Comparison of effects of clodronate and zoledronic acid on the repair of maxilla surgical wounds - Histomorphometric, RANKL, OPG, von Willebrand factor and caspase-3 evaluation

Comparison of effects of clodronate and zoledronic acid on the repair of maxilla surgical wounds - Histomorphometric, RANKL, OPG, von Willebrand factor and caspase-3 evaluation

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ABSTRACT

BACKGROUND. The aim of this study was to compare clodronate and zoledronic acid regarding their influence on the repair of surgical wounds in maxillae (soft tissue wound and tooth extraction) and their relation to osteonecrosis.

MATERIAL AND METHODS. Thirty-four Wistar rats were allocated into 3 groups according to the treatment received: (1) 12 animals treated with zoledronic acid; (2) 12 animals treated with clodronate; and (3) 10 animals that were given saline solution. All animals were subjected to tooth extractions and surgically induced soft tissue injury. Histological analysis of the wound sites was performed by means of hematoxylin-eosin (H&E) staining and immunohistochemical staining for RANKL, OPG, von Willebrand factor and caspase-3.

RESULTS. The zoledronic acid group showed higher incidence of non-vital bone than did the clodronate group at the tooth extraction site. At the soft tissue wound site, there were no significant differences in non-vital bone between the test groups. RANKL, OPG, von Willebrand factor and caspase-3 did not show significant differences between the groups for both sites of surgical procedures.

CONCLUSION. Both of the bisphosphonates zoledronic acid and clodronate are capable of inducing maxillary osteonecrosis. Immunohistochemical analysis suggests that the involvement of soft tissues as the initiator of osteonecrosis development is less probable than has been pointed out.

Key words: bisphosphonates; zoledronic acid; clodronate; osteonecrosis

Introduction

Bisphosphonates are classified as nitrogen- and non-nitrogen bisphosphonates according, respectively, to the presence or absence of nitrogen in their chemical structure. These drugs are associated with osteonecrosis of the jaws, an important adverse effect first described in 2003. The disease is characterized by exposure of bone to the oral cavity that does not heal within eight weeks, in patients who have received bisphosphonates without history of radiotherapy in the head and neck region (1, 2). In some cases, the condition is refractory to treatment, where it is only possible to preserve the patient's quality of life by controlling pain and infection, as well as preventing the occurrence of new areas of necrosis (3).

Bisphosphonates inhibit bone resorption through direct and indirect effects on osteoclasts, which undergo apoptosis or become unable to differentiate from hematopoietic stem cells (4-6). It is important to point out that nitrogen- and non-nitrogen-containing bisphosphonates differ in their mechanisms of action. Once internalized by osteoclasts during the resorption process, nitrogen-containing bisphosphonates inhibit the mevalonate pathway, preventing protein prenylation, which is an essential process for normal functioning of vital intracellular proteins. On the other hand, non-nitrogen ones become ATP analogues, which impair mitochondrial function. Both mechanisms result in osteoclast apoptosis (3). Other effects of these drugs, such as impairment of both angiogenesis (7-11) and epithelial cell proliferation (12), support the hypothesis that osteonecrosis of the jaws has a multifactorial etiology.

The pathway of receptor activator of nuclear factor-kB ligand (RANKL) and osteoprotegerin (OPG) is one of the main regulators of the molecular mechanisms involved in the development and function of osteoclasts. Studies on bisphosphonates, *in vitro* and *in*

vivo, have shown controversial results when these proteins are used as immunohistochemical, genetic and serum markers (13, 14). Also, considering the effects of bisphosphonates on apoptosis and angiogenesis (4-11), and the important role of caspase-3 and von Willebrand factor as markers of these processes, it seems that these are important aspects to be analyzed. It was demonstrated that nitrogen-containing bisphosphonates are able to cause toxicity to the cells of oral epithelium, raising concerns whether disease onset could occur in bone tissue or in oral mucosa (15, 16). Moreover, most studies evaluating the effects of the drug on body tissues have been performed with nitrogen-containing bisphosphonates, which generate many concerns about tissue behavior when non-nitrogen-containing ones are used (17).

