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Effect of tyrosine kinase inhibitor *sunitinib* on tissue repair at tooth extraction sites

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Abstract

Background: Both zoledronic acid, a potent bisphosphonate, and the antiangiogenic drug sunitinib are included in anticancer protocols and have also been associated with jaw osteonecrosis. Our aim was to compare the effect of these drugs on tissue repair at tooth extraction sites.

Methods: Wistar rats were allocated into four groups: (1) sunitinib; (2) sunitinib/zoledronic acid; (3) zoledronic acid; (4) control group. The animals underwent tooth extractions and maxillae were macro- and microscopically analyzed.

Results: On macroscopic evaluation, the zoledronic acid group showed a significantly higher frequency of oral mucosal lesion; lesions in the sunitinib/zoledronic acid group were larger, albeit not significantly so. The sunitinib/zoledronic acid group had significantly less epithelium than the zoledronic acid and control group, but showed no significant difference compared to the sunitinib group. The sunitinib/zoledronic acid and zoledronic acid groups did not differ from each other, but had significantly less connective tissue and more non-vital bone and microbial colonies than sunitinib and control groups, whereas these latter two groups did not significantly differ from each other. Vital bone and inflammatory infiltrate did not significantly differ between groups.

Conclusion: Sunitinib alone is not associated with non-vital bone, whereas the sunitinib/zoledronic acid combination and zoledronic acid alone are.

KEYWORDS
bisphosphonates, osteonecrosis, sunitinib, tooth extractions, wound healing

1 | INTRODUCTION

Medication-related osteonecrosis of the jaw (MRONJ) is an adverse effect that has been reported in cancer patients subjected to different anticancer drug therapies (Yarom et al., 2019). The first cases of MRONJ were related to bisphosphonates in 2003 (Marx, 2003), and since then, other anticancer drugs have been associated with the disease (Toriumi et al., 2020; Troeltzsch et al., 2012; Zhang et al., 2016). Local factors play a significant role in MRONJ etiology, where tooth extraction is a major one, with 45% to 61% of patients reporting this intervention before lesion onset (Fehm et al., 2009; McGowan et al., 2018; Vahtsevanos et al., 2009).

Currently, three groups of drugs are known to be MRONJ-related: bisphosphonates, denosumab and antiangiogenics (Fusco et al., 2020; Ramírez et al., 2015). The main action of antiangiogenics is the inhibition of vascular endothelial growth factor (VEGF), which is expressed in the majority of malignant tumors (Lugano et al., 2020), and tumor neoangiogenesis is thereby suppressed. This group comprises bevacizumab, sunitinib, cabozantinib, everolimus, lenalidomide, pazopanib, ramucirumab, sorafenib,

aflibercept, thalidomide and sirolimus. Sunitinib is a receptor tyrosine kinase (RTK) inhibitor launched on the market in 2006 (Pfizer, New York, NY, USA; Schmid & Gore, 2016). RTK inhibition blocks multiple targets including VEGFR-1, VEGFR-2, fetal liver tyrosine kinase 3 (FLT3), PDGFRα and PDGFRβ in cellular and biochemical assays, which in turn, inhibits cell proliferation, migration and differentiation and neoangiogenesis and cancer cell invasion (Lugano et al., 2020; Oudard et al., 2011). Sunitinib has been indicated in the treatment of stromal gastrointestinal carcinoma, metastatic renal cell cancer and pancreatic neuroendocrine tumor (Oudard et al., 2011).

The risk of MRONJ increases for patients being treated with sunitinib combined with intravenous bisphosphonate, showing a prevalence of 0.9% to 2.4% (Ramírez et al., 2015). This happens because VEGFR inactivation and consequent angiogenesis blockade impairs tissue healing (Hoefert & Eufinger, 2010), hampering bone healing and remodeling (Gordon et al., 2009). Several reports in the literature corroborate the notion of increased risk of MRONJ in such patients (Beuselinck et al., 2012; Bozas et al., 2010; Brunello et al., 2009; Hoefert & Eufinger, 2010; Ramírez et al., 2015; Toriumi et al., 2020). Some authors, (Abel Mahedi Mohamed et al., 2018; Fleissig et al., 2012; Koch et al., 2011; Melloni et al., 2016) in turn, reported cases of patients undergoing only sunitinib therapy, who developed MRONJ after tooth extraction. These authors pointed to sunitinib as a possible causative factor of MRONJ, even when used in single drug therapy. Accordingly, Abuohashish et al., 2019 reported a marked decrease in bone volumetric mass in healing extraction sockets in rabbits after angiogenesis suppression by bevacizumab therapy. Nevertheless, in the literature, cases of zoledronic acid-induced MRONJ are much more reported than those related to antiangiogenics (Schiodt et al., 2018; Toriumi et al., 2020). Overall, most reported patients who had sunitinib-induced osteonecrosis had a previous or current history of zoledronic acid therapy, either simultaneously with sunitinib or not. Moreover, in some reports, it is not clear whether the patient really did not previously use bisphosphonate. That is, the literature does not seem to be definitive about sunitinib being an independent MRONJ-associated factor.

