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ASSOCIATION BETWEEN BISPHOSPHONATES AND JAW OSTEONECROSIS: A STUDY IN WISTAR RATS

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Running Title: Jaw osteonecrosis and bisphosphonates

Abstract

Background: This work aimed at determining whether bisphosphonate therapy produces a sufficient condition for jaw osteonecrosis after tooth extraction.

Methods: Rats were allocated into 3 groups: (1)11 rats treated with alendronate; (2)10 rats treated with zoledronic acid; (3)10 control rats. The animals were submitted to tooth extractions, and at the end of bisphosphonate therapy, they were euthanized. Histological sections of the surgical site were processed and analyzed.

Results: The zoledronic acid group showed higher incidences of osteonecrosis, inflammatory infiltrate and microorganisms. There was no significant difference for epithelial or connective tissue, root fragments, vital bone and positive staining for vascular endotheial growth factor (VEGF) among the groups.

Conclusions: Zoledronic acid is associated with jaw osteonecrosis, whereas alendronate did not produce a condition sufficient for osteonecrosis after tooth extraction. Neither zoledronic acid nor alendronate was associated with a reduced immunohistochemical expression of VEGF in vital bone at the tooth extraction site.

Key words: Bisphosphonates; Jaw osteonecrosis; Angiogenesis; Zoledronic acid; Alendronate.

INTRODUCTION

The first synthesis of bisphosphonates for industrial application occurred in Germany in the mid-nineteenth century, in 1865. Their use in humans began about 40 years ago.[1] These compounds contain a P-C-P bond as part of their chemical structure, and they act as effective inhibitors of bone resorption mediated by osteoclasts. Therefore, bisphosphonate drugs are indicated for the management of calcium and bone metabolism disorders[2] characterized by increased bone resorption, such as Paget's disease, hypercalcemia, bone metastases of malignant neoplasms, primary and secondary hyperparathyroidism and osteoporosis.[3]

Since 2003, there have been case reports of osteonecrosis of the jaws in patients undergoing therapy with bisphosphonates, mainly after tooth extraction.[4-8] Some peculiar findings, such as the restriction of the lesion to the jaws without affecting other bones of the skeleton,[9] and the challenge that the therapeutic approach represents, have stirred the interest of the scientific community.[5,10-12]

There are reports indicating that oral use of these drugs is associated with a lower risk of osteonecrosis, while intravenous administration represents an elevated risk.[12,13] Corticotherapy, chemotherapy,[14,15] radiotherapy, anemia, coagulopathies, infections, pre-existing oral diseases,[16] family history, lifestyle, and use of alcohol and tobacco,[11] among others, are pointed out as risk factors. However, the real role of these factors in the development of lesions is still a controversial subject. Currently available information is mostly derived from clinical investigations in patients with the condition. The aim of the present study was to determine in an animal model, whether therapy with bisphosphonates, administered by either the oral or parenteral route, could be a sufficient condition for the occurrence of maxillary osteonecrosis in rats submitted to tooth extractions. Attempts were also made to evaluate the macro- and microscopic events that characterize the area submitted to exodontia.

MATERIALS AND METHODS

Animals

The present study was approved by the Research Ethics Committee of the Pontifical Catholic University of Rio Grande do Sul. The sample comprised 31 female rats (*Rattus*

norvegicus, Wistar strain) from the animal facility of the Federal University of Pelotas (UFPEL, RS), which had a mean age of 140 days and mean weight of 240.66 g. The animals were randomly allocated into 3 groups: (A) alendronate group: 11 animals that were given the nitrogen-containing bisphosphonate alendronate orally by gavage and that were submitted to tooth extractions; (B) zoledronic acid group: 10 animals that were administered the nitrogen-containing bisphosphonate zoledronic acid intraperitoneally and that were submitted to tooth extractions; and (C) control group: 10 animals that were submitted to tooth extractions, without receiving bisphosphonates. The animals were kept in suitable plastic cages, which were labeled and placed in ventilated racks (Alesco, Monte Mor, SP, Brazil), with controlled temperature (22 ± 1^{o}C) and 12-h light-dark cycle (lights on at 7 a.m. and off at 7 p.m.). The bedding was changed three times a week, and food (Nuvilab, Colombo, PR, Brazil) and filtered water were provided *ad libitum*.

