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Effect of resveratrol on the progression of experimental periodontitis in an ovariectomized rat model of osteoporosis: Morphometric, immune-enzymatic, and gene expression analysis

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Abstract

Objective: This study aimed to determine the role of resveratrol (RESV) on the progression of experimental periodontitis (EP) in ovariectomy rats (OVT).

Background: Estrogen deficiency is the main cause of osteoporosis and is related to higher periodontal attachment loss and reduction of alveolar bone. Zoledronate (ZLD) is an antiresorptive drug used to control osteoporosis but can lead to osteonecrosis of the jaw. RESV, a natural product, can reduce bone loss and control and prevent osteoporosis. Thus, this study aimed at investigating the effect of RESV on the progression of EP in estrogen-deficient rats.

Material and Methods: The animals were subjected to the OVT or sham surgery to induce estrogen-deficiency and then were divided into the groups: OVT + RESV (n: 10); OVT + PLAC (n: 10): OVT + placebo; OVT + ZLD +PLA (n: 10); OVT + RESV +ZLD (n: 10): OVT + RESV and ZLD; SHAM (n: 10): non-ovariectomized animals + placebo. To induce estrogen deficiency, the rats were subjected to ovariectomy. Experimental periodontitis was induced by the placement of a ligature at the second maxillary molars. Daily administration of the placebo solution, resveratrol (10 mg/kg), and ZLD (0.1 mg/kg) was carried out for a period 42 days prior to initiation of EP, and then for another 28 days following ligature placement. After euthanasia, the specimens were processed for micro-CT and morphometric analysis of bone loss (linear measurement), and the gingival tissue surrounding the maxillary second molar was collected for the quantification of inflammatory markers using Luminex/MAGPix, of oxidative stress markers using ELISA assay, and gene expression analysis of bone markers, by real-time PCR.

Results: Morphometric and micro-CT analysis showed higher bone loss and lower bone density, respectively, in OVT + PLAC when compared to the other groups ($P < .05$). ZLD treated groups had lower alveolar bone loss, as well as, higher density and percentage of bone volume, when compared to OVT + RESV and SHAM + PLAC

groups ($P < .05$). IL-4 levels were significantly lower in the OVT + PLAC group versus OVT + ZLD +RESV and SHAM + PLAC ($P < .05$). NADPH oxidase (nicotinamide adenine dinucleotide phosphate oxidase) levels were significantly lower OVT + RESV group when compared to OVT + PLAC ($P < .05$). OPG mRNA levels were lower in OVT + PLAC compared with the SHAM + PLAC group ($P < .05$).

Conclusion: It can be concluded that resveratrol modulated alveolar bone loss during experimental periodontitis progression in estrogen-deficient rats by downregulating NADPH oxidase levels.

KEYWORDS

alveolar bone loss, anti-inflammatory, estrogen deficiency, inflammation, periodontitis, resveratrol, zoledronic acid

1 | INTRODUCTION

Estrogen deficiency is factor of osteoporosis,[1] which is defined as a systemic skeletal disease characterized by low bone mass and micro-architectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture.[2] Periododontitis is multifactorial inflammatory chronic disease associated with dysbiotic plaque biofilms and mediated by host response, resulting in periodontal tissue destruction and tooth-loss.[3,4]

Several studies have demonstrated that estrogen deficiency can develop an important role on the periodontal disease.[5-7] A recent study showed that women diagnosed with osteoporosis on the post-menopause period present higher periodontal attachment loss and significantly reduction of alveolar bone, when compared to health women.[5] Chang et al[6] demonstrated that osteoporosis patients present higher risk of periodontitis. Recently, Dai et al[7] concluded, in an experimental rat model, that the osteoporotic phenotype has the potential to accelerate the progression of periodontal disease.

Zoledronate (ZLD) is an anti-resorptive agent used in the control of osteoporosis. ZLD can control bone resorption and increase the apposition of this tissue.[8] Animal [9-11] and clinical[12-14] studies have demonstrated that anti-resorptive drugs reduce periodontitis alveolar bone loss. ZLD is a potent biphosphonate used in annual doses for osteoporosis treatment. Studies have shown the positive effect of zoledronate on bone density in patients with osteoporosis.[15,16] However, higher doses of ZLD, aiming to mimic the effect of prolonged use of the substance, demonstrated the occurrence of osteonecrosis, an important side effect of the substance, which is exacerbated in the presence of periodontitis.[17,18]