It is known that tissue wound healing demands high metabolism, with increased rates of angiogenesis, which would support the association of antiangiogenics with MRONJ. Anyway, considering that the role of these drugs as an independent factor for MRONJ development still has some obscure points, and also that tooth extraction is one of the major risk factors for this disease, the aim of the present study was to investigate the effect of sunitinib on tissue repair at tooth extraction sites in animal models, comparing it with the action of zoledronic acid alone or in combination with sunitinib.

2 | MATERIAL AND METHODS

All the procedures 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; Percie du Sert et al., 2020). The present study was approved by the Ethics Committee on Animal Use of Pontifical Catholic University of Rio Grande do Sul (CEUA-PUCRS) under protocol #8305. The sample was composed of 52 female rats (*Rattus novergicus*, Wistar strain) from the Central Facility (CEMBE/PUCRS), with age ranging from 65 to 75 days and weight ranging from 230 to 250 g. The calculation of the sample size, with a margin of error of 1%, significance level of 5% and power of 80%, based on Maahs et al., 2011, indicated the need for 11 rats per group (software WinPepi, version 11.28). This number was increased by 2 per group (20%) considering possible losses during the experiment period.

The animals were kept in microisolator cages with controlled temperature (23 ± 1°C) and 12-h light–dark cycle, with lighting of 300 lux in the center of the room and 60 lux inside the cages. The cages were cleaned and exchanged according to the facility center protocol, and feed (Nuvilab, Colombo, PR, Brazil) and filtered water were provided *ad libitum*. The animals were randomly allocated into 4 groups: (1) 13 animals that were given sunitinib (SU11248; Sutent; Pfizer, Inc., New York, NY, USA); (2) 13 animals that were given sunitinib and zoledronic acid (Novartis Pharma, Basel, Switzerland); (3) 13 animals that were given zoledronic acid; and (4) control group: 13 animals with no drug. The first administration of both drugs was carried out at the beginning of the experiment, after labeling and weighing of the animals. Sunitinib was administered by gavage at a dose of 6 mg/kg/day for 35 days, and zoledronic acid was administered by the intraperitoneal route (IP) at a dose of 0.3 mg/kg/week for a total of 5 doses. In the control group, 6 rats received IP saline at the amount of 1 ml/kg/week, and 7 rats received filtered water, 1 ml/kg/day by gavage. The animals were weighed every 7 days to adjust the doses.

2.1 | Tooth extractions

Tooth extractions were performed 15 days after beginning the experiment, respecting a 3-day wash-out period (48 h before and 24 h after the tooth extractions) for sunitinib. The procedure was performed under deep anesthesia with a mixture of 5% ketamine hydrochloride (70 mg/kg; Syntec, Cotia, SP, Brazil) and 2% xylazine hydrochloride (7 mg/kg; Syntec, Cotia, SP Brazil) administered IP, with the animal in dorsal decubitus (Koth et al., 2020). The right upper molars were extracted using a lever movement with a #3s Hollenback carver (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil) and pediatric forceps (Edlo, Canoas, RS, Brazil) whose functional portion was adapted to the size of the teeth. Right after the tooth extractions, the animals were returned to the cages where they remained on a sterile surgical pad and under controlled body temperature until the anesthetic effect subsided. During the postoperative period, the animals received dipyrone IP at a dose of 200 mg/kg every 24 h for three days, and mashed chow was provided. A total of 5 animals were lost due to complications during the surgical procedure:

2 animals from the sunitinib group, 2 animals from the sunitinib/zoledronic acid group and 1 animal from the zoledronic acid group. Six rats from the sunitinib group and 5 rats from the sunitinib/zoledronic acid group developed skin desquamation and necrosis, as well as edema of the extremities.

2.2 | Euthanasia, macroscopic evaluation, and preparation of the specimens

The animals were sedated by IP administration of 5% ketamine hydrochloride at a dose of 70 mg/kg and 2% xylazine hydrochloride at a dose of 7 mg/kg and subjected to cardiac puncture and exsanguination. After exsanguination, an overdose of the ketamine and xylazine mixture was also administered. After euthanasia, the maxilla was dissected and subjected to macroscopic evaluation to determine the presence/absence of oral mucosal lesion in the area of tooth extractions by using a #5 dental explorer (SSWhite, Duflex, Rio de Janeiro, RJ, Brazil). When present, the lesion was measured with a Williams periodontal probe (SSWhite, Duflex), determining its length and greatest width in millimeters, to calculate the area (mm^2). The observer was blinded to the group examined. The specimens (maxillae) were then fixed for 24 h in 10% buffered formalin. After fixation, the osteotomized segment comprising the tooth extraction area was cut in the middle in a buccal–lingual direction into two pieces, both of them displaying the area of interest at the cut surface.