The bisphosphonates used were sodium alendronate (Galena Química & Farmacêutica, Campinas, SP, Brazil) and zoledronic acid (Zometa®, Novartis Pharma AG, Basel, Switzerland). The first administration of both drugs was carried out at the beginning of the experiment, after labeling and weighing of the animals. Later, alendronate was administered weekly, at a dose of 0.05 mg/kg by gavage,[17] for 23 weeks. The animals of the zoledronic acid group received 5 doses of 0.6 mg/kg, intraperitoneally, at intervals of 28 days.[18] The animals were weighed every 28 days in all groups to adjust the doses.

Clinical evaluation

To determine the presence/absence of oral lesions, a blinded observer performed a careful oral examination after anesthesia, prior to tooth extractions.

Tooth extractions

Tooth extractions were performed 45 days after the onset of experiments, under deep anesthesia with a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg), administered intraperitoneally.[19,20] The animal was placed in the dorsal decubitus position, the mouth was kept opened with the help of rubberbands (string type) anchored on the upper and lower incisors, and stretched and fixed to the operating table. The upper molars on the right side were extracted using a lever movement with a 3s spatula (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) adapted for luxation and pediatric forceps (Edlo, Canoas, RS, Brazil) whose functional portion was adapted to the size of the teeth. During the surgical procedure, the area was irrigated continuously with saline. There was no need for postoperative suturing because of the small amount bone exposed.

Euthanasia, macroscopic evaluation and preparation of specimens

Euthanasia was performed by isoflurane inhalation in an appropriate anesthesia chamber[20-22] (Cristalia, Porto Alegre, RS, Brazil), after 150 days from the start of the experiment. After euthanasia, the maxilla was dissected and submitted to macroscopic evaluation in order to determine the presence/absence of integrity of the oral mucosa in the area of tooth extractions, by probing with a dental explorer no.5 (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil). The observer was blinded to the group examined. The specimens (maxillae) were then fixed for 24 h in 10% buffered formalin made from 37% formaldehyde (TopGlass, Porto Alegre, RS, Brazil), distilled water (TopGlass, Porto Alegre, RS, Brazil), monobasic sodium phosphate (Cromoline, Diadema, SP, Brazil) and dibasic sodium phosphate (Synth, Diadema, SP, Brazil).

After fixation, the maxillae were sectioned in the latero-lateral direction, obtaining a cross-section that included the area of the tooth extractions as well as the opposite area in which the molars were preserved. The aim of such procedure was to obtain a specimen of adequate size and with sufficient parameters for the identification of the sites and anatomic repairs by embedding in paraffin and histological examination. The specimen was then divided into two fragments in the latero-lateral direction with the help of a steel-sanding disk at low speed. The internal surface of each fragment was identified and positioned as the cutting surface during paraffin embedding, which guaranteed the inclusion of the area of tooth extractions in the histological sections. Subsequently, the specimens were submitted to decalcification in formic acid solution for 24 h. This solution was composed of 780 ml of 10% tribasic sodium citrate P.A. (Cromoline, Diadema, SP, Brazil) and 220 ml of 85% formic acid P.A. (Synth, Diadema, SP, Brazil). After the decalcification step, the specimens were processed using the paraffin technique.

Histological processing

The specimens were embedded in paraffin, and 62 blocks were prepared from the 31 animals. Two sections of 4-µm thickness were obtained from each paraffin block for the preparation of the respective histological slides. One of the slides was submitted to hematoxylin and eosin (H&E) staining and the other one to immunohistochemical processing. In the latter, the slides were submitted to antigen retrieval in a 99ºC waterbath for 30 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 sections were incubated with anti-rat VEGF monoclonal antibody (clone VG1, ZymedR Laboratories, South San Francisco, CA, USA), diluted 1:300. The detection

system utilized was the Dako LSAB Kit (Dako, Carpinteria, CA, USA). Color development was with the chromogen 3,3`- diaminoazobenzidine (DAB) and PBS containing 0.002% hydrogen peroxide, and slides were then stained with hematoxylin, dehydrated, cleared and coverslipped. Sections of colon carcinoma were used as the positive control, and sections of the study sample were processed in the absence of antibody for the negative control.