There are many case reports and some animal model studies in which the use of zoledronic acid is associated with jaw osteonecrosis. Nevertheless, even though clodronate is the most prescribed non-nitrogen-containing bisphosphonate, there are a few case-reports in the literature on osteonecrosis of the jaws induced by this drug (18). In some of them, patients had used nitrogen-containing bishosphonates before the treatment with non-nitrogen ones. Nor has there been a study about clodronate effects using animal models. The aim of this work was to compare the effects of clodronate and zoledronic acid on the repair of surgical wounds of maxillae. Microscopic features of tooth extraction and soft tissue injury areas were evaluated by means of hematoxylin-eosin (H&E) staining and immunohistochemical detection of RANKL, OPG, vWF and caspase-3.

Material and methods

Animals

The present study was approved by the Ethics Committee for Animal Use of the Pontifical Catholic University of Rio Grande do Sul, and the procedures were carried out in accordance with institutional guidelines for animal care and use. The sample comprised 34 female rats (*Rattus norvegicus,* Wistar strain) from the animal facility of the Federal University of Pelotas, which had a mean age of 120 days and a mean weight of 230 g. Animals were individually identified on the tails and housed in plastic cages (5 per cage) placed in ventilated racks (Alesco, Monte Mor, SP, Brazil) at a temperature of 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*. The animals were randomly allocated into 3 groups, according to the bisphosphonate used: (1) zoledronic acid group: 12 animals treated with the nitrogen-containing bisphosphonate zoledronic acid (Novartis Pharma AG, Basel, Switzerland) intraperitoneally (0.6 mg/kg, every 28 days); (2) clodronate group: 12 animals treated with the non-nitrogen-containing bisphosphonate clodronate (Jenahexal Pharma GmbH, Thuringia, Germany), intraperitoneally (20 mg/kg, every 28 days); and (3) control group: 10 animals that were given saline solution (0.9% sodium chloride), intraperitoneally every 28 days.

Surgical procedures

All animals were subjected to tooth extractions and surgical-induced soft tissue injury as described below. Oroscopy was performed after the anesthesia and before the surgical procedures to certify that there were no previous oral lesions.

Tooth extractions

Tooth extractions were performed 60 days after the beginning of the experiment. Animals were anesthetized with a single intraperitoneal injection of a mixture of ketamine hydrochloride 5% (100 mg/kg; Vetbrands, Jacareí, SP, Brazil) and xylazine hydrochloride 2% (10 mg/kg; Vetbrands). The three upper right molars were extracted using an adapted 3s spatula (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) for luxation and a pediatric forceps (Edlo, Canoas, RS, Brazil) whose functional portion was adapted to the size of the tooth.

Surgically induced soft tissue wound

Immediately after the tooth extractions, a surgical wound was made on the mucosa of the hard palate at the opposite side (left side) of the maxilla, with reference to the second left upper molar, and using a surgical scalpel with no. 3 Bard-Parker handle (Solidor, São Paulo, SP, Brazil) and a no. 15 blade (Solidor). The incision was elliptical, 3 mm long and 1 mm deep.

Euthanasia of the animals, macroscopic evaluation and dissection of the maxillae

After completing 102 days of drug administration, the animals were euthanized by deep anesthesia with isoflurane (Cristalia, Porto Alegre, RS, Brazil) in an appropriate anesthesia chamber. The specimens were then dissected and examined by means of a no.5 clinical probe (SS White, Duflex, Rio de Janeiro, RJ, Brazil) to determine the presence/absence of oral mucosal lesion. Afterwards, the maxillae were fixed for 24 h in 10% buffered formalin (TopGlass, Porto Alegre, RS, Brazil).

Histological processing

The specimen was cut into two fragments in the coronal direction, using a steel-sanding disc at low speed and subjected to decalcification in ethylenediaminetetraacetic acid

(EDTA, Biodinâmica, Ibiporã, PR, Brazil) solution for 30 days. Next, they were paraffin-embedded, cut into 4-µm sections and stained with hematoxylin and eosin (H&E) as well as immunohistochemically processed.