After decalcification in 10% nitric acid for 8 h, the specimens were washed in tap water and subsequently dehydrated and cleared respectively with sequential ethanol and xylene immersions by using an automated tissue processor (Histocore Pearl Leica, Leica Biosystems, Wetzlar, Germany). Next, the specimens were embedded in paraffin, and 4-µm-thick sections were obtained, processed and stained with hematoxylin and eosin (H&E).

2.3 | Capture of the images and histological analysis

Histological images were captured with an Olympus BX-43 light microscope (Olympus, Tokyo, Japan), connected to a computer with Olympus DP-73 digital camera (Olympus). Five fields of each slide were captured using a 10x objective, so as to include the whole area of the tooth extraction. The images were stored as uncompressed TIFF (tag image file format). The analysis was carried out by using the Image Pro-Plus 5.1 software (Media Cybernetics, Bethesda, MD, USA; Amenábar et al., 2006), where epithelium, connective tissue, vital bone, non-vital bone, inflammatory infiltrate, microbial colonies, and tooth fragment were quantified. Readings were performed by using the manual point-counting technique with a grid of 520 points, which was overlaid on each histological image. The variables were then coded and analyzed, where each point of the grid was counted, determining which histological feature it matched. This procedure was done by clicking the mouse on the desired points, and the information was processed by Image Pro-Plus (Media Cybernetics). The software itself calculated the points counted for each analyzed feature in absolute and relative (%) values (Figure 1; Amenábar et al., 2006; Maahs et al.,

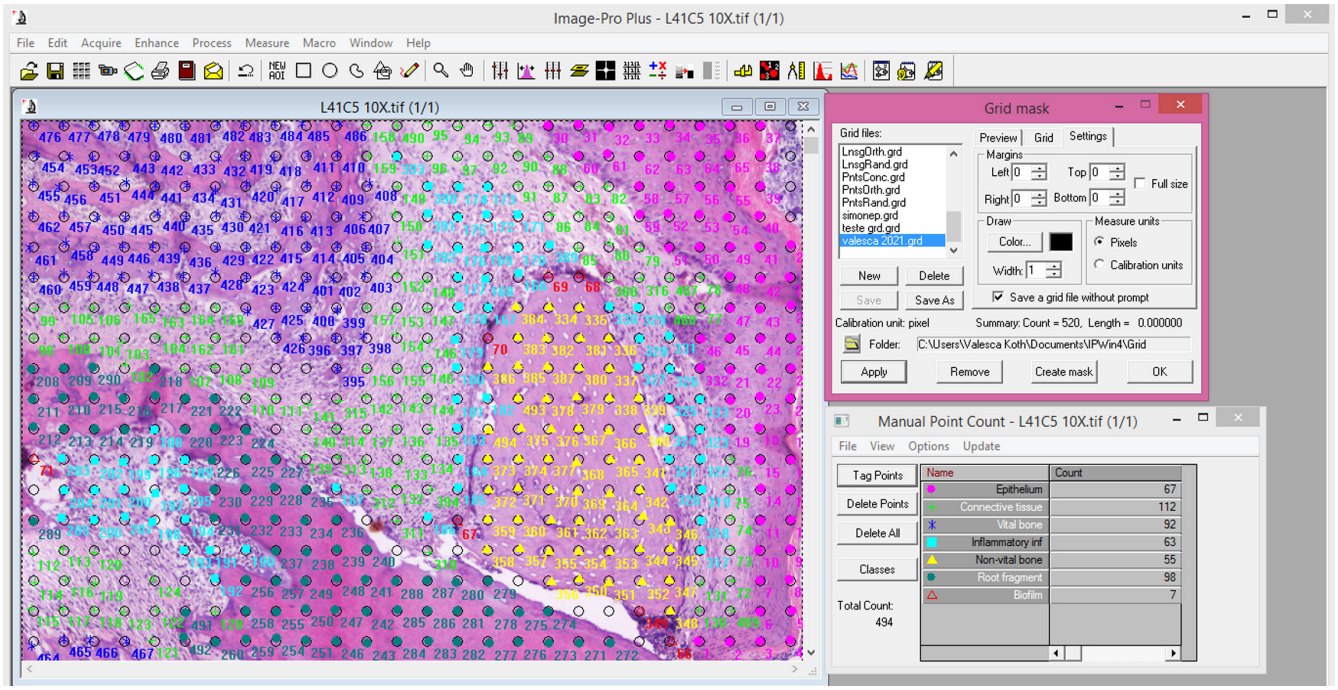

FIGURE 1 Histological analysis by using the manual point-counting technique in the Image Pro-Plus 5.1 software (Media Cybernetics, Bethesda, MD, USA). Each color represents a specific variable