Histological analysis

Image capture was carried out using a Zeiss Axioskop 40 light microscope (Zeiss, Oberkohen, Germany), connected by a Roper Scientific videocamera (Media Cybernetics, Silver Spring, USA) to a pentium IV 2.2 GHZ computer with 512 MB RAM, 160 GB hard drive and Image Pro Capture Kit Plattform (Media Cybernetics, Silver Spring, USA). The images were captured using 5x (H&E) and 10x (VEGF) objectives and stored in TIFF (True Image Format File) format. Histological analysis was carried out by a calibrated and blinded observer. The calibration consisted of an evaluation of a series of 20 histological images at two different times. The results of the evaluation were submitted to a paired *t* test and Pearson's correlation test, which showed respectively the absence of a significant difference between the analyses ($p>0.05$) and a strong correlation ($r=0.9$).

In the slides stained with H&E, a quantitative analysis (proportion) was made of the variables osteonecrosis, inflammatory infiltrate, microbial colonies, epithelial tissue, connective tissue, root fragments and vital bone at the site of tooth extractions. In each slide, 4 fields were selected in a standardized manner in order to include the whole area of the tooth extraction. Readings were carried out using Image Pro Plus 6.0 software, applying a grid of 798 points on each selected field, and measuring each of the histological variables by the manual point counting technique. In this technique, each one of the points of the grid

is counted determining which histological feature (osteonecrosis, vital bone, epithelial tissue, connective tissue, inflammatory infiltrate, microbial colonies, and root fragment) it matches with. This procedure is done by clicking the mouse and the information is processed by the software. The software itself gives the points counted for each feature in absolute and relative (%) values (Figure 1). Therefore, the analysis provided the proportion of each variable in the area of tooth extractions visualized in 4 microscopic fields. It is also important to point out that the areas of bone without osteocytes and showing microbial colonies in the periphery, in the medullary spaces and in the adjacent sites were regarded as areas of osteonecrosis. In the same manner, a quantitative analysis of the immunohistochemical expression of VEGF was performed in the field of vital bone, in the area of tooth extractions, which was selected in a standardized manner in all images.

Statistical analysis

The results were analyzed by means of descriptive statistics and Kruskal-Wallis, ANOVA and chi-squared tests, at a level of significance of 5%. The parameters loss of mucosal integrity, osteonecrosis, vital bone, inflammatory infiltrate, microbial colonies, connective tissue, epithelial tissue, root fragments and VEGF expression were compared among the groups (alendronate, zoledronic acid and control). The chi-squared and Kruskall-Wallis tests were complemented respectively by analysis of adjusted residuals and by the multiple comparisons test.

RESULTS

Clinical and macroscopic evaluation

On oral examination prior to tooth extractions, no animal in the three groups exhibited lesions of the oral mucosa. Table 1 presents the results of macroscopic evaluation, carried out after euthanasia. By means of the chi-squared test (χ^2), complemented by analysis of adjusted residuals (α=0.05), the zoledronic acid group was found to be associated with loss of mucosal integrity (p<0.001).

Histological evaluation

Table 2 shows the results of the frequency (presence/absence) of the histological features in the groups evaluated. By means of the χ^2 test complemented by analysis of adjusted residuals (α=0.05), it was seen that: (1) the zoledronic acid group was associated with the presence and the alendronate and control groups with the absence of osteonecrosis (p<0.001); and (2) the zoledronic acid group was associated with the presence of microbial colonies (p=0.008).

Table 3 presents the results of the quantification of the histological features in the area of tooth extractions for the three groups evaluated. By means of the Kruskal-Wallis test complemented by its multiple comparisons test (α=0.05), the occurrence of osteonecrosis, inflammatory infiltrate and microbial colonies was significantly greater in the zoledronic acid group compared to the other groups (p<0.05). While the zoledronic acid group showed higher frequency of loss of mucosal integrity as presented in Table 1, the quantitative analysis for epithelial tissue, connective tissue, root fragments and vital bone, did not show significant differences among the groups evaluated (Table 3, p>0.05).