Antigen retrieval was carried out in a 100°C water bath for 40 min, using Tris/EDTA buffer, pH 9 (20 mM Tris/0.65 mM EDTA). Endogenous peroxidase was blocked with a 3% solution of hydrogen peroxide in methanol for 30 min. The blocking of nonspecific binding was done with the commercial solution Protein Block Serum-Free (Dako, Carpinteria, CA, USA) for 30 min at room temperature. The immunohistochemical staining method based on capillary action was used (Thermo, Shandon, CA, USA), where the sections were incubated overnight at 2°C with the following antibodies: anti-RANKL (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:500; anti-OPG goat polyclonal antibody (SC8468 - Santa Cruz Biotechnology) at 1:100; anti-vWF (Biocare, Concord, Massachusetts, USA); and anti-caspase-3 (Novocastra, Newcastle, UK) diluted at 1:500. For amplification of the antigen-antibody reaction, the Picture Max system (Invitrogen, Carlsbad, CA, USA) was used according to manufacturer's recommendations. The slides were then washed with PBS and incubated with diaminobenzidine solution for 5 min. The detection system used was Dako LSAB Kit (Dako). Color development was carried out with the chromogen 3'-diaminoabenzidine and phosphate buffer solution containing 0.002% hydrogen peroxide. All markers had external and internal controls.

Histological analysis

The histological sections were digitized using a Zeiss Axioskop 40 light microscope (Zeiss, Göettingen, Germany), connected to a Roper Scientific video camera (Media Cybernetics, Bethesda, MD, USA) and a Pentium IV 2.2 GHZ computer with 512 MB RAM, 160 GB

hard drive, and Image Pro Capture Kit Platform (Media Cybernetics). The images were captured using 5x (H&E stain) and 10x (immunohistochemistry) objectives and stored in Joint Photographic Expert Group (JPEG) format. For H&E analysis, 5 fields were selected in a standardized manner in each slide to include the whole area of tooth extractions and soft tissue wound. For immunohistochemistry, 2 fields were selected in a standardized manner to include the epithelial and connective tissue at both sites evaluated.

The images were analyzed by a calibrated and blinded examiner. The calibration consisted of evaluating a series of 20 histological images, for each technique (H&E and immunohistochemistry) twice, at two different moments. The results of these two evaluations were subjected to a paired t test and Pearson's correlation coefficient, showing the absence of a significant difference ($p > 0.05$) and a strong correlation ($r > 0.8$).

H&E analysis

The H&E images were analyzed using the manual counting technique in the Image Pro Plus 4.5.1 software (Media Cybernetics). A quantitative analysis was made for the variables epithelial tissue, connective tissue, root fragments, microbial colonies, inflammatory infiltrate, non-vital bone, and vital bone. A point-grid of 532 points was superimposed to each image and each point was counted according to the matching morphological structure (Fig. 1).

Immunohistochemical analysis

The immunohistochemical expression of RANKL, OPG, vWF and caspase-3 was quantified by means of semi-automated segmentation technique (19) in the Image Pro Plus 4.5.1 software (Media Cybernetics, Bethesda, MD, USA; Fig. 2).

Statistical analysis

The data were analyzed by descriptive statistics, chi-squared test complemented by analysis of adjusted residuals, and Kruskal-Wallis test complemented by its multiple comparisons test, setting the level of significance at 5%. The statistics were processed by the SPSS 17.0 software (Statistical Package for the Social Sciences, Chicago, IL, USA).

Results

Clinical and macroscopic evaluation

On oral examination before tooth extractions, no animal exhibited oral mucosal lesions. Table 1 presents the results of macroscopic evaluation (after euthanasia) for tooth extraction and soft tissue wound sites (Fig. 3). Zoledronic acid was associated with the loss of mucosal integrity in the tooth extraction site ($p<0.001$). Neither zoledronic acid nor clodronate was associated with loss of mucosal integrity at the soft tissue wound site ($p=0.151$).

H&E analysis

Table 2 shows the results for the frequency of non-vital bone. It was observed that (1) zoledronic acid was associated with non-vital bone at the tooth extraction site ($p<0.001$); (2) at the soft tissue wound site, both zoledronic acid and clodronate were associated with non-vital bone ($p<0.001$).