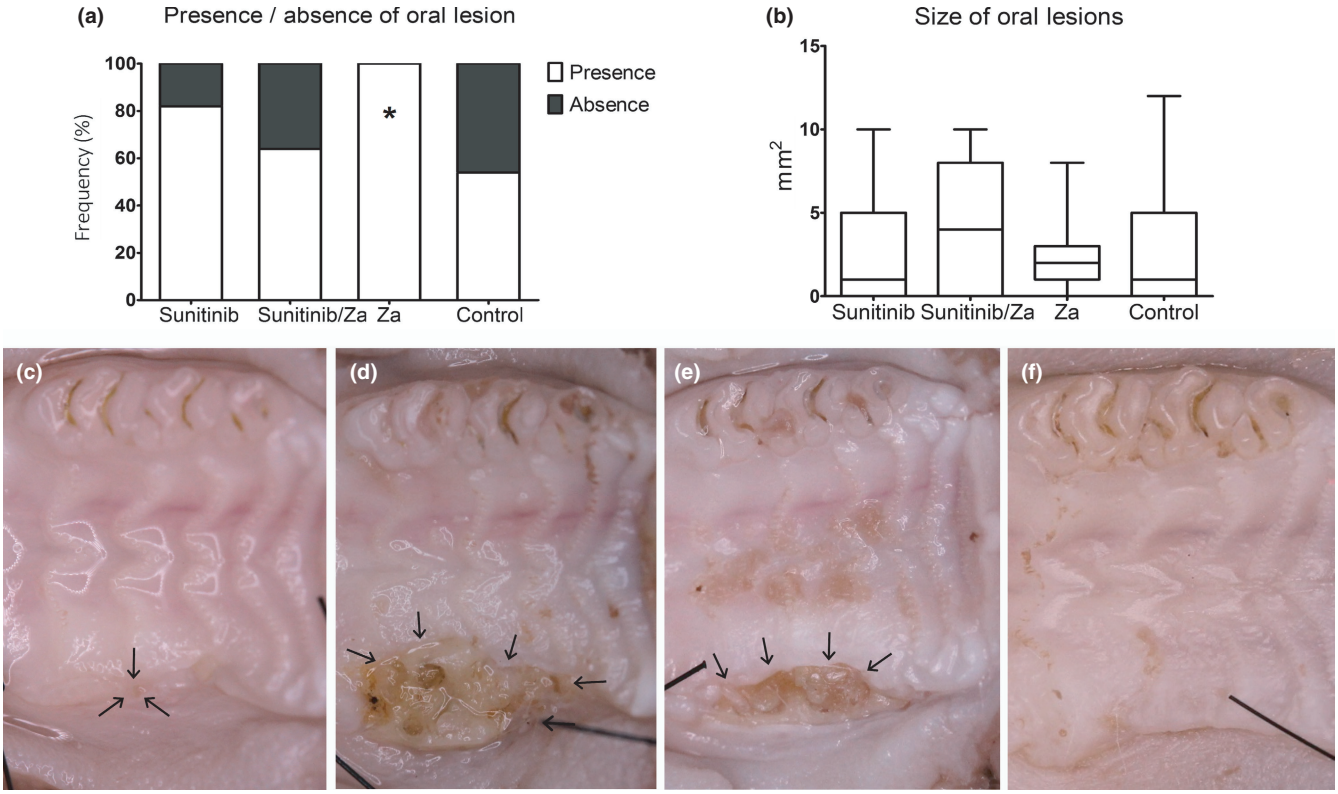

FIGURE 2 Macroscopic analysis. (a) Presence/absence of oral mucosal lesion (Za = zoledronic acid, $^*p < 0.05$, chi-square test, adjusted residual analysis). (b) Size of the oral lesions ($p > 0.05$, Kruskal–Wallis). Arrows indicate the oral lesions in sunitinib group (c); sunitinib/zoledronic acid group (d) and zoledronic acid group (e). f: Absence of oral lesion in the control group

2011). All variables present in the image were consecutively quantified. Bone was classified as vital if there were lacunae filled with osteocytes with or without interposed areas of bone marrow. Non-vital bone criterion was lack of osteocytes (empty lacunae) with or without microbial colonies replacing bone marrow (Berti-Couto et al., 2014). The observer was blinded (not knowing the group to which each image belonged) and calibrated. Calibration consisted of analyzing a series of 35 images, twice, at two different moments. The results of these analyses were tested by intraclass correlation coefficient, which showed $r = 0.9$.

2.4 | Statistical analysis

Data were analyzed with descriptive and inferential statistics. The chi-square test was used to compare oral mucosal lesions and non-vital bone frequencies between the groups. The Kolmogorov–Smirnov test was used to analyze data distribution, which required the Kruskal–Wallis test with the Student–Newman–Keuls post hoc test to compare the size of the oral lesions and the measurement of the histological variables. The Spearman coefficient tested the relationship between the variables. Statistical analysis was performed in SPSS 17.0 (Statistical Product and Service Solutions, SPSS Inc, USA) at a significance level of 5%.

3 | RESULTS

3.1 | Macroscopic analysis

The zoledronic acid group showed a significantly higher frequency of oral mucosal lesion than the other groups ($p = 0.046$). There was no difference in oral mucosal lesion occurrence between the sunitinib, sunitinib/zoledronic acid and control groups (chi-square, adjusted residual analysis, Figure 2a), and there was only a tendency toward greater lesion size in the sunitinib/zoledronic acid group (Kruskal–Wallis, $p = 0.670$, Figure 2b). Figure 2 illustrates the macroscopic analysis according to the group analyzed.