Table 4 shows the proportion of immunohistochemical expression of VEGF in the areas of vital bone in the zoledronic acid, alendronate and control groups. Based on

ANOVA, no significant difference was demonstrated in the positive VEGF staining between the different groups (p=0.860).

Descriptive analysis (H&E)

Osteonecrosis identified on histological examination showed areas of non-vital bone, as well as microbial colonies in the periphery, in medullary spaces and in adjacent areas. Besides lymphocytes and plasma cells, there was infiltrate of polymorphonuclear neutrophils close to the microbial colonies (Figure 2 and Figure 3). Most microbial colonies were morphologically compatible with *Actinomyces* sp. (Figure 4). In the specimens in which there was exodontic wound repair without osteonecrosis, connective tissue and vital bone were observed, and the overlying mucosa exhibited keratinized stratified squamous epithelium (Figure 5).

DISCUSSION

The finding that no animal in the three groups studied exhibited oral mucosa lesions prior to tooth extractions supports the idea that the lesions detected afterward were associated with the surgical procedure and with the use of bisphosphonates. Macroscopic examination after euthanasia revealed that all animals in the zoledronic acid group exhibited loss of mucosal integrity, while in the alendronate and control groups, this frequency was respectively 8 of 11 and 1 of 10 animals. Our data shows that 8 of 10 animals in the zoledronic acid group exhibited osteonecrosis on histological examination, and this might be well correlated with the loss of mucosal integrity. Accordingly, the mucosa is incapable of epithelization and of uniting the edges of the wound in the areas of osteonecrosis.[23] On the other hand, in the alendronate group, in which 8 animals showed

loss of mucosal integrity by macroscopic examination, no animal exhibited osteonecrosis by microscopy. Neither this group showed a greater frequency of root fragments compared to the other groups. Uncommon adverse effects such as damage to the gastric mucosa have been reported for nitrogen-containing bisphosphonates.[11,24,25,26] However, the most important aspect to be considered is the fact that these compounds likely inhibit keratinocyte growth, which is involved in the healing of the oral mucosa as pointed out by Landesberg et al.[23] Such inhibitory effect could play a significant role in the initiation of osteonecrosis in the jaws. In fact, it is not well elucidated if the lesion begins in the oral mucosa or derives from the subjacent bone. In this regard, Landesberg et al.[23] demonstrated that therapeutic doses of pamidronate are toxic to epithelial cells *in vitro*. This drug caused the detachment of the epithelial cells, an event that indicates the possible induction of necrosis.[23] Interestingly, the possible damage to oral mucosa cells despite the absence of osteonecrosis is an issue that deserves to be investigated in further *in vivo* studies.

Osteonecrosis was the focus of the present study and was demonstrated on microscopic examination in 80% of the animals in the zoledronic acid group and in none of the animals in the alendronate and control groups. Such findings confirm the association between osteonecrosis and the use of zoledronic acid, already shown in clinical studies.[5,7,10-12] Also, from our data, it was evident that the use of the drug combined with trauma caused by exodontia constitutes a sufficient condition for the occurrence of osteonecrosis, without any additional related risk factor or co-morbidity as suggested by some previous studies.[9,12,14]

In the alendronate group, none of the animals showed osteonecrosis, despite the presence of root fragments, inflammatory process, microbial colonies and loss of mucosal integrity. Still, it is necessary to consider some important factors, since there are reports in

the literature of patients who developed osteonecrosis of the jaws with the use of alendronate.[5,7,10-12,27] The lower absorption of the drug with oral administration[28,29] can account for our findings in rats. Besides, the presence of two nitrogen atoms in a heterocyclic ring makes zoledronic acid more potent than alendronate.[30] Thus, it exerts earlier effects on bone tissue.[31] Therefore, our results confirm and extend previous data showing that intravenous administration of nitrogen-containing bisphosphonates represents a greater risk of osteonecrosis than oral administration.[12,13]

