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ORIGINAL ARTICLE

Resveratrol for preventing medication-related osteonecrosis of the jaws in rats

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Abstract

This study evaluated the effect of resveratrol (RES) on the prevention of medication-related osteonecrosis of the jaws (MRONJ) in ovariectomized (OVX) rats treated with zoledronate (ZOL). Fifty rats were distributed in five groups: SHAM ($n = 10$): non-ovariectomy + placebo; OVX ($n = 10$):ovariectomy + placebo; OVX + RES ($n = 10$):ovariectomy + resveratrol; OVX + ZOL ($n = 10$):ovariectomy + placebo + zoledronate; and OVX + RES + ZOL ($n = 10$):ovariectomy + resveratrol + zoledronate. The mandibles left sides were analyzed with micro-CT, histomorphometry, and immunohistochemistry. On the right side, bone markers gene expression was analyzed by qPCR. ZOL increased the percentage of necrotic bone and reduced the neo-formed bone compared to groups not receiving ZOL ($p < 0.05$). RES impacted the tissue healing pattern in OVX + ZOL + RES, reduced inflammatory cell infiltrate, and improved bone formation in the extraction site. Osteoblasts, alkaline phosphatase (ALP)-, and osteocalcin (OCN)-immunoreactive cells were lower in OVX-ZOL than in SHAM, OVX, and OVX-RES. The OXV-ZOL-RES had fewer osteoblasts and ALP- and OCN-cells than the SHAM and OVX-RES. The tartrate-resistant acid phosphatase (TRAP)-positive cells were reduced in the presence of ZOL ($p < 0.05$), while the TRAP mRNA levels increased with ZOL treatment, with or without resveratrol, compared with the other groups ($p < 0.05$). RES alone increased superoxide dismutase levels compared to OVX + ZOL and OVX + ZOL + RES ($p < 0.05$). In conclusion, resveratrol reduced the tissue impairment severity induced by ZOL; however, it could not prevent the occurrence of MRONJ.

KEYWORDS
bisphosphonates, osteonecrosis, osteoporosis, ovariectomy, resveratrol, zoledronic acid

1 | INTRODUCTION

Bisphosphonates have been extensively used for treating bone-related diseases, like osteoporosis or cancer with bone metastasis (Kuźnik et al., 2020). However, they are related to adverse effects such as medication-related osteonecrosis of the jaws (MRONJ) (Ruggiero et al., 2022). MRONJ was defined by the American Association of Oral and Maxillofacial Surgeons as bone exposure or bone that can be probed through an intraoral or extraoral fistula in the maxillofacial region, persisting for longer than 8 weeks in a patient who has been using (or has used in the past) antiresorptive medication alone or combined with immune modulators or antiangiogenic

medications and with no history of radiotherapy in the jaw region or evident metastatic disease to the jaws (Ruggiero et al., 2022). Another definition also describes "a progressive destruction and death of bone that affects the mandible and maxilla of patients exposed to the treatment with medications known to increase the risk of disease, in the absence of a previous radiation treatment, independently from the presence of exposed necrotic bone or bone probing" (Campisi et al., 2020). Its occurrence has been related to many local risk factors related to infection and inflammation, such as alveolar bone surgery, tooth extraction, and dental-periodontal lesions (Campisi et al., 2020). Histologically, MRONJ lesions presented necrotic bone with surrounding residual vital bone, inflammatory cellular elements (especially macrophages), fibrous tissue areas, and osteoclasts (Basso et al., 2013).

Zoledronate (ZOL) is a third-generation nitrogenated bisphosphonate commonly used to treat and prevent osteoporosis due to postmenopausal estrogen deficiency. It is the most potent bisphosphonate, acting as an antiresorptive agent (Reid et al., 2020) and the most frequently associated with MRONJ (Silverman & Landesberg, 2009). ZOL also has an antiangiogenic effect that inhibits endothelial cell proliferation, modulates its adhesion and migration, causes apoptosis, and reduces angiogenesis (Fournier et al., 2002). All these conditions may increase the chance of bacterial tissue colonization and infection at the healing site and bone necrosis (Aghaloo et al., 2011).

There are two modalities of treatment suggested for MRONJ: non-surgical and surgical. The non-surgical treatments include chlorhexidine, antibiotic therapy, and local wound care (Dos Santos Ferreira et al., 2021; Hadaya et al., 2018). The surgical therapy consists of necrotic bone removal (Favia et al., 2018). However, there is no effective curative treatment established for MRONJ (Beth-Tasdogan et al., 2022). The increasing prevalence and high morbidity of these lesions (Dos Santos Ferreira et al., 2021) call attention to the need for new and effective approaches to treating and preventing MRONJ. It would be desirable to use therapies with minimal adverse effects to prevent and treat bone disorders related to systemic medical disorders treatment (such as bone cancer and osteoporosis).

It was hypothesized that resveratrol (3,5,4′-trihydroxystilbene), a plant-derived polyphenol with antioxidant, antitumor, anti-inflammatory, and antiresorptive effects (Bo et al., 2013), could be an interesting alternative to prevent MRONJ occurrence. Resveratrol (RES) can enhance bone repair, upregulation of bone remodeling, and downregulation of osteoclastogenic markers (Casarin et al., 2014; Franck et al., 2018; Molez et al., 2020; Pino et al., 2017; Wong et al., 2020). Additionally, RES can inhibit apoptosis (Kumar et al., 2015), directly affecting osteonecrosis control. RES has modulated the proliferation and differentiation of ZOL-treated osteoblasts and presented a protective effect on those cells in an in vitro study (Borsani et al., 2018). Furthermore, treatment with RES in a steroid-induced osteonecrosis animal study has reduced osteonecrosis by modulating the angiogenic events (Zhai et al., 2016). Regarding bisphosphonate-induced osteonecrosis, RES has reduced the osteonecrosis event in rats treated with alendronate and dexamethasone, and no alteration in newly formed bone was observed between groups (Movahedian Attar et al., 2020). However, little is known about the RES effect of ZOL treatment on bone makers and cellular behaviors.

The antioxidant, anti-inflammatory, antiresorptive effects, and apoptosis control characteristics of resveratrol could be important to prevent and control the occurrence of MRONJ associated with bisphosphonates. Thus, this study aimed to evaluate the potential of resveratrol administration in preventing MRONJ post-exodontia in ovariectomized rats treated with zoledronate.