3.2 | Histological analysis

3.2.1 | Frequency of vital and non-vital bone in the sample

Figure 3a displays the sample distribution in the groups according to the presence of vital and non-vital bone. There was no difference between the groups in frequency of vital bone. The sunitinib/zoledronic acid group showed association with non-vital bone, whereas the sunitinib and control groups did not. Although the zoledronic

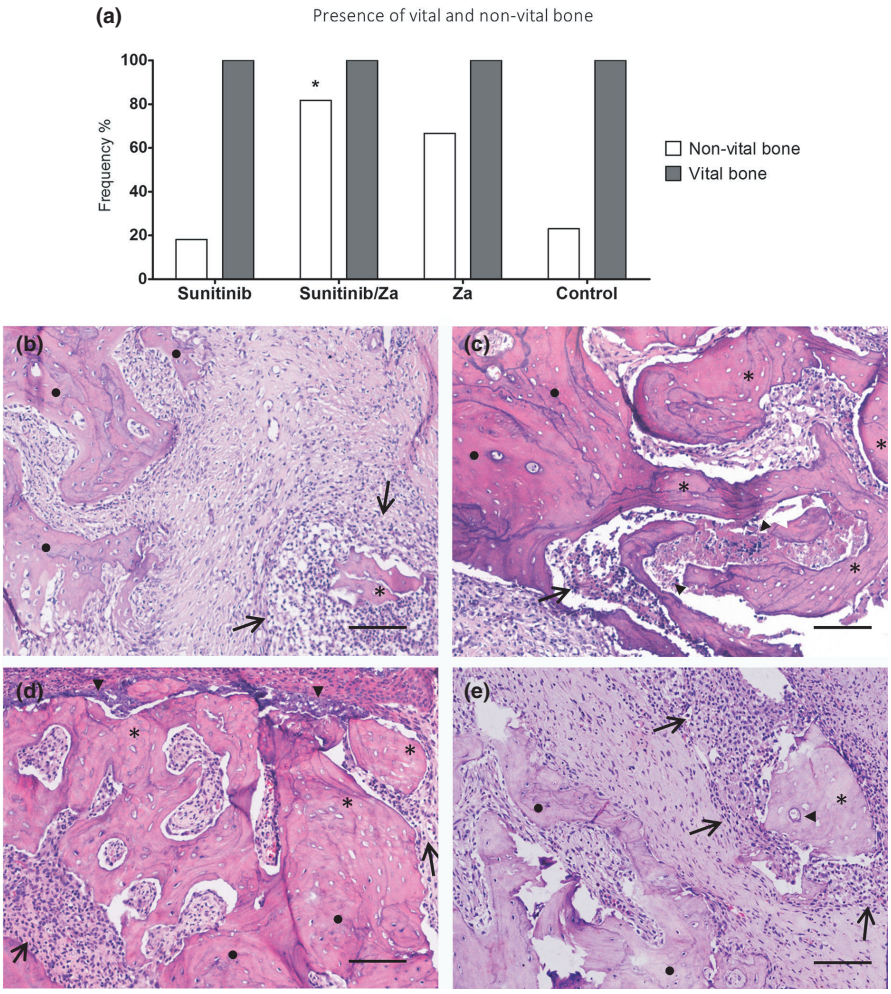

FIGURE 3 (a) Frequency of vital and non-vital bone (*$p < 0.05$, chi-square test, adjusted residual analysis. Za = zoledronic acid); (b) sunitinib group; (c) sunitinib/zoledronic acid group; (d) zoledronic acid group; (e) control group. Inflammatory infiltrate (arrows), microbial colonies (arrow heads); vital bone (●); non-vital bone (*). H&E, scale bar = 100 μm

acid group showed 66.7% of animals with non-vital bone, this was not statistically significant (chi-square, adjusted residual analysis, $\alpha = 0.05$, Figure 3).

3.2.2 | Quantitative analysis of the histological variables

The sunitinib/zoledronic acid group had significantly less epithelium than the zoledronic acid group and the control, but showed no significant difference with regard to the sunitinib group. There was no significant difference in this variable between the other groups. The sunitinib/zoledronic acid and the zoledronic acid groups did not differ from each other, but had significantly less connective tissue and more non-vital bone and microbial colonies than the sunitinib and the control groups, where the latter two groups did not significantly differ from each other with regard to these variables. Vital bone ($p = 0.328$), inflammatory infiltrate ($p = 0.117$) and tooth fragment ($p = 0.309$) did not significantly differ between the groups evaluated (Kruskal–Wallis, Student–Newman–Keuls, $\alpha = 0.05$, Figure 4). Figure 5 illustrates the histological variables according to the group analyzed.