The factors duration of use and dose of alendronate could be related to the absence of osteonecrosis in this group. It was previously shown in clinical studies that periods of up to 3 years of alendronate use were not associated with lesions, and that there was a significant risk of osteonecrosis after longer exposure to alendronate.[9,32] In the present study, tooth extractions were performed 45 days after the onset of drug administration, and the animals were kept on therapy for 5 months. Perhaps, a more prolonged period of administration of alendronate prior to tooth extractions would have produced different results. Nonetheless, it is worth mentioning that animal toxicology studies with up to one month of drug administration are able to detect 90% of the toxic effects of most drugs,[33] which supports the idea that the time of treatment used in the present study was adequate.

Another aspect to consider is the dose of alendronate used. Each animal was given a dose of 0.05 mg/kg once a week for 23 weeks. On the basis of the equivalent therapeutic dose used in humans, which would be 0.05 mg/kg/day,[17] and also considering the fact that the elimination of the drug is more rapid in rats than in humans,[34] the alendronate dose could have been increased. Moreover, there are authors who defend the use of higher doses of alendronate in animal models.[35] Actually, it is possible that higher doses are capable of inducing osteonecrosis, as well as other types of lesions. However, the dose chosen in the

present work was based on the therapeutic dose prescribed for humans and adjusted according to rodents' metabolic rates[17] and to the longer period of treatment (150 days). According to the literature,[9,32] the potential of alendronate to induce osteonecrosis is related to the length of treatment. In fact, an important feature of our study was the longer period of time during which the animals were kept on bisphosphonate therapy. As alendronate can induce serious adverse effects such as gastric ulcerations, and considering the relevance of treament duration in the development of osteonecrosis, we opted for a lower dose, but keeping the animals under treatment for a longer time. Since alendronate is not metabolized and because it accumulates in the bone, the long-term treatment (150 days) supported the idea that the cumulative dose would be sufficient. However, as alendronate did not induce osteonecrosis, we are conducting new studies using higher doses of this drug, in order to clarify this point.

It is suspected that smoking,[32] chronic use of alcohol,[32,36] diabetes mellitus,[11] hypoproteinemia,[4] corticotherapy, chemotherapy,[6,12,14,15,37,38] immunosuppressive therapy,[12,36] endodontic lesions, periodontal disease, abscesses,[9] poor oral hygiene,[32] and renal insufficiency[27] are co-factors in the development of osteonecrosis by bisphosphonates. Therefore, it is necessary to consider that in the case of alendronate, one or more co-factors could exert an essential role in the development of osteonecrosis. Studies to assess this possibility are currently important, since the monitoring of co-factors that can be controlled could be a form of preventing lesions, or co-factors could constitute a contra-indication for the use of bisphosphonates.

The high frequency of microorganisms in the zoledronic acid group probably resulted from osteonecrosis, which occurred in this group. The alendronate group did not

significantly differ from the control group regarding the occurrence of microbial colonies. Nevertheless, alendronate group showed a high frequency of animals without microbial colonies. Maybe if either a larger sample size or other methods of analysis such as culture protocols or PCR procedures had been employed, this result would not have been obtained. Anyway, it is also reasonable to consider that some aspects of the mechanism of action of bisphosphonates remain to be elucidated,[7,39] including the reports of their effects on some protozoans[40] and bacterial enzymes.[41]

In the present work, there was no a significant difference in the immunohistochemical expression of VEGF among zoledronic acid, alendronate and control groups. This result suggests that the drugs evaluated did not cause inhibition of angiogenesis and that this was not a factor associated with osteonecrosis observed in the animals of the zoledronic acid group. Such findings are not in complete agreement with reports in the literature. Although the mechanism of action of bisphosphonates is still poorly understood,[7,39] some studies have demonstrated their inhibitory effect on angiogenesis.[31,42,43] Zoledronic acid is able to inhibit the proliferation of human endothelial cells[31] as well as to inducing apoptosis and diminishing the formation of capillary tubes *in vitro*.[42] Alendronate was found to be able to reduce intratumor neoangiogenesis, although it did not significantly alter the immunohistochemical expression of VEGF, an effect that results, in part, from the direct antiangiogenic action on intratumor endothelial cells by inhibition of geranylgeranylation of Rho.[44] This finding could explain the lack of difference in the immunohistochemical expression of VEGF between the alendronate and control groups. However, it does not explain the same finding in the zoledronic acid group.