2 | MATERIALS AND METHODS

2.1 | Animals

Fifty adults female Wistar rats (200–300 g - Butantan Institute, Butantã, São Paulo, Brazil) were used. The rats were acclimatized for 15 days before use, and they were kept in temperature-controlled cages, exposed to a 24-h light–dark cycle of equal time, and had free access to water and food ad libitum (Labina, Purina, Paulínia, São Paulo, Brazil) in the Bioterium of Paulista University. The experimental procedure was approved by the Paulista University Institutional Animal Care and Use Committee (094/16 CEP/ICS/UNIP).

2.2 | Experimental design

2.2.1 | Treatment groups

This research was a controlled, placebo, single-blinded animal study aiming to evaluate the use of RES in the prevention of MRONJ. The experimental design can be observed in Figure 1. The animals were assigned to the following groups: SHAM ($n = 10$): non-ovariectomy surgery + placebo; OVX ($n = 10$): ovariectomy + placebo; OVX + RES ($n = 10$): ovariectomy + resveratrol; OVX + ZOL ($n = 10$): ovariectomy + placebo + zoledronate; and OVX + RES + ZOL ($n = 10$): ovariectomy + resveratrol + zoledronate.

The detailed study protocol, medication, rat osteonecrosis model, and the following analysis are described in Appendix S1, and a brief description is provided below.

The rats underwent ovariectomy to induce estrogen deficiency (Shuai et al., 2015). Bilateral ovariectomies (OVX) were performed in 40 rats, and SHAM surgeries (in which the ovaries were lifted and returned intact to the original position) were performed on the remaining ten rats (Chen et al., 2015).

Resveratrol (10 mg/kg) and placebo were administrated daily by gavage for 140 days (Casati et al., 2013; Corrêa et al., 2018). Zoledronic acid solution (0.1 mg/kg) was prepared using PBS and was administered intraperitoneally twice weekly (Yanık et al., 2016). Administration of RES or placebo was started 84 days before teeth extraction and then for another 56 days following MRONJ induction.

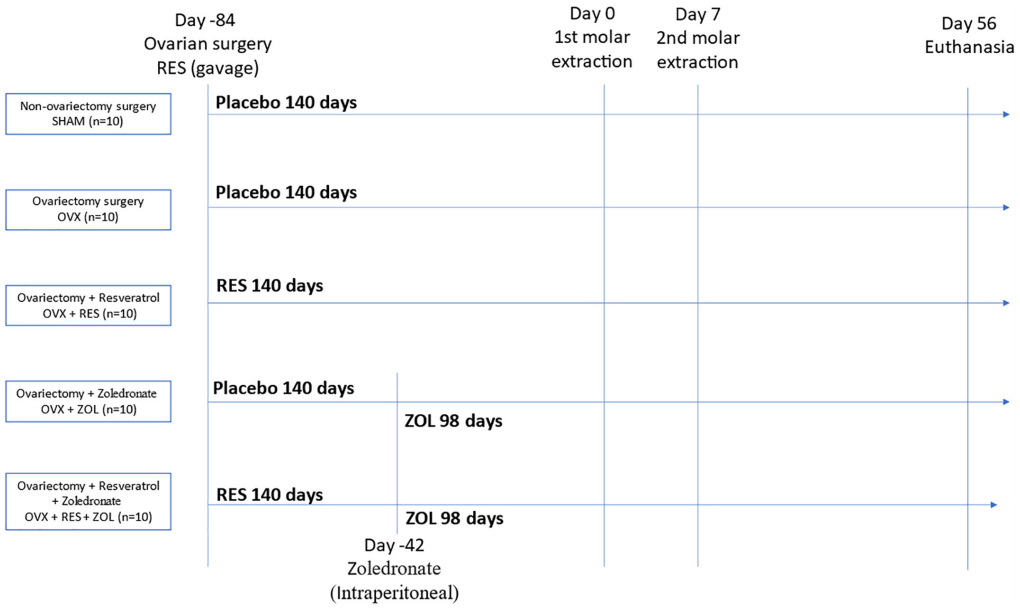


FIGURE 1 Schematic illustration of experimental design.

ZOL treatment started 42 days before teeth extraction and followed 56 days after this procedure.

2.2.2 | Rat osteonecrosis model

The induction of osteonecrosis lesions followed the protocol established by Howie et al., 2015. The first bilateral mandibular molars were extracted at Day 0 using Adson surgical forceps. Seven days later, the second bilateral molars were extracted. At each extraction time, the defects were standardized with a 1.0 mm round bur at 15,000 RPM. The animals were euthanized by CO_2 inhalation 56 days after teeth extraction. The mandibles were excised for clinical-macroscopic evaluation of the extraction sockets healing. The left side of the specimens was processed for micro-Ct, histopathological, histometric, and immunohistochemical analysis. The right side of the specimens was collected for mRNA quantification of bone-related markers.

2.2.3 | Clinical-macroscopic evaluation of extraction sockets healing

After euthanasia, standardized photographs were taken with a 6.1-megapixel digital camera (EOS 40D; Canon, New York, NY, USA) placed on a tripod to keep the camera parallel to the ground at a minimum focal distance, thereby ensuring reproducibility of image acquisition. The specimens were stabilized in wax with their occlusal planes parallel to the ground and long axes perpendicular to the camera. The photographs were analyzed using the post-extraction sites healing degree: 1- closed wound; 2- open wound without bone exposure; 3- open wound with bone exposure (Howie et al., 2015). The measurements were performed after intra-examiner calibration by evaluating ten images not taken for this study. The single-blinded examiner (MV) classified all photographs twice within 24 h (89% intra-class correlation).

2.2.4 | Micro-CT analysis

The specimens were scanned using a cone-beam micro-CT system (SkyScan 1272, Bruker, Kontich, Belgium). The images were reconstructed via the N-RECON v.1.6.3.3 program. After reconstruction, the images were processed by the C-TAN v1.10.11.0 program to obtain the sagittal sections. The DATAVIEWER v1.4.3 program analyzed the bone microarchitecture at the first and second lower molars regions. The three-dimensional specimens' images were obtained and visualized by CT-VOX v2.0.0.0 software. The region of interest (a parallelepiped of 4 mm width, 8 mm length, and 4 mm height) was positioned in the mesial of the third molar at the cementum–enamel junction. The volumetric measurements of this region were performed with the C-TAN program. The architectural parameters evaluated were: bone volume (BV/TV - %), trabeculae thickness (Tb.Th - mm^2), number of trabeculae (Tb.N - mm^2), and separation between trabeculae (Tb.Sp - mm).

