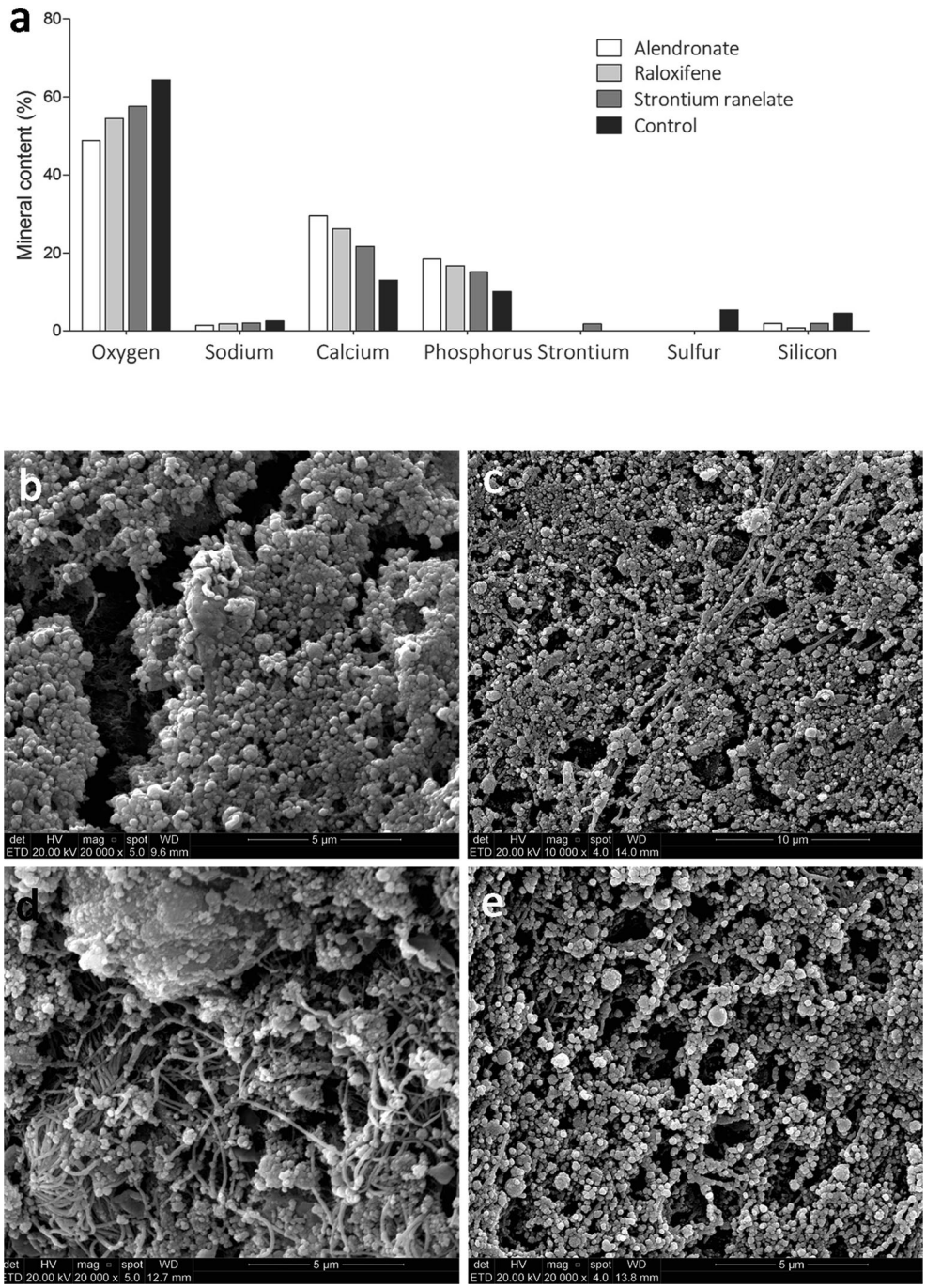

Fig. 6 Analysis of mineral content of alveolar bone. Chemical elements of alveolar bone estimated by energy dispersive spectroscopy (EDS) shows a higher level of calcium and phosphorus in the alendronate group and higher level of oxygen, sodium and silicon in the control group (**a**); images of scanning electron microscopy (20,000x) show the proportion between organic and inorganic content of bone: the alendronate group shows a highly mineralized bone surface and a microcrack area (**b**); collagen fibrils with mineral particle deposits are seen in the raloxifene and control groups (**c**, **e**); high amount of collagen fibrils is seen in the strontium ranelate group partially mineralized (**d**)

EDS

A representative analysis of chemical elements in the alveolar bone is shown in Fig. 6. The alendronate sample showed higher levels of calcium and phosphorus while control showed higher levels of oxygen, sodium, and silicon. Strontium was detected only in the strontium ranelate group and sulfur in the control group.