The microscopic field chosen for VEGF evaluation corresponded to vital bone in the region of tooth extraction, independent on the occurrence of osteonecrosis. Such choice

was based on the fact that bisphosphonates have an affinity for bone tissue at sites of active metabolism.[45] In addition, VEGF increases with hypoxia, and angiogenesis occurs in specific physiological situations.[46] The healing of exodontic wounds involves a process of high metabolic activity,[47,48] which suggests that it requires increased angiogenesis. Maybe, it would have been more useful to determine the expression of VEGF in connective tissue or even in the serum of these animals, as performed in other studies.[49,50] Knowing that there are greater metabolic requirements in the initial phase of the wound healing process,[47,48] it is also necessary to consider if the evaluation of VEGF should have been done in an earlier period, soon after tooth extractions.

The present study was conducted in Wistar rats, and it is presumed that the response of these animal models, barring inherent restrictions to peculiarities of the species, simulates the situation in humans. In the animal model used, no difference in the immunohistochemical expression of VEGF was observed in vital bone among the groups evaluated. This result is in accordance with Landesberg et al.[23] who argued that the antiangiogenic effect of bisphosphonates *in vivo* was not yet proven. Pampu et al.,[51] in turn, observed an increase and not a decrease in blood vessels in the bone tissue of rabbits treated with zoledronic acid, in a histomorphometric study with H&E staining.

There are about 40 endogenous inducers and inhibitors of angiogenesis,[52] but VEGF is the predominant pro-angiogenic factor.[53] Studies have demonstrated that neoformed endothelial cells of vessels are strongly dependent on VEGF expression, but not pre-existing cells in tumors.[54] Since bisphosphonates are indicated as inhibitors of angiogenesis, the evaluation of VEGF expression was considered relevant to the present study. On the other hand, it would also be important to analyze the expression of other markers of angiogenesis, including using other methods besides immunohistochemistry.

We have already made a comment about the dose of alendronate used in this study, but it is also worth pointing out some considerations regarding the zoledronic acid dose. In clinical practice this drug is commonly administered by intravenous route, but here, the animals were treated by intraperitoneal injection, which is safer and easier to manage. In general, this route results in plasma concentrations lower than the intravenous one. Hence, the dose administered was stated as 0.6 mg/kg/28 days. There are many reported studies using intraperitoneal zoledronic acid, where doses vary greatly among them. In the study of Ottewell et al.,[55] rats were given zoledronic acid at 0.1 mg/kg/week, by the intraperitoneal route, for 6 weeks. According to these authors, this dose is equivalent to the human dose of 4 mg. Although the 0.6 mg/kg dose may be considered somewhat high, it should be noted that it was administered monthly, and its safety is supported by *in vivo* preclinical studies.[18] Accordingly, zoledronic acid does not cause any significant effect on the urinary excretion rate or electrolyte secretion in rats at intravenous doses up to 1 mg/kg.

Bisphosphonates are effective to treat pathological conditions whose main characteristic is increased bone resorption,[2,3] but at the same time, their use is accompanied by the risk of jaw osteonecrosis. The intensification of research to find out answers to many aspects that remain obscure would help the proper use of these drugs.

CONCLUSIONS

The administration of the nitrogen-containing bisphosphonate zoledronic acid by the parenteral route is associated with maxillary osteonecrosis and is a condition sufficient for this to occur in rats submitted to tooth extractions. The nitrogen-containing bisphosphonate alendronate, administered orally, does not produce a condition sufficient

for the occurrence of maxillary osteonecrosis in rats submitted to tooth extractions. The administration of nitrogen-containing bisphosphonates alendronate or zoledronic acid is not associated with decreased immunohistochemical expression of VEGF in vital bone tissue in the area of tooth extractions.