Table 1 displays the values for "r" in correlation analysis between the variables using Spearman coefficient. Epithelium was negatively correlated with tooth fragment ($r = -0.423$); connective tissue was negatively correlated with vital bone ($r = -0.407$), non-vital bone ($r = -0.537$), inflammatory infiltrate ($r = -0.417$), and microbial colonies ($r = -0.387$); vital bone was negatively correlated with inflammatory infiltrate ($r = -0.454$). Non-vital bone was positively correlated with inflammatory infiltrate ($r = 0.523$) and with microbial colonies ($r = 0.603$). Inflammatory infiltrate was positively correlated with microbial colonies ($r = 0.401$).

4 | DISCUSSION

The zoledronic acid group was the only one that showed an association with oral mucosal lesion on macroscopic analysis, whereas lesion frequency in the sunitinib groups did not significantly differ compared to control. The odd finding here was that the sunitinib/zoledronic acid group had no association with lesion, where it was expected to have at least the same rate as the zoledronic acid group. However, it is important to point out that since the control group had similar results as the experimental ones, it is more plausible that

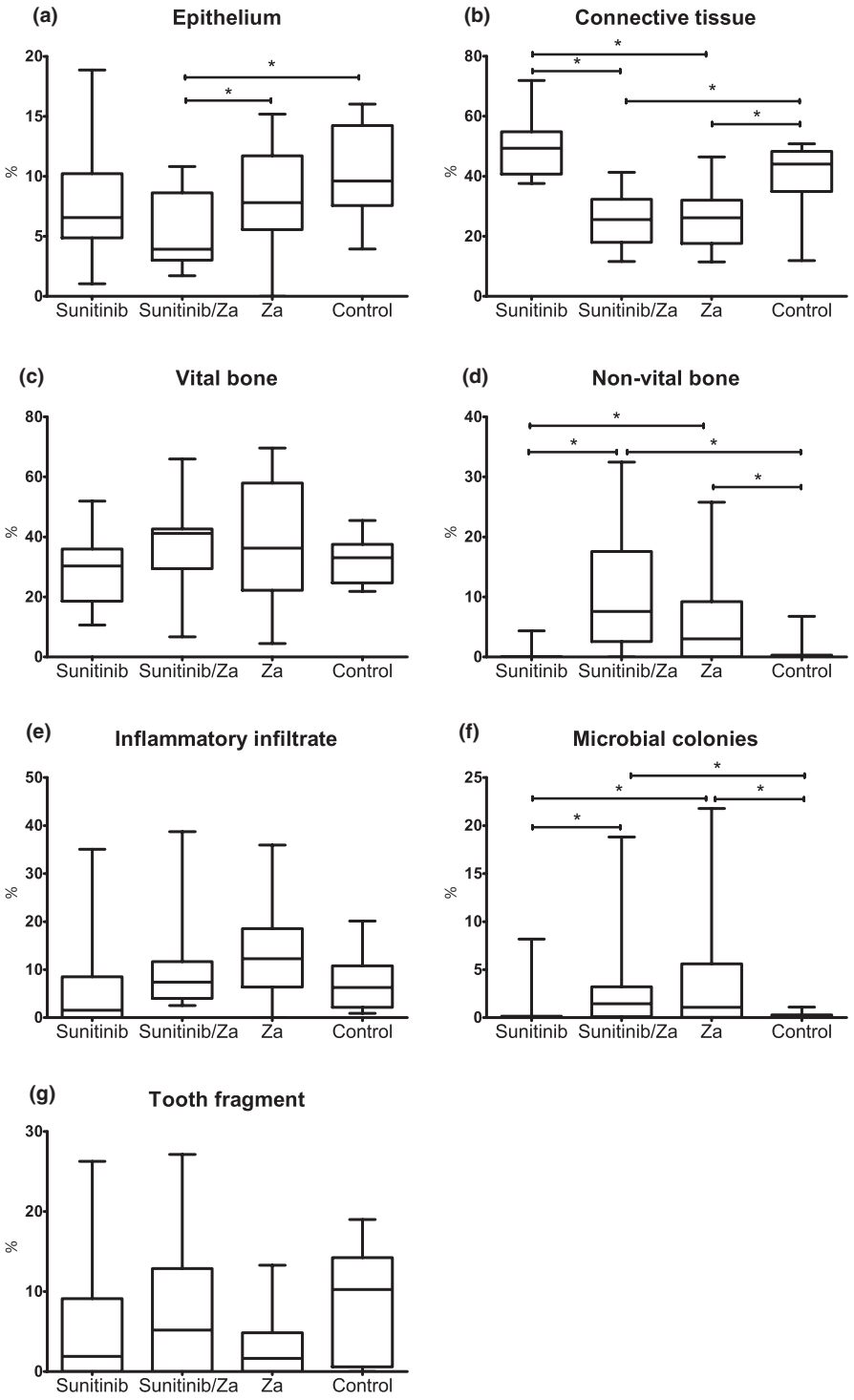

FIGURE 4 Quantitative analysis of the histological variables according to the group: epithelium (a), connective tissue (b), vital bone (c), non-vital bone (d), inflammatory infiltrate (e), microbial colonies (f) and tooth fragment (g). *$p < 0.05$, Kruskal–Wallis, Student–Newman–Keuls. Za = zoledronic acid group

some of the macroscopic lesions could have resulted from the tooth fragments persisting at the extraction site and not as a consequence of the drug used, which agrees with the results for this variable in the histological analysis. These facts reinforce the great importance of microscopic evaluation.