Discussion

Bisphosphonates are prescribed as first-line therapy for osteoporosis, since they increase bone mineral mass and decrease the risk of bone fractures, meanwhile having a reasonable dosage and low cost [5, 32]. Nevertheless, these drugs exert considerable suppression of bone metabolism, which alters bone properties and leads to microcrack accumulation [33].

These effects associated with surgical procedures in the jaws predispose to MRONJ, whose treatment demands withdrawing the antiresorptive drug [1, 3]. In severe cases of osteoporosis though, the treatment cannot be interrupted, and an alternative therapy is required. The present study was conceived with the aim of investigating whether the antiresorptives raloxifene, and strontium ranelate would interfere with bone repair after tooth extraction in a similar way to bisphosphonates.

The alendronate group showed a greater amount of non-vital bone, inflammatory infiltrate and biofilm on H&E analysis, and larger mucosal lesion on macroscopic examination as well. It is important to recall that all these features have been associated with MRONJ [1, 3, 11, 30]. In agreement with that, in the correlation analysis, non-vital bone, inflammatory infiltrate, and oral mucosal lesion were negatively correlated with vital bone. In this regard, long-term use of bisphosphonate can cause an excessive suppression of bone turnover, which sometimes leads to jaw osteonecrosis, especially if bone trauma is involved [3, 11, 33]. The scarce detection of bone cells and Howship lacunae and lower levels of oxygen in the alendronate group determined by SEM analysis were indicative of such suppressive effect, which was reinforced by the lower expression of RANKL in this group.

Bisphosphonates accumulate in bone by binding to hydroxyapatite and then induce osteoclast apoptosis [7, 33]. Even after therapy withdrawal, their antiresorptive effect persists until the whole drug is eliminated. The longer the patient takes the drug, the higher is the cumulative dose [33], leading to remodeling suppression, microcrack accumulation, and brittleness. This accumulation of microcracks in the jaws has been one of the proposed causative factors of MRONJ [34, 35]. Accordingly, in our study, numerous microcracks were detected all over the bone surface in the alendronate group, where they were longer and deeper than in the other groups. These findings represent an excessive suppression of bone turnover and corroborate the capacity of alendronate to impair bone healing after tooth extraction, which demands special care and follow-up for patients taking this medication. Like bisphosphonates, strontium ranelate accumulates in bone by binding to hydroxyapatite [20], which was detected in our EDS analysis. In agreement with some reports, after drug withdrawal, it can take up to 3 years to be completely eliminated from bone [20]. Raloxifene, in turn, exerts its effects through the activation of β-estrogen receptor, with no cumulative deposits in bone [23]. This behavior along with a shorter half-life (27.7 hours) is an advantage of raloxifene over bisphosphonates and strontium ranelate. Also, there are only three reported cases of MRONJ in patients taking raloxifene [12–14], where one of them had a history of bisphosphonate use. Eventually, our data suggest a lack of association between raloxifene and MRONJ, which is corroborated by some robust studies [36, 37].

Strontium ranelate has already been reported as a substitute for bisphosphonate in MRONJ patients [15–18]. This drug has anabolic effects and exerts a boost in bone formation, which would improve necrotic bone healing. Anyway, even though we did not expect it, non-vital bone did occur in this group. Another unexpected finding in the strontium ranelate group was the lower expression of OPG in spite of reports on its upregulation and osteoblast stimulation being associated with the drug [20, 21]. Considering that OPG inhibits osteoclastogenesis, it is reasonable to infer that these results depict a weaker antiresorptive effect of strontium ranelate in osteoporosis treatment, compared with the other two drugs tested. Besides, this group showed non-vital bone, which suggests that replacing bisphosphonate with strontium ranelate in MRONJ patients would not be a plausible alternative.

The alendronate group showed more biofilm than did the raloxifene and strontium ranelate groups. Similarly, Kos et al. [38] reported that bisphosphonates enhance bacterial adhesion to bone hydroxyapatite, another important event in jaw osteonecrosis. Bacterial invasion of alveolar bone has been reported as a major reason for medication-related osteonecrosis occurrence exclusively in the jaws [39, 40]. Less biofilm and absence of non-vital bone in the raloxifene group suggest no interference of this drug with alveolar bone healing, which agrees with Luvizuto et al. [27]. Moreover, there are reports of antibacterial effects of raloxifene [41, 42], which could have favored these results. Taking these points into account, raloxifene would be more suitable than strontium ranelate to replace bisphosphonates in MRONJ patients.