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FIGURE LEGENDS

FIGURE 1. Quantification of histological features in one of the 4 fields examined at the site of tooth extraction using Image Proplus 6.0 software (Media Cybernetics, Silver Spring, USA) (H&E, x5 objective).

FIGURE. 2. Osteonecrosis, microbial colonies and inflammatory infiltrate (H&E, x100).

FIGURE 3. Osteonecrosis: non-vital bone and microbial colonies (H&E, x200).

FIGURE 4. Microbial colonies compatible with *Actinomyces* sp. and inflammatory infiltrate (H&E, x400).

FIGURE 5. Wound repair: vital bone, connective tissue and keratinized stratified squamous epithelium (H&E, x100).

(The figures are not available in this version).

Table 1. Sample distribution according to presence/absence of loss of mucosal integrity on macroscopic examination (tooth extraction site)

Group	Loss of mucosal integrity			
	Present		Absent	
	No. of animals	%	No. of animals	%
Zoledronic acid	**10***	100.0	0	0.0
Alendronate	8	72.7	3	27.3
Control	1	10.0	**9***	90.0

Chi-squared test, χ^2=18.01; p<0.001
*Analysis of adjusted residuals: p<0.05

Table 2. Sample distribution according to presence/absence of histological features evaluated at tooth extraction site

Histological features	Group												*p*
	Zoledronic acid (n=10)				Alendronate (n=11)				Control (n=10)				
	Present		Absent		Present		Absent		Present		Absent		
	n	%	n	%	n	%	n	%	n	%	n	%	
Osteonecrosis	**8***	80	2	20	0	0	**11***	100	0	0	**10***	100	<0.001
Inflammatory infiltrate	10	100	0	0	7	63.6	**4***	36.4	10	100	0	0	0.015
Microbial colonies	**10***	100	0	0	4	36.4	**7***	63.6	5	50	5	50	0.008
Root fragments	6	60	4	40	6	54.5	5	45.5	**10***	100	0	0	0.047
Connective tissue	10	100	0	0	11	100	0	0	10	100	0	0	-
Epithelial tissue	10	100	0	0	11	100	0	0	10	100	0	0	-
Vital bone	10	100	0	0	11	100	0	0	10	100	0	0	-

p= minimum level of significance of chi-squared test (χ^2)
*Analysis of adjusted residuals: p<0.05
Abreviation: n= number of animals

Table 3. Quantification of histological features (H&E) at tooth extraction site in zoledronic acid, alendronate and control groups

Histological features	Zoledronic acid (%)			Alendronate (%)			Control (%)		
	Mean	SD	Median	Mean	SD	Median	Mean	SD	Median
Osteonecrosis	10.96	11.47	**7.8***	0	0	0	0	0	0
Inflammatory infiltrate	4.34	4.12	**2.5***	1.27	2.11	0.7	1.18	1.78	0.6
Microbial colonies	4.82	7.60	**1.4***	0.28	0.75	0	0.26	0.54	0.1
Epithelial tissue	13.07	5.01	11.3	16.88	6.05	15.3	15.30	6.32	13.2
Connective tissue	30.77	5.90	31	37.68	9.36	37.9	36.70	7.67	36.3
Root fragments	4.33	7.10	3.4	3.43	5.48	3.1	4.72	6.42	4.6
Vital bone	31.68	16.41	36.8	40.42	13.12	41.2	41.81	11.30	42.9

Abreviation: SD= Standard deviation; H&E= Hematoxylin and eosin
*Kruskal-Wallis test complemented by multiple comparisons test, $p<0.05$

Table 4. Immunohistochemical expression of VEGF at vital bone site in zoledronic acid, alendronate and control groups

Group	n	Immunohistochemical Expression of VEGF (%)			
		Mean	SD	Minimum	Maximum
Zoledronic acid	10	21.79	5.84	11.59	29.90
Alendronate	11	22.36	6.27	9.83	34.60
Control	10	23.28	6.22	15.84	32.99

ANOVA, $P=0.860$
Abreviation: VEGF= vascular endothelial growth factor; SD= Standard deviation; n= sample size