Considering the frequency of animals having non-vital bone on microscopic examination, the groups treated with zoledronic acid whether or not in combination with sunitinib showed the highest prevalence, although only the sunitinib/zoledronic acid group showed a statistically significant difference. This finding indicated that sunitinib could potentiate the effect of zoledronic acid, whereas sunitinib alone would not be capable of causing the lesion. Another point to consider is that this was a dichotomous analysis in a relatively small sample, where non-vital bone criterion was bone tissue with empty lacunae (with no osteocytes; Bacci et al., 2011; Nicolatou-Galitis et al., 2020; Tresguerres et al., 2020) with or without microbial colonies replacing bone marrow. This analysis did not take into account the extent of this and the other features usually observed in MRONJ lesions, such as microbial colonies and inflammatory infiltrate (Boff et al., 2014; Shuster et al., 2019). We know that empty

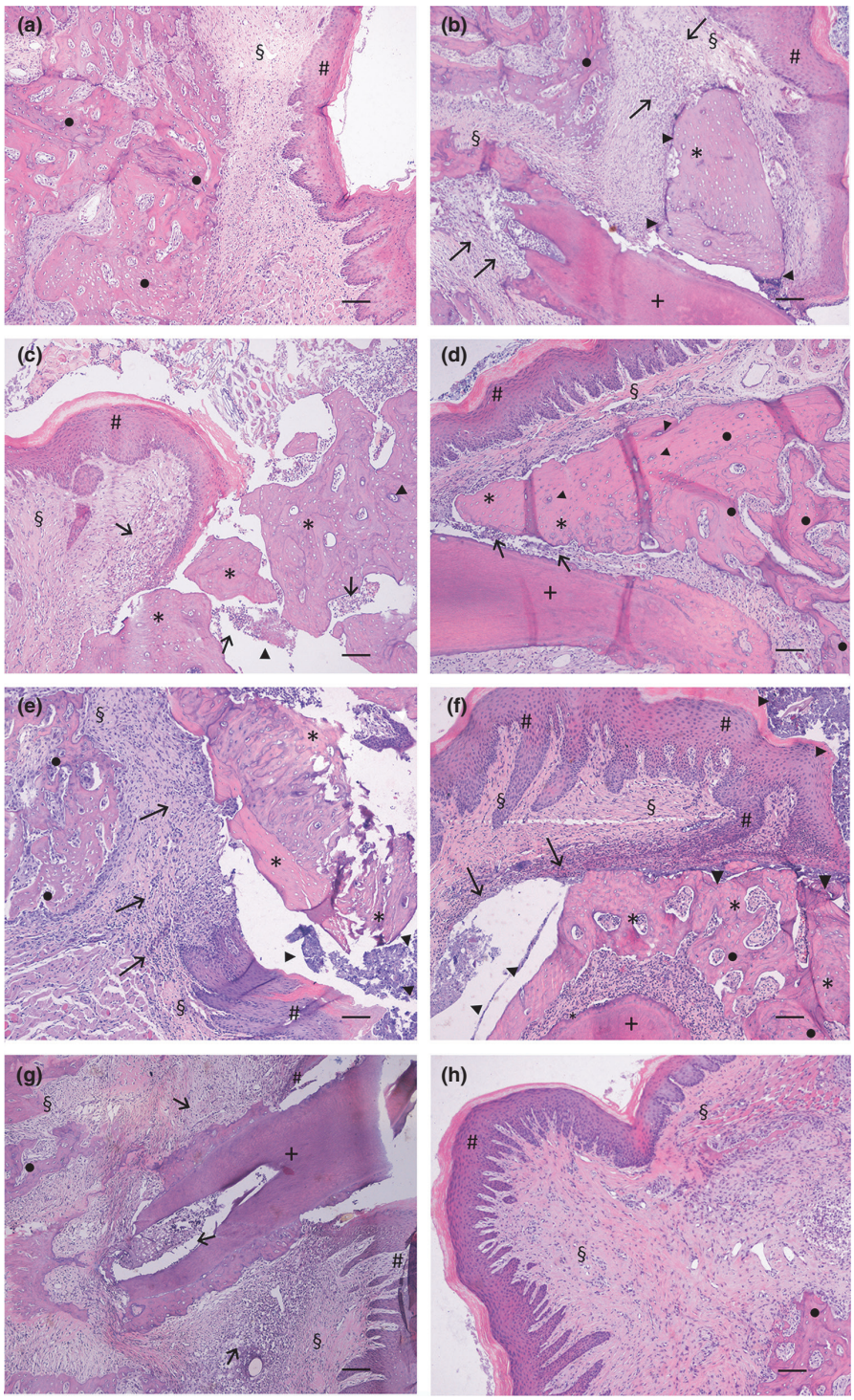

FIGURE 5 Representative images of the histological analysis (H&E, scale bar = 100 μm). (a, b) sunitinib group; (c, d) sunitinib/zoledronic acid group; (e, f) zoledronic acid group; (g, h) control group. Epithelium (#), connective tissue (§), vital bone (●), non-vital bone (*), inflammatory infiltrate (arrows), microbial colonies (arrow heads), tooth fragment (+). In a, sunitinib group shows complete healing of the surgical wound and in b, tooth fragment and an area of non-vital bone. In c, sunitinib/zoledronic acid group shows non-healing surgical wound with loss of epithelial integrity, adjacent to non-vital bone, and in d, vital bone adjacent to non-vital bone. In e and f, zoledronic acid group shows non-healing surgical wound with microbial colonies surrounding non-vital bone. In g, control group shows loss of epithelial integrity due to tooth fragment, whereas in h, there is complete healing of the surgical wound

lacunae can sometimes be an artifact resulting from the histological process (Schaffler et al., 2014). These factors could impart a bias in this evaluation, and therefore, to have an accurate evaluation, the quantitative analysis of the histological features must be considered.

The sunitinib/zoledronic acid group had significantly less epithelium, which agrees with the results for non-vital bone in this group, since oral mucosa is incapable of re-epithelialization and of uniting the edges of the wound in areas of osteonecrosis (Landesberg et al., 2008; Ravosa et al., 2011). Our findings are also in agreement with the literature, in that the initial damage induced by sunitinib in the oral cavity may affect not only vascular tissue but also keratinocytes (Mignogna et al., 2009). Accordingly, it is believed that oral mucositis caused by sunitinib could progress to osteonecrosis (Hoefert & Eufinger, 2010; Troeltzsch et al., 2012). Connective tissue levels, in turn, were significantly less in the sunitinib/zoledronic acid and zoledronic acid groups and also negatively correlated with non-vital bone, inflammatory infiltrate and microbial colonies, indicating that its lower levels in these groups were a result of the occurrence of osteonecrosis. These same groups (sunitinib/zoledronic acid and zoledronic acid) had significantly greater amounts