The results for the proportions of the histological variables (Fig. 4) are presented in Tables 3 and 4. At the tooth extraction site, the proportion of non-vital bone and microbial colonies was significantly greater in the zoledronic acid group compared to the clodronate and control groups, but the latter two did not differ significantly from each other. Neither vital bone nor root fragment was associated with any group evaluated. The proportion of

connective tissue was significantly greater in the clodronate compared to the zoledronic acid group, but they did not differ significantly from the control group (Table 3).

At the soft tissue wound site, the proportions of nonvital bone and microbial colonies were significantly greater in the zoledronic acid and clodronate groups than in the control. The proportion of connective tissue was significantly greater in the control group compared to the clodronate and zoledronic acid groups, whereas the proportion of vital bone was significantly greater in the control group compared to the clodronate group, but these two did not differ significantly from the zoledronic acid group. There was no significant difference in epithelial tissue and inflammatory infiltrate between the groups (Table 4, Fig. 5 A-D).

Immunohistochemical analysis

Immunohistochemical quantification for RANKL, OPG, caspase-3 and vWF at tooth extraction and soft tissue wound sites did not differ significantly between the groups (Tables 5 and 6, Fig. 5 E-H).

Discussion

On macroscopic examination, an association was observed between loss of mucosal integrity at the tooth extraction site and zoledronic acid use. The occurrence of osteonecrosis and root fragments seems to explain this finding in both test groups and control. At the soft wound site, however, this association was not observed in any group, even with all animals in the test groups showing non-vital bone. This finding is in agreement with reports suggesting that bisphosphonate-associated jaw osteonecrosis can occur without bone exposure (20-22), even though its clinical definition refers to exposed

bone in the oral cavity that does not heal (1, 2). Still, it disagrees with the hypothesis that soft tissues could occupy the first position in the pathophysiology of osteonecrosis (23-26).

The frequency of non-vital bone was significantly higher at the soft tissue wound site than at the tooth extraction site. The anatomical specificities of the sites subjected to the surgical procedures, as well as the type of lesion induced, could have contributed to this result. After a tooth extraction, the alveolar socket is first filled with the clot, which gives rise to a neoformed connective tissue, rich in fibroblasts and capillaries, which in turn promotes healing through the formation of well-organized bone trabeculae (27). The buccal and lingual bone walls remain covered by mucosa, healing occurs by secondary intention (28), and growing epithelial cells restore the epithelial continuity of the mucosa (29). In the case of bisphosphonate users, this process would be impaired by diminished vascularization (7) and reduced bone neoformation (16). Moreover, the hard palate has poor vascularization and its mucosa is firmly adhered to the periosteum (30). The wound at this site seems to have the potential of exposing a wider area of bone tissue, with less blood supply, if compared to the tooth extraction site, which could explain its higher prevalence of osteonecrosis. Therefore, the results of non-vital bone for the soft tissue wound site suggest that oral mucosal lesions, depending on their location, vascularization, submucosal thickness and relationship with the subjacent bone tissue, constitute a sufficient risk factor for the occurrence of osteonecrosis associated with bisphosphonates, with no necessity of more invasive interventions such as tooth extractions. According to our results, this seems to be true not only for nitrogen-containing bisphosphonates but also non-nitrogenous ones.

Nevertheless, it is important to recall that the maxilla, because of its higher vascularization (31) and turnover, is less prone to bisphosphonate osteonecrosis compared to the mandible is (32). Thus, it is plausible to infer that if we had done the tooth

extractions in the mandible instead of maxilla, the prevalence of osteonecrosis could have been higher at this wound site than at the soft tissue wound site.