2.2.5 | Histological analysis

The histological analysis was performed according to (Toro et al., 2019). Briefly, after Micro-CT acquisition, the identical specimens were histologically (histopathological, histometric, and immunohistochemical) evaluated. The samples were demineralized for 60 days and included in paraffin for conventional histological processing. The microtomy was performed from lingual to vestibular, and the histological sections of the dental socket previously occupied by the mesial and distal roots of the first and second lower

molars were collected. The histological sections of the dental extraction site and adjacencies were stained with hematoxylin–eosin (HE) for the histopathological and histometric analysis.

The histological sections were deparaffinized in xylol for immunohistochemical analysis and hydrated in decreasing ethanol series. The histological slides were prepared for antigen retrieval and immunohistochemical reaction. The slides from each experimental group were divided into three batches incubated with one of the primary antibodies (Santa Cruz Biotechnology®): anti-tartrate-resistant acid phosphatase (TRAP), anti-alkaline phosphatase (ALP), and anti-osteocalcin (OCN). The sections were incubated with biotinylated secondary antibody for 2 h and streptavidin-horseradish peroxidase conjugate for 1 h. A negative control accompanied all samples.

A certified and blinded histologist (EE) performed the histological and histometric analyses. The region of interest (ROI) consisted of a 4 mm × 4 mm area encompassing the portion of the socket previously occupied by the mesial root and the distal root of the second lower molar and its adjacencies.

2.2.6 | Histometric analysis

The images were captured in the ROI using a digital camera (AxioCam®, Carl Zeiss, Gottingen, Germany) coupled to the optical microscope (AxioLab®) and connected to a microcomputer. The total amount of bone tissue was measured by Axiovision 4.8.2® program (Carl Zeiss, Gottingen, Germany), and the percentage of neo-formed bone tissue, percentage of necrotic bone tissue, and the number of osteoblasts per mm of vital bone tissue were calculated. The histometric was performed according to (Toro et al., 2019).

2.2.7 | Immunohistochemical analysis

Images of the histological sections immunolabelled with TRAP, ALP, and OCN were acquired similarly for the histometric analysis. The number of TRAP-, ALP-, and OCN-positive cells per mm^2 of vital bone tissue was expressed as mean ± standard deviation. The immunohistochemical analysis was performed according to (Toro et al., 2019).

2.2.8 | Gene expression of bone and apoptotic markers

Bone tissue from the osteonecrosis lesions was stored in RNAlater® at −70°C. The mRNA of DEAD-box helicase 5 (Ddx5), Tartrate-resistant acid phosphatase 5b (TRACP 5b), osteoprotegerin (OPG), Receptor activator of nuclear factor kappa-B ligand (RANKL), β-catenin, superoxide dismutase 1 (SOD1) e glutathione-disulfide reductase (GSH), were evaluated using qRT-PCR (LightCycler® 96 Instrument, Roche Diagnostics GmbH, Mannheim, Germany), as previously described (Ribeiro et al., 2017).

2.3 | Statistical analysis

A blinded statistician performed statistical analysis using SAS software (Program Release 9.3). The data were examined for normality using the Shapiro–Wilk test, and the data that achieved normality were analyzed using parametric methods. The data were then analyzed by Kruskal–Wallis for comparison between the experimental groups. The percentage of necrotic bone tissue was considered the primary outcome, and a significance level of 5% was adopted for all evaluations.

3 | RESULTS

3.1 | Clinical-macroscopic evaluation of extraction sockets healing

The clinical appearance of extraction socket healing was observed in Figure 2a–e. The percentage of the post-extraction healing degree scores of each group is described in Table 1. No statistical differences were observed in the clinical-macroscopic evaluation of extraction sockets healing ($p > 0.05$).

3.2 | Micro-CT analysis

The microtomographic analysis showed lower bone volume and lower trabeculae thickness in OVX groups compared with the other groups ($p < 0.05$) (Figure 2k,l, respectively). When resveratrol was administered isolated, lower bone volume and trabeculae thickness was observed compared with the groups receiving zoledronate and SHAM ($p < 0.05$) (Figure 2k,l, respectively). Considering the number of trabeculae, the ovariectomized groups that did not receive ZOL presented fewer trabeculae compared with SHAM and the groups receiving zoledronate ($p < 0.05$) (Figure 2m). Regarding the separation between trabeculae, OVX presented higher values than SHAM and the groups receiving zoledronate ($p < 0.05$) (Figure 2n).

3.3 | Histological analysis

3.3.1 | Osteoblasts, neo-formed and necrotic bone tissue

The histomorphometric analysis showed lower percentages of neo-formed bone tissue in OVX + ZOL and OVX + RES + ZOL than