TABLE 1 "*r*" values in correlation analysis between the variables using Spearman coefficient

Variable	Epithelium	Connective tissue	Vital bone	Non-vital bone	Inflammatory infiltrate	Microbial colonies	Tooth fragment
Epithelium	1						
Connective tissue	0.231	1					
Vital bone	−0.213	**−0.407***	1				
Non-vital bone	−0.231	**−0.537***	−0.084	1			
Inflammatory infiltrate	0.030	**−0.417***	**−0.454***	**0.523***	1		
Microbial colonies	−0.214	**−0.387***	−0.198	**0.603***	**0.401***	1	
Tooth fragment	**−0.423***	−0.182	0.129	−0.155	−0.073	0.009	1

*Correlation is significant at the 0.01.

of non-vital bone and microbial colonies than did the sunitinib and control groups, where these latter two groups did not significantly differ from each other. This is an important finding, where sunitinib seemed to have detrimental effects on bone repair only when combined with zoledronic acid. On the other hand, zoledronic acid combined or not with sunitinib was capable of impairing the healing of the surgical wound, as previously reported (Kolpakova et al., 2017; Maahs et al., 2011). This would suggest that sunitinib causes non-vital bone only if combined with zoledronic acid, and considering that the sunitinib/zoledronic acid group did not show significantly greater levels of this variable than the zoledronic acid group, sunitinib did not potentiate the effect of zoledronic acid. This is corroborated by the finding that non-vital bone did not differ between the sunitinib group and control.

Our results suggest that the association of MRONJ with antiangiogenics still leaves some doubts, considering that these drugs are often administered in combination with bisphosphonates (Beuselinck et al., 2012) and denosumab (Sivolella et al., 2013), either concomitantly or sequentially. Maybe the growing number of case reports of antiangiogenic-related MRONJ should be critically considered, especially making sure that the patient has not undergone bisphosphonate therapy in preceding years, since this drug (bisphosphonate) has such a long half-life and long-lasting effects over the time elapsed (Cremers et al., 2019; Yarom et al., 2019).

ETHICS

The present study was approved by the Ethics Committee on Animal Use of Pontifical Catholic University of Rio Grande do Sul (CEUA #8305). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

Bruna Ratzkowski: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing-original draft; Writing-review & editing. Valesca Koth: Data curation; Formal analysis; Methodology; Writing-review & editing. Alan Azambuja: Data curation; Methodology; Writing-review & editing. Fernanda Salum: Conceptualization; Methodology; Writing-review & editing. Maria Antonia Figueiredo: Conceptualization; Methodology; Writing-review & editing. Karen Cherubini: Conceptualization; Data curation; Formal analysis; Methodology; Supervision; Writing-original draft; Writing-review & editing.

PEER REVIEW

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