On microscopic examination, the tooth extraction site showed non-vital bone in 100% of the animals in the zoledronic acid group. This finding is in agreement with other studies, according to which the trauma constitutes a sufficient risk factor for osteonecrosis in zoledronic acid users (31, 33, 34). On the other hand, at the same site, the clodronate group was not statistically associated with non-vital bone, even though 5 (41.6%) out of 12 animals showed this feature. Considering that there was an association between this variable and clodronate at the soft tissue wound site, it is possible that a larger sample size would have given us a different result for the tooth extraction site in the clodronate group. In humans, the lower prevalence of osteoncerosis associated with non-nitrogen bisphosphonates, especially clodronate, could be explained by the fact that these compounds have lower potency, and are less prescribed than nitrogen-containing ones (35).

At the tooth extraction site, the zoledronic acid group had a higher proportion of microbial colonies than the clodronate and control. This is in accordance with the higher proportion of non-vital bone found in the zoledronic acid group, since osteonecrosis is characteristically accompanied by microbial infection, especially by *Actinomyces* sp. (36). Nevertheless, there are reports on the antimicrobial effect of clodronate on *Staphylococcus aureus* and *Pseudomonas aeruginosa* (37). Zoledronic acid effects on microorganisms are also reported, although in an opposite direction, as this drug has been shown to improve, *in vitro,* the adhesion of *S. mutans* to the bone hydroxyapatite, promoting bacterial growth in culture dishes (12). Therefore, the greater occurrence of microbial colonies in the zoledronic acid group when compared to clodronate could be related to the specific interactions of each one of these two bisphosphonates with microorganisms.

At the site of soft tissue wound, the clodronate and zoledronic acid groups showed a lower proportion of fibrous connective tissue when compared to the control group. These findings are in agreement with studies showing that bisphosphonates are able to decrease fibroblast proliferation (38). Curiously, at this same site, vital bone proportion was significantly lower in the clodronate than in the control group, whereas the latter and the zoledronic acid group did not differ from each other. It is known that bisphosphonates promote nearly complete suppression of bone turnover (39, 40), but in this case we do not know why clodronate had a greater effect than did zoledronic acid.

The quantification of RANKL, OPG, vWF and caspase-3 did not show significant differences between the groups evaluated, either at the soft tissue wound site or the tooth extraction site. The bisphosphonate effects on the cells of the oral mucosa are poorly understood, and until now, it has not been determined if osteonecrosis starts in bone tissue or in oral mucosa (15, 25, 38).

We quantified caspase-3 in epithelial and connective tissues aiming to determine if higher rates of apoptosis in these tissues could favor osteonecrosis development. The results are in accordance with the reports of no increase in caspase-3 expression in oral keratinocytes and gingival fibroblasts treated with nitrogen-containing bisphosphonates (15, 38). The lack of a difference in caspase-3 expression between the groups suggests that if the soft tissues are the target of the onset of osteonecrois (41), this involvement is not related to increased apoptosis of these cells.

The lack of significant differences in vWF expression between the groups suggests that bisphosphonates are not capable of inhibiting vascularization in oral soft tissues. This agrees with the reports of Wehrhan et al. (42), who found that the immunohistochemical expression of CD31 in mucoperiosteal tissue did not differ between bisphosphonate users

with and without osteonecrosis. However, studies investigating the bisphosphonate effects on angiogenesis show conflicting results.

According to some *in vitro* studies, zoledronic acid inhibits human endothelial cell differentiation (43), reduces their proliferation (44, 45), induces their apoptosis (11) and decreases the formation of capillary tubes (43). Similarly, other *in vitro* studies report that clodronate inhibits endothelial cell proliferation (45, 46). On the other hand, immunohistochemical expression of VEGF in bone tissue at the tooth extraction site did not change in rats treated with bisphosphonates (33). There are also reports of a significant reduction in serum levels of VEGF in cancer patients treated with bisphosphonates (47, 48). The disagreements between the studies, either *in vitro* or *in vivo,* reflect the difficulties in comparing them because of the different methods applied.

vWF is an important marker of vascularization, but it does not differentiate new vessels from preexisting ones (49). One could infer that by using a marker capable of indentifying new vessels, such as VEGF, we could have found a different result. Nevertheless, we should consider the phase of healing in which the immunohistochemical analysis was performed here. In rats, the healing process at tooth extraction sites is completed by 40 days, and neoformed vessels should be detected at 7 days (27), but not at the time at which we performed the evaluation. At this moment, the differentiation between new and preexisting vessels does not seem to influence the results anymore. Considering the conditions of the present study, as well as the controversial results with VEGF and also the fact that vWF immunostaining pattern is more uniform if compared with VEGF and CD31 (50), we chose vWF as the marker of vascularization in our study.