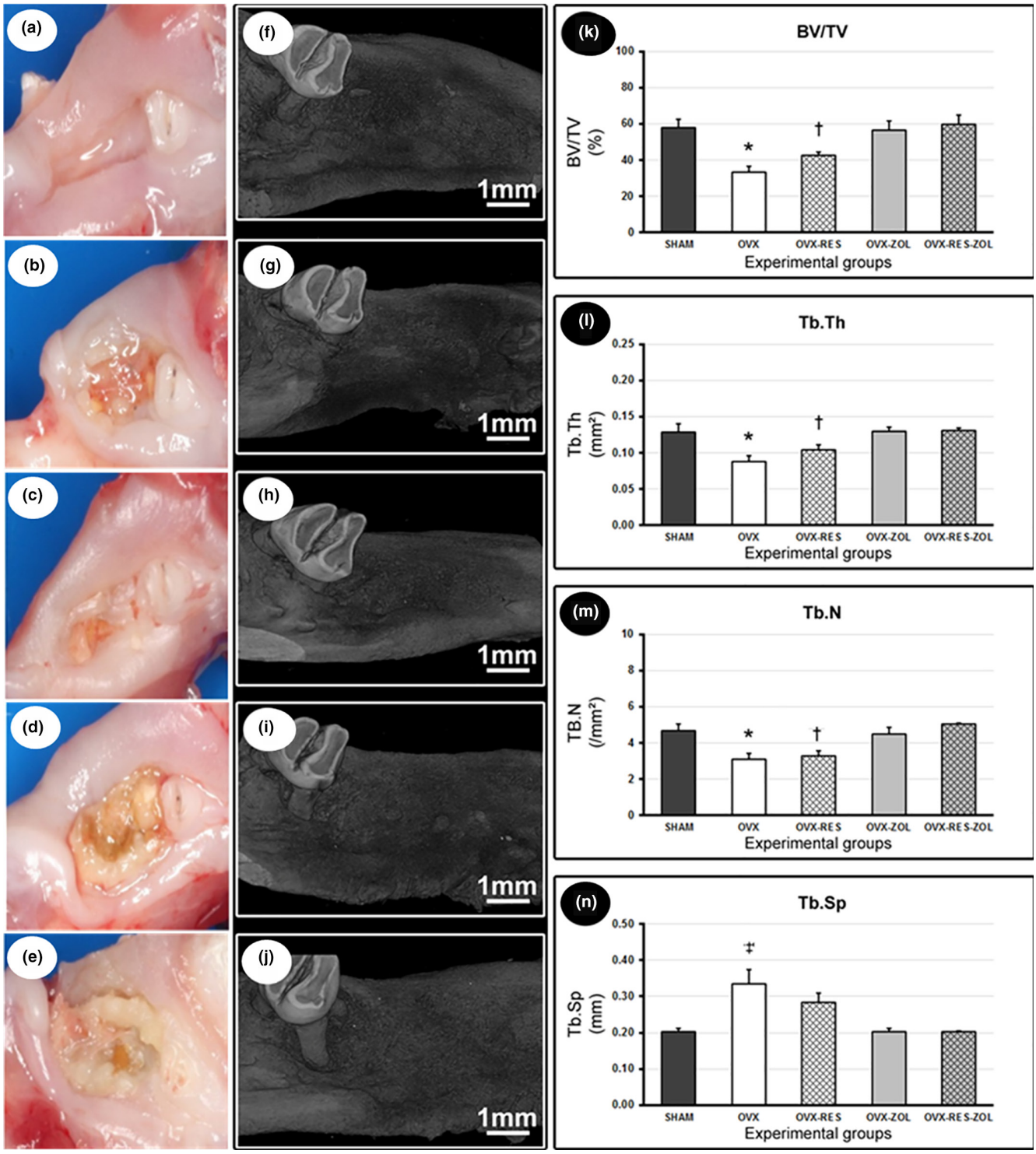


FIGURE 2 Clinical aspect (a–e) and representative micro-CT images (f–j) of each group: SHAM (a and f), OVX (b and g), OVX+RES (c and h), OVX+ZOL (d and i), OVX+ZOL+RES (e and j). Micro-CT analysis is represented by mean ± standard deviation (k–n). *Indicates statistically significant difference to SHAM, OVX+RES, OVX+ZOL, and OVX+ZOL+RES (ANOVA/Tukey – $p<0.05$); †Indicates statistically significant difference to OVX+ZOL, OVX+ZOL+RES and SHAM (ANOVA/Tukey – $p<0.05$); ‡Indicates statistically significant difference to OVX+ZOL+RES and SHAM (Kruskal-Wallis/ Dunn – $p<0.05$).

SHAM, OVX, and OVX+RES ($p<0.05$) (Figure 3a). Regarding the necrotic bone, OVX+ZOL, and OVX+RES+ZOL presented higher percentages compared with SHAM, OVX, and OVX+RES ($p<0.05$) (Figure 3b).

The number of osteoblasts in OVX+ZOL was lower than in the SHAM, OVX, and OVX+RES groups. The OXV+ZOL+RES group showed fewer osteoblasts than the SHAM and OVX+RES groups (Figure 3c).

	SHAM	OVX	OVX + RES	OVX + ZOL	OVX + ZOL + RES
Score 1 (% sites)	54.55	14.30	22.22	0	0
Score 2 (% sites)	27.27	28.26	22.22	16.66	27.28
Score 3 (% sites)	18.18	57.14	55.56	83.33	72.72

Note: Score 1: Closed wound; Score 2: Open wound with no bone exposure; Score 3: Open wound with bone exposure.

TABLE 1 Clinical-macroscopic evaluation of the extraction sockets healing.

3.3.2 | Histopathological results

Regarding the lamina propria overlying the extraction site, fibrous connective tissue with rare inflammatory cells was observed in SHAM (Figure 3d). In OVX (Figure 3e), although the presence of inflammatory cells is small, the connective tissue is moderately dense, whereas the treatment with resveratrol (Figure 3f) resulted in much more fibrous connective tissue in OVX + RES, with small amounts of inflammatory cells. In OVX + ZOL (Figure 3g), an intense inflammatory infiltrate observed in the lamina propria, whereas in OVX + ZOL + RES (Figure 3h), a discrete inflammatory infiltrate was observed in moderately dense connective tissue.

In the first molar alveolus, bone tissue composed of thick bony trabeculae filling the entire tooth socket was observed in SHAM (Figure 3i). In OVX (Figure 3j) and OVX + RES (Figure 3k), bony tissue was observed filling the dental alveolus; however, the bone trabeculae were much thinner than in SHAM. A total absence of neo-formed bone tissue was observed in OVX + ZOL (Figure 3l), which did not occur in the OVX + ZOL + RES (Figure 3m), which had a bone tissue composed of thin bony trabeculae partially filling the dental socket.

Regarding the bone tissue at the periphery of the alveolus, vital bone tissue was observed in the SHAM (Figure 3n), OVX (Figure 3o), and OVX + RES (Figure 3p), where there are gaps occupied by osteocytes in both preexisting bone tissue in the alveolus wall (left side) and the neo-formed bone trabeculae (right side). In OVX + ZOL (Figure 3q), only necrotic bone tissue was observed in the alveolus wall, composed of bone trabeculae filled with osteocytes and circumscribed by necrotic remains and bacteria. In OVX + ZOL + RES (Figure 3r), both the preexisting bone tissue in the alveolar wall (left side) and the neo-formed bone trabeculae (right side) are concomitantly composed of bone trabeculae with and without of osteocytes. A complete description of the histological findings is provided in Table S1, and using RES in OVX + ZOL + RES statistically reduced the local inflammatory intensity and extension and improved the epithelial and connective tissue structure compared to OVX + ZOL ($p < 0.05$).