The alendronate group showed a higher proportion of root fragment, as already reported [29]. This seems to represent the difficulty of tooth extraction in patients under bisphosphonate therapy [43], where changes in mineral/collagen ratio make bone less expandable during luxation movements [44, 45]. For that reason, this group had greater amounts of minerals on both EDS and SEM analysis. The other groups, especially strontium ranelate, seemed to have more collagen fibrils with mineral particle deposits. These findings suggest more organic bone component in the strontium ranelate group, whereas in the alendronate the inorganic component prevailed. It is reasonable to infer that these changes in bone properties in the alendronate group would be responsible for both the difficulty in tooth extraction and MRONJ risk. Moreover, one could claim that root fragments may interfere with the healing process. It is important to note that even though remnant periodontal ligament has been reported to favor tooth extraction healing [46, 47], alendronate group was the one with more of this variable (root fragment) and, in spite of that, also more non-vital bone. On the other hand, if we consider root fragment impairing wound healing, it is important to remark that even having a high level of this variable, the raloxifene group did not show non-vital bone. Therefore, root fragments could

explain the high frequency of oral mucosal lesion in this group, but they would not be responsible for the non-vital bone in the other groups.

Strontium ranelate has been associated with thromboembolic and cardiac events, skin reactions, hepatitis, and blood disorders [5, 48, 49]. Therefore, its use was not approved by the US Food and Drug Administration (FDA). However, it had been commercially available in other countries under the names Protos®/Protelos®/Osseor®/Bivalos® (Servier), but these products have recently been removed from the market. Nevertheless, the generic formulation of strontium ranelate is still commercially available [50–52]. It is important to emphasize that, in the present study, there were considerable side effects of strontium ranelate, culminating in the death of a substantial number of animals. Considering the known adverse effects, one could manifest some concern about giving the animals this drug. In this regard, it is important to make it clear that at the time this research was conducted, Protos® was still commercially available, being prescribed for patients. In fact, this work was motivated by a patient who asked about the safety of strontium ranelate regarding MRONJ. This patient had developed MRONJ while taking alendronate, and her doctor had prescribed strontium ranelate to replace the bisphosphonate [15–18]. Also, at the same time that some clinical trials reported an increased risk of vascular events with this therapy [48], Martín-Merino et al. [53] reported that their data did not support an increased risk of venous thromboembolism for strontium ranelate compared with other anti-osteoporotic therapies. Besides, preclinical and other in vivo studies have not reported such adverse events in rats [28, 54, 55]. It is possible that the intensity of the side effects in the present study was a result of the interaction between strontium ranelate and general anesthesia, which should be a warning to researchers who intend to work with this drug. Maybe the drug washout we used, which was one day, should have been longer. Therefore, it is crucial to report this complication when considering its possible administration either to animals or humans. In view of the serious adverse effects and no scientific proof of alveolar bone improvement in the case of MRONJ, we believe that the use of strontium ranelate should be carefully pondered.

The present study investigated MRONJ by using an experimental animal model, where the drugs were administered at human equivalent doses. A comprehensive analysis of data was *conducted*, including macro- and microscopic features in H&E, immunohistochemistry, and SEM. However, there is no perfect animal model, and therefore, the effects of the drugs in patients could differ a little from what is observed in rats in some aspects, considering the specificities of humans either in general or individually. Therefore, although the use of raloxifene seems promising in cases of MRONJ, further cohort studies evaluating patients undergoing treatment with this medication are needed to weigh the risks and benefits compared with other antiresorptive drugs such as bisphosphonates and denosumab.

Conclusion

Sodium alendronate is associated with histological and structural features compatible with MRONJ and reduced bone metabolism as well. Strontium ranelate is associated with non-vital bone, whereas raloxifene is not associated with MRONJ features.

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Compliance with ethical standards

Conflict of interest Valesca Sander Koth declares that she has no conflict of interest. Fernanda Gonçalves Salum declares that she has no conflict of interest. Maria Antonia Zancanaro de Figueiredo declares that she has no conflict of interest. Karen Cherubini declares that she has no conflict of interest.

Ethical approval This study was approved by the Ethics Committee on Animals Use of the Pontifical Catholic University of Rio Grande do Sul (PUCRS, protocol #7700). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

Informed consent For this type of study, formed consent is not required.

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