The lack of significant differences in the expression of OPG and RANKL between the zoledronic acid, clodronate and control groups would suggest that bisphosphonates are

not capable of increasing the OPG/RANKL ratio. In our study, these proteins, which are also expressed in endothelial cells and fibroblasts, were quantified in connective tissue, 6 weeks after the surgical procedures, corresponding to the time of complete bone formation (51). This site of evaluation was chosen because we wanted to analyze the alterations in soft tissue that could be related to the onset of osteonecrosis and also because connective tissue is the matrix of bone neoformation. At this site, growth factors, cytokines and prostaglandins exert their effects on the recruitment, replication and differentiation of bone precursor cells (52).

It is also important to consider that most of the *in vivo* studies evaluating the effects of bisphosphonates on the behavior of these proteins have measured their serum levels (53, 54). Maybe this analysis does not represent what actually occurs in the environment of bone cells, oral keratinocytes and fibroblasts (54). Moreover, we have to consider that the serum levels of RANKL reflect only its soluble form, excluding the major mediator of osteoclastogenesis, which is its form as a cytokine of cell membrane (55). Studies comparing the expression of these two forms of RANKL would be helpful.

It is worth recalling that the immunohistochemical analysis was conducted in epithelial and connective tissues, not in bone tissue. Considering this point, the absence of any significant result for the markers used in the test groups suggests that the major bisphosphonate effects occur in bone tissue. The high bone affinity of these drugs (40) corroborates this idea. According to the literature, 70% of the zoledronic acid administered binds to hydroxyapatite crystals, whereas the rest of the dose is eliminated unaltered by renal excretion (56). The situation with clodronate is similar except for the fact that the absence of the hydroxyl group in this compound reduces its ability to bind to hydroxyapatite crystals, compared to zoledronic acid (57).

In conclusion, both bisphosphonates zoledronic acid and clodronate are capable of inducing maxillary osteonecrosis. The findings of immunohistochemical analysis suggest that the involvement of soft tissues as the initiator of osteonecrosis development is less probable than has been pointed out.

Acknowledgements

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Table 1. Sample distribution according to presence/absence of loss of mucosal integrity on macroscopic examination of the tooth extraction site and soft tissue wound site.

Group	Loss of mucosal integrity							
	Tooth extraction site				Soft tissue wound site			
	Present		Absent		Present		Absent	
	n	%	n	%	n	%	n	%
Zoledronic acid (n=12)	**12***	**100***	0	0	9	75	3	25
Clodronate (n=12)	10	83.3	2	16.7	9	75	3	25
Control (n=10)	1	10	9	90	4	40	6	60
Total	23	67.6	11	32.3	22	64.7	12	35.2

n = number of animals
*Significant difference; chi-square test; analysis of adjusted residuals; p<0.001

Table 2. Sample distribution according to presence/absence of non-vital bone at the tooth extraction and soft tissue wound site on microscopic examination

Group	Non-vital bone							
	Tooth extraction site				Soft tissue wound site			
	Present		Absent		Present		Absent	
	n	%	n	%	n	%	n	%
Zoledronic acid (n=12)	**12***	**100***	0	0	**12***	**100***	0	0
Clodronate (n=12)	5	41.6	7	58.3	**12***	**100***	0	0
Control (n=10)	0	0	10	100	4	40	6	60
Total	**17***	**50**	17	50	**28***	**82.3**	6	17.6

n = number of animals
*Significant difference; chi-square test; analysis of adjusted residuals; p=0.007

Table 3. Quantification of histological features (hematoxylin-eosin stain) at the tooth extraction site in the zoledronic acid, clodronate and control groups.