3.3.3 | Immunohistochemical analysis

Immunohistochemical analysis revealed that ALP- and OCN-immunoreactive cells in OVX-ZOL were lower than in SHAM, OVX, and OVX-RES groups. The OXV + ZOL + RES group showed less immunolabeling for ALP and OCN than the SHAM and OVX + RES groups (Figure 4a–l).

The groups treated with zoledronate showed a lower number of TRAP-positive cells compared with SHAM, OVX, and OVX + RES ($p < 0.05$) (Figure 4m–s).

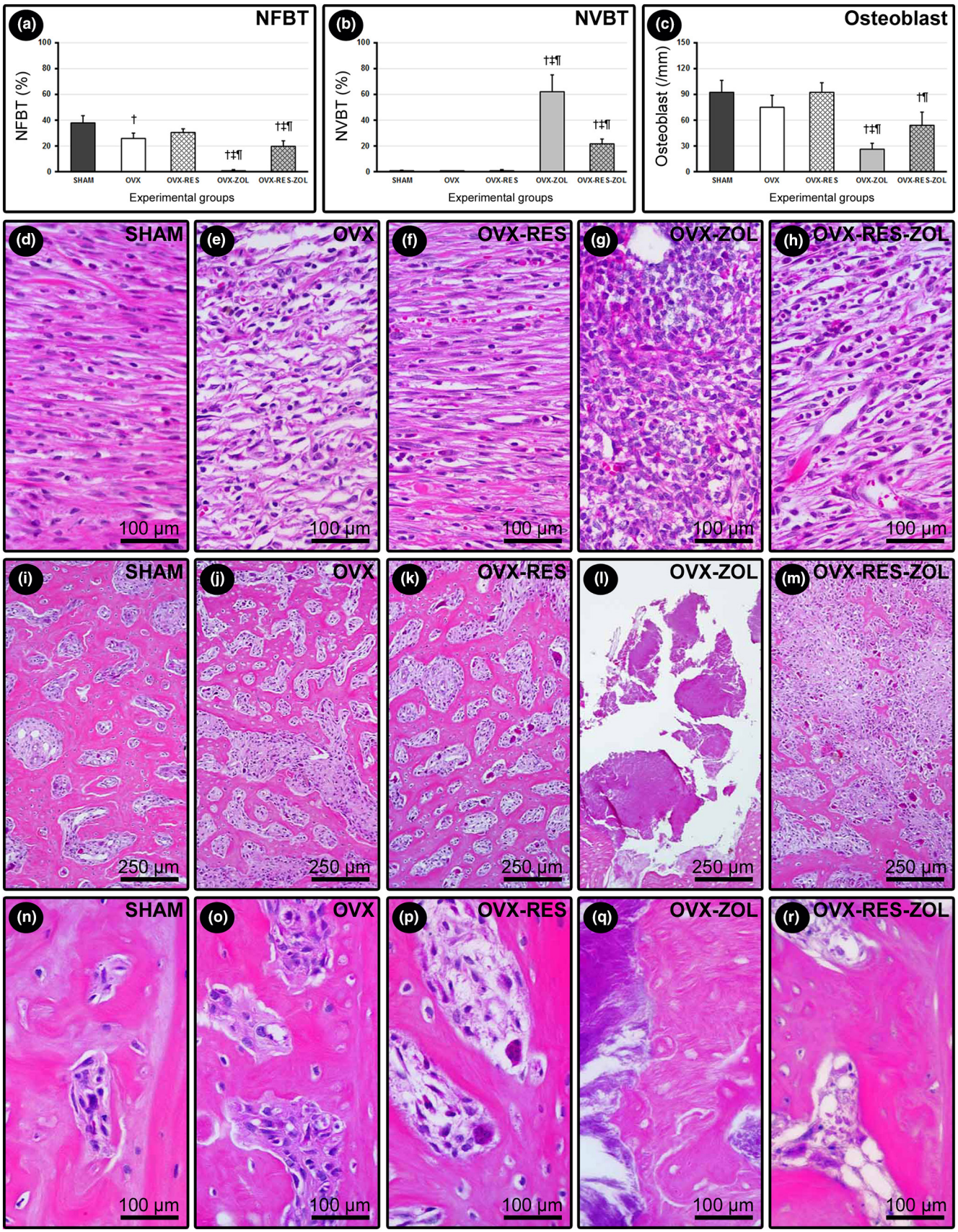
3.3.4 | Gene expression analysis

Ovariectomized animals presented lower OPG mRNA expression compared with SHAM ($p < 0.05$), whereas SHAM showed higher β-catenin compared with ovariectomized animals treated with a placebo ($p < 0.05$). The SHAM and OVZ + RES groups revealed higher SOD levels than OVX, OVX + ZOL, and OVX + ZOL + RES ($p < 0.05$). Furthermore, in the presence or absence of RES, the treatment with ZOL promoted higher levels of TRAP mRNA than in all the other groups ($p < 0.05$). RANKL, DDX-5, and GSR levels were not affected by the treatments ($p > 0.05$) (Figure 5).

4 | DISCUSSION

Zoledronic acid is a potent antiresorptive agent used to treat and prevent osteoporosis due to postmenopausal estrogen deficiency. However, it is often related to medication-related osteonecrosis of the jaw. Different treatment protocols for MRONJ have been investigated, but an effective therapeutic approach has not yet been established in the literature (Beth-Tasdogan et al., 2022). This study proposed resveratrol as a treatment option to prevent osteonecrosis lesion-like in rats treated with zoledronate due to its host modulation potential and anti-inflammatory, antiresorptive, and antioxidant effect. Even though resveratrol could not clinically prevent MRONJ

FIGURE 3 Histopathological results. (a) Newly formed bone tissue (NFBT) in the tooth extraction site; (b) necrotic bone tissue (NVBT) in areas adjacent to the tooth extraction site; (c) the number of osteoblasts per mm of vital bone tissue was calculated. (d–h) Representative photomicrographs of the lamina propria of the mucosa overlying the dental extraction sites (hematoxylin and eosin [H&E], original magnification ×400. Bar = 100 μm). (i–m) Representative photomicrographs of the interior of the first mandibular molar alveolus (hematoxylin and eosin [H&E], original magnification ×100. Bar = 250 μm). (m–r) Representative photomicrographs of the bone tissue at the periphery of the dental alveolus of the lower first molar (hematoxylin and eosin [H&E], original magnification ×400. Bar = 100 μm). Symbols: †, statistically significant difference to SHAM; ‡, statistically significant difference to OVX; ¶, statistically significant difference to OVX-RES.



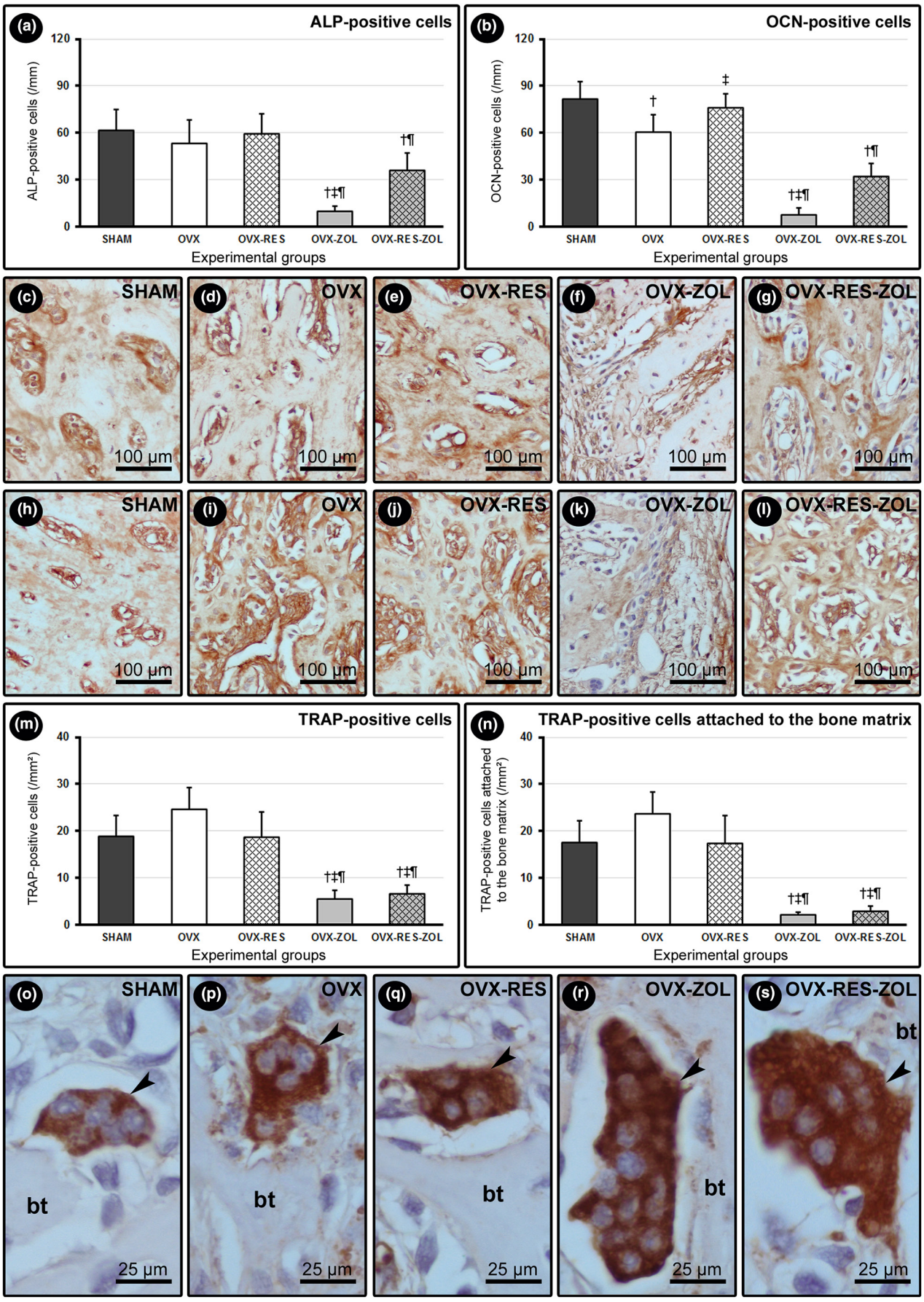


FIGURE 4 Immunohistochemistry results. (a) Immunolabeling for ALP; (b) Immunolabeling for OCN; (c–g) Photomicrographs showing ALP-positive cells in SHAM (c), OVX (d), OVX-RES (e), OVX-ZOL (f), and OVX-RES-ZOL (g). (h–l) Photomicrographs showing OCN-positive cells in SHAM (h), OVX (i), OVX-RES (j), OVX-ZOL (k), and OVX-RES-ZOL (l). (m) Immunolabeling for TRAP-positive cells and (n) TRAP-positive cells attached to the bone matrix in the tissue of the extraction site. (o–s) Photomicrographs showing TRAP-positive cells in SHAM (o), OVX (p), OVX-RES (q), OVX-ZOL (r), and OVX-RES-ZOL (s). Symbols: †, statistically significant difference to SHAM; ‡, statistically significant difference to OVX; ¶, statistically significant difference to OVX-RES.