Histological feature	Zoledronic acid (%)			Clodronate (%)			Control (%)		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
Non-vital bone	15.38	10.02	**13.78**[A]	1.28	2.12	**0**[B]	0	0	**0**[B]
Epithelial tissue	18.74	5.90	18.37	16.20	3.46	16.73	18.47	4.70	18.82
Connective tissue	28.13	8.23	**30.44**[B]	41.73	10.44	**42.42**[A]	37.48	8.68	**34.49**[AB]
Vital bone	16.39	13.84	13.42	24.97	15.74	20.58	31.13	14.63	31.33
Inflammatory infiltrate	8.04	6.36	5.74	7.97	7.57	5.37	8.41	7.88	6.17
Microbial colonies	3.46	2.77	**3.32**[A]	0.49	0.60	**0.21**[B]	0.38	0.72	**0.08**[B]
Root fragments	9.83	7.89	8.37	7.33	5.04	7.97	3.40	4.79	0.79

* Bold printed medians, followed by different letters, indicate features that differed significantly between groups; Kruskal-Wallis test complemented by multiple comparisons test, $p \leq 0.05$
SD = Standard deviation

Table 4. Quantification of histological features (hematoxylin-eosin stain) at the soft tissue wound site in the zoledronic acid, clodronate and control groups.

Histological feature	Zoledronic acid (%)			Clodronate (%)			Control (%)		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
Non-vital bone	31.35	8.89	**30.79**[A]	21.99	6.55	**23.26**[A]	3.65	5.61	0
Epithelial tissue	12.21	3.65	11.44	13.96	5.6	13.11	12.62	2.10	12.98
Connective tissue	31.22	5.95	**30.43**[C]	41.03	9.10	**43.99**[B]	52.11	9.33	**51.96**[A]
Vital bone	11.49	8.73	**11.32**[AB]	3.73	3.57	**3.51**[B]	22.95	16.44	**27.77**[A]
Inflammatory infiltrate	10.12	8.87	8.47	16.33	7.67	15.89	8.00	7.49	8.92
Microbial colonies	3.58	3.34	**2.35**[A]	2.94	2.60	**2.29**[A]	0.34	0.85	**0**[B]

** Bold printed medians, followed by different letters, indicate features that differed significantly between groups; Kruskal-Wallis test complemented by multiple comparisons test, $p \leq 0.05$
SD = Standard deviation

Table 5. Immunohistochemical quantification of RANKL, OPG, von Willebrand factor (vWF) and caspase-3 at tooth extraction site in the zoledronic acid, clodronate and control groups.

Marker	Group									$P*$
	Zoledronic acid (mm^2)			Clodronate (mm^2)			Control (mm^2)			
	Mean	SD	MD	Mean	SD	MD	Mean	SD	MD	
RANKL	0.38	0.32	0.35	0.34	0.38	0.25	0.42	0.44	0.38	0.76
OPG	0.31	0.30	0.22	0.35	0.20	0.31	0.26	0.20	0.21	0.55
vWF	0.50	0.35	0.39	0.77	0.61	0.58	0.49	0.27	0.41	0.58
Caspase 3	1.72	2.11	1.10	1.03	0.97	0.89	1.33	1.15	1.11	0.74

*Kruskal-Wallis test, $\alpha=0.05$
SD = Standard deviation; MD = Median

Table 6. Immunohistochemical quantification of OPG, RANKL, von Willebrand factor (vWF) and caspase-3 at soft tissue wound site in the zoledronic acid, clodronate and control groups.

Marker	Group									P
	Zoledronic acid (mm^2)			Clodronate (mm^2)			Control (mm^2)			
	Mean	SD	MD	Mean	SD	MD	Mean	SD	MD	
RANKL	0.35	0.40	0.13	0.41	0.34	0.32	0.52	0.55	0.30	0.77
OPG	0.36	0.33	0.30	0.29	0.26	0.16	0.59	0.32	0.71	0.08
vWF	0.97	0.93	0.67	0.61	0.58	0.30	0.79	0.71	0.60	0.46
Caspase 3	1.41	1.18	1.27	1.52	2.20	0.82	1.30	0.92	1.14	0.75

*Kruskal-Wallis test, $\alpha=0.05$
SD = Standard deviation; MD = Median