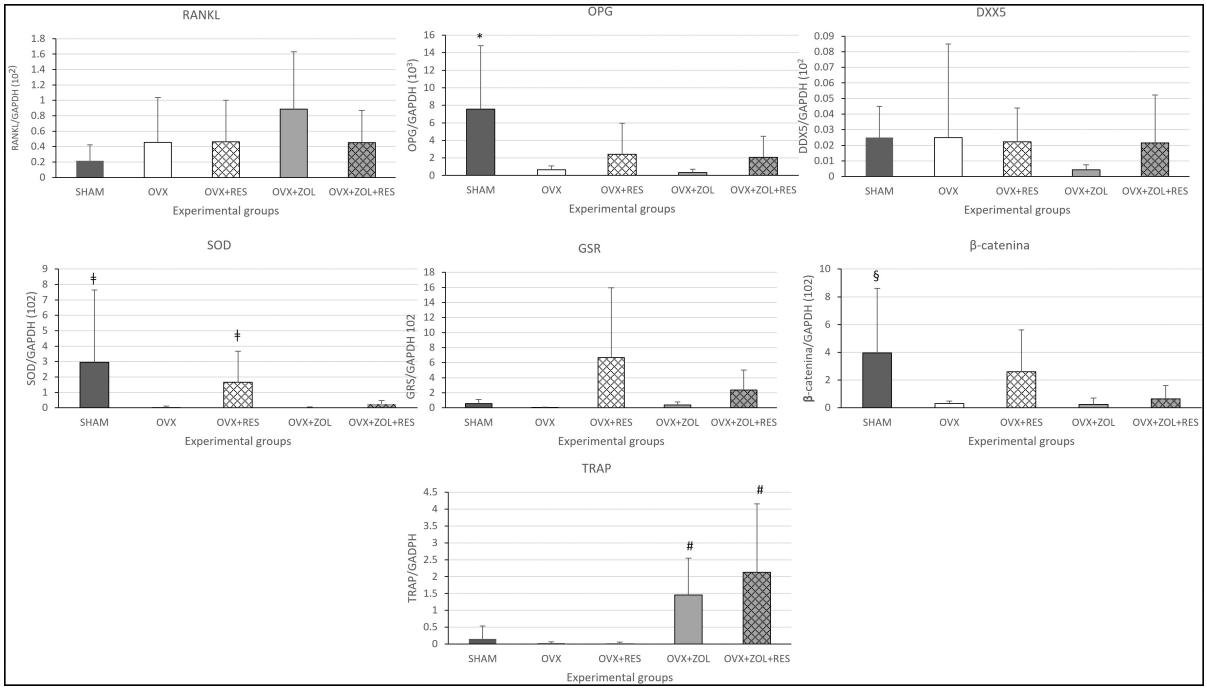


FIGURE 5 Relative levels of mRNA for all genes (mRNA gene/mRNA GAPDH). *Indicates statistically significant difference to OVX + ZOL (Kruskal–Wallis/Dunn – $p < 0.05$); ‡Indicates statistically significant difference to OVX, OVX + ZOL, OVX + ZOL + RES, and SHAM (Kruskal–Wallis/Dunn – $p < 0.05$); §Indicates statistically significant difference to OVX (Kruskal–Wallis/Dunn – $p < 0.05$); #Indicates statistically significant difference to OVX, OVX + RES e SHAM (Kruskal–Wallis/Dunn – $p < 0.05$).

occurrence, interesting histological results regarding the bone formation and healing process were observed.

Clinical-macroscopic evaluation of extraction sockets did not reveal statistical differences in the prevention of MRONJ among the studied groups; however, a tendency for numerical differences is noticed between treatments. The groups treated with ZOL had higher numerical values of an open wound with bone exposure (score 3) compared with the non-treated groups, and the group treated with the combination of ZOL + RES presented approximately 10% fewer lesions under this classification. Experimental studies in rats show that MRONJ clinical confirmation presents variable rates of 0–100% (Aguirre et al., 2012; Biasotto et al., 2010; Hokugo et al., 2010; Sharma et al., 2013). The variability can be a limitation of the MRONJ animal model that should not be evaluated solely by the clinical aspect (Mitsimponas et al., 2016). Thus, even though no differences were observed in the clinical findings of the MRONJ, it was possible to histologically verify the treatment's effect on MRONJ, confirming the model's effectiveness.

The histological findings of the present study indicated the presence of an intense inflammatory infiltrate on the lamina propria, a total absence of newly formed bone in the alveoli, and peripheral bone of the dental alveolus with necrotic bone in the alveolus wall (composed of bone trabeculae devoid of osteocytes and circumscribed by necrotic remains and bacteria) in OVX + ZOL. According to the previously discussed definition of osteonecrosis, the histological aspect, the presence of necrotic bone, fibrous tissue, and osteoclasts is of great importance for MRONJ characterization (Ruggiero et al., 2022). When RES was associated with ZOL, a reduction in the inflammatory infiltrate, and a moderately dense connective tissue on lamina propria, the bone formation and the presence of thin trabeculae and osteocytes in the alveolus suggests that RES can potentially modulate the healing process and the local necrosis.

The antiresorptive effect expected for ZOL was demonstrated by the increase in bone volume, thickness, and the number of trabeculae and the lower separation between trabeculae visualized in the micro-CT. This information corroborates a previous study that showed an increase in bone density in rats treated with ZOL for 6 months (Vermeer et al., 2017). Bisphosphonates generally act by inhibiting the prenylation of essential proteins in the mevalonate pathway and, consequently, by reducing osteoclast activity and bone resorption (Coscia et al., 2010). The improvement in bone architecture observed with ZOL can be further justified by its effect

on osteoblasts observed in in vitro studies, increasing its differentiation and activity (Ishtiaq et al., 2015).

The ZOL inhibitory effect on osteoclasts (Lin et al., 2021) is also in line with our results regarding the number of TRAP-positive cells, which was lower in the groups treated with ZOL. Interestingly, the gene expression analysis evidenced higher levels of TRAP 5b mRNA in the ZOL-treated groups, regardless of the presence of resveratrol. The TRAP 5b activity has been correlated to the number of osteoclasts and negatively correlated to bone mineral density (Ivanova et al., 2021; Rissanen et al., 2008). In addition, TRAP has been linked to the regulation of osteoclast migration in an environment with osteopontin substrate with post-translational modifications (Ek-Rylander & Andersson, 2010). Thus, the up-regulation in mRNA expression of TRAP 5b can be an answer to address the reduction in the number of TRAP-positive cells.

Another interesting finding is the percentage of necrotic bone tissue, which was higher in the ZOL groups than in the other experimental groups. Zoledronate is known to be internalized by osteoclasts, inducing its apoptosis, a mechanism by which this substance acts in the interruption of bone resorption/remodeling. However, this same process of apoptosis can compromise wound repair since osteoclasts are fundamental cells in bone repair and remodeling (Magopoulos et al., 2007). Therefore, the higher percentage of necrotic bone tissue in the presence of zoledronate may be justified, in part, by the apoptotic process of osteoclasts induced by this substance. Another possible explanation for the increased occurrence of necrosis in the presence of zoledronate would be the antiangiogenic effect of this substance. ZOL inhibits endothelial cell proliferation, modulates the adhesion and migration of these cells, and causes their apoptosis, reducing vascular neoformation (Gao et al., 2017). A recent study using a similar model of osteonecrosis induction, associating zoledronate and exodontia, found a revascularization impairment after exodontia when using ZOL (Kün-Darbois et al., 2018).

Resveratrol was proposed to prevent the negative effect of ZOL in the healing process. The groups of animals submitted to ovariectomy and treated with ZOL or ZOL + RES had a higher percentage of necrotic bone tissue and a lower percentage of new bone formation than those non-treated with ZOL, regardless of the presence of RES. Notwithstanding the absence of a statistical difference between OVX + ZOL and OVX + ZOL + RES, it is important to highlight a tendency to increase newly formed bone and decrease necrotic bone in OVX + ZOL + RES. This tendency can also be supported by the histopathological results, which suggest some RES benefits in bone formation. A reduction of the inflammatory infiltrates in the connective tissue overlying the extraction site was observed with RES and increased infiltrate in the presence of the ZOL alone. Likewise, bone tissue composed of bone trabeculae partially filling the dental alveolus was described in ZOL + RES, whereas only necrotic bone tissue was seen in the alveolus wall in the ZOL group. Thus, although RES did not positively affect histometric results, the histological analysis of tissue repair shows a reduction in the deleterious effect of ZOL on bone tissue even when necrosis is present, suggesting that RES promoted a repairing and, somehow, a protective effect on MRONJ lesions-like.

Previous studies have shown the positive effect of resveratrol in inflammatory diseases, such as periodontitis (Corrêa et al., 2017, 2018; Ribeiro et al., 2017), and in bone repair, increasing the gene expression of the bone morphogenetic protein (BMP)-2, BMP-7, osteopontin RANKL/OPG, DKK1 and osterix in repaired bone tissue (Casarin et al., 2014; Pino et al., 2017). RES also affected the proliferation and differentiation of ZOL-treated osteoblasts and presented a protective effect on those cells by positively modulating sirtuin 1, BMP-2, and OPG in an in vitro study (Borsani et al., 2018). Thus, it significantly increased cell proliferation, vitality, and calcium deposition (Borsani et al., 2018).

Additionally, an animal model study of steroid-induced osteonecrosis has shown that treatment with resveratrol reduced osteonecrosis by increasing vascular endothelial growth factor production, reducing the production of thrombomodulin, increasing blood flow, and reducing thrombosis formation by inhibition of transcription tissue factor (a molecule that initiates the coagulation cascade and is a marker of endothelial cell injury) (Zhai et al., 2016). The etiopathogenesis of osteonecrosis is not fully understood, but there is evidence that endothelial injury, intravascular thrombosis, and insufficient vascularization may be related to its onset (Zhang et al., 2009). Thus, the pro-angiogenic effect of RES modulating the Trx-1-HO-1-VEGF pathway and other factors such as nuclear factor (NF)-κB (Fukuda et al., 2006) could potentially improve tissue repair. This effect could have contributed to the histological findings where discrete inflammatory infiltrates and higher newly formed bone tissue was observed when RES was associated with ZOL, compared to ZOL alone, where only necrotic bone tissue was observed in the alveolus wall. Thus, it can be hypothesized that RES could modulate the local bone metabolism and locally reduces the osteonecrosis effect.

The treatment with ZOL in ovariectomized animals reduced OPG expression (important in the bone repair process) compared with the SHAM group, whereas ovariectomized animals treated with RES, in the presence or absence of ZOL, did not present a significant reduction in these bone markers. This finding corroborates previous studies showing greater bone repair in peri-implant tissue and critical calvaria defects when RES was used therapeutically for the optimization of bone metabolism modulating important molecules (OPN, DKK1, and RANKL/OPG ratio) (Casarin et al., 2014; Franck et al., 2018; Pino et al., 2017), which can potentially improve the bone metabolism and repair.

Furthermore, higher levels of SOD1 mRNA were observed in OVX + RES and SHAM groups compared to the others. SOD1 is an antioxidant enzyme that degrades superoxide and oxygen radicals. RES is an antioxidant (positively modulates SOD and sirtuin 1 production) and, at the same time, reduces the levels of important oxidative process initiators (Corrêa et al., 2018). Thus, the elevation of SOD1 could reduce oxidative stress and, consequently, bone tissue degradation in the face of osteoporosis. In contrast, treatment with

ZOL increases the oxidizing enzymes and reduces the antioxidant enzymes, leading to oxidative stress and tissue degradation (Bagan et al., 2014). Thus, it may be speculated that resveratrol can enhance bone formation by producing antioxidant factors and reducing oxidizing agents.

Although RES could not prevent the clinical occurrence of MRONJ, it produces a beneficial effect on histological parameters and the expression of some regulatory molecules. Thus, it could be speculated that using higher doses of RES and more extended therapy periods may optimize its modulatory effect in the presence of ZOL, which should be evaluated in future studies. The possibility of using RES as a treatment after the MRONJ establishment should also be tested once the histologic benefits observed in this study can also potentialize bone repair and necrosis control and reduce the healing time of MRONJ lesions. Furthermore, it would be interesting to evaluate the local application of resveratrol to verify if this substance could have a superior effect compared to that obtained with systemic treatment.

In conclusion, resveratrol was unable, per se, to prevent the occurrence of MRONJ; however, it reduced the severity of tissue impairment induced by treatment with ZOL in ovariectomized rats.

AUTHOR CONTRIBUTIONS

Marcelo Vitale: Methodology; writing – review and editing; investigation. **Mônica Grazieli Corrêa:** Methodology; investigation; visualization; writing – original draft; formal analysis. **Edilson Ervolino:** Investigation; formal analysis; visualization; methodology; writing – review and editing. **Fabiano Ribeiro Cirano:** Conceptualization; investigation; methodology; writing – review and editing. **Fernanda Vieira Ribeiro:** Conceptualization; investigation; methodology; writing – review and editing. **Mabelle Freitas Monteiro:** Formal analysis; writing – original draft; validation; investigation. **Marcio Zaffalon Casati:** Conceptualization; investigation; writing – review and editing; methodology. **Suzana Peres Pimentel:** Conceptualization; investigation; funding acquisition; writing – review and editing; methodology; supervision.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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