Considering that estrogen deficiency can modify the host response and that the use of drugs like bisphosphonates can lead to osteonecrosis of the jaw, and estrogen therapy has non-skeletal adverse effects, including vascular events and breast carcinoma,[19] immunomodulation is an interesting therapeutic approach, mainly focusing on natural substances. In this context, resveratrol (3,5,4'-trihydroxystilbene), a plant-derived polyphenol with antioxidant, antitumor, and anti-inflammatory effects, has been demonstrating benefit in the prevention and treatment of several systemic diseases.[20,21] A recent systematic review and metanalysis showed that, in preclinical studies, RESV can prevent EP progression.[22] Some preclinical studies have demonstrated the effect of resveratrol on the prevention and treatment of osteoporosis,[23,24] showing improvement in bone mass and density and restoration of alkaline phosphatase levels,[24] suggesting that resveratrol may be used as adjunct therapy in the treatment and prevention of osteoporosis. In addition, the positive effective of systemic administration of resveratrol also has been demonstrated on the reduction of alveolar bone loss, even in the presence of systemic illness.[25-28]

In view of the potential of resveratrol in reducing bone loss and controlling and preventing osteoporosis, it would be interesting to investigate the possible benefits of resveratrol in the progression of experimental periodontitis in the presence of estrogen deficiency and use of ZLD. Thus, this study aimed at investigating the effect of resveratrol on the progression of experimental periodontitis in estrogen-deficient rats and to elucidate some molecular data about the effect of resveratrol in the immune-inflammatory cascade and in the bone metabolism process during EP in the presence of osteoporosis.

2 | MATERIAL AND METHODS

2.1 | Animals

Fifty adult female Wistar rats (200-300 g- Butantan Institute, Butantan, Sao Paulo, Brazil) were used. The rats were acclimatized for 15 days before use and they were kept in temperature-controlled cages (5 animals/cage), exposed to a 24-hours light-dark cycle of equal time and had free access to water and food ad libitum (Labina, Purina, Paulínia) 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

The experimental design can be observed in Figure 1. The animals were assigned to the following groups: OVT + RESV (n: 10): OVT + resveratrol; OVT + PLAC (n: 10): OVT + placebo; OVT + ZLD +PLA (n: 10): OVT + zoledronate +placebo; OVT + RESV +ZLD (n: 10): OVT + RESV and ZLD; SHAM (n: 10): non-ovariectomized animals + placebo. The number of animals included in the present study was based on previous studies that had found significant differences in the levels of bone loss, inflammatory markers, and gene expression.[25-28] To induce estrogen deficiency, the rats were subjected to ovariectomy.[29] Bilateral ovariectomies were performed in 40 rats, and sham surgeries (in which the ovaries were lifted up and returned intact to the original position) were performed on the remaining 10 rats.[30] This procedure was performed under general anesthesia by the intramuscular administration of ketamine hydrochloride (0.5 mL/kg) and xylazine hydrochloride (10 mg/kg). Postoperatively, the animals received antibiotic given as a single intramuscular injection (1 ml/kg) (Pentabiotico; Wyeth-Whitehall Ltda, Sao Paulo, SP, Brazil).

Resveratrol (10 mg/kg of RESV) [26,28] and placebo were daily administrated by gavage for 70 days. A stock solution of RESV (molecular weight: 228.2) (0,1 mg/kg) (R5010-500MG—Sigma-Aldrich Ltda) was prepared using PBS (P381- Sigma-Aldrich Ltda) was prepared in Tween-80 (P4780—100 ML, Sigma-Aldrich) and further diluted in water for working concentrations. The placebo solution was composed of the same quantities of Tween-80 and water as used in the preparation of RESV. Zoledronic acid solution (SML-0223, Sigma-Aldrich) and was administered intraperitonially twice weekly.[31] Administration of RESV or PLAC and ZLD was started 42 days prior to initiation of EP, and then for another 28 days following ligature placement. Administration of substances was done always in the morning.

2.3 | Rat periodontitis model

To induce EP, the second maxillary molars in each animal were assigned to receive a cotton ligature (Coats Corrente no. 10) knotted subgingivally at the cementoenamel junction (CEJ). The ligatures were kept in position in order to allow biofilm accumulation over 28 days thereby providing for initiation of EP. Twenty-eight days after EP induction the animals were euthanized by CO_2 inhalation. The maxillae were excised for micro-CT and morphometric analysis, and the gingival tissues surrounding the molars were collected for quantification of biomolecules. The gingival tissues from the right second maxillary molars were collected for measurement of IL-1β, IL-4, IL-6, IL-17, and INF-γ by Luminex/MAGpix assay. These tissues were also subjected to superoxide dismutase (SOD1) e nicotinamide adenine dinucleotide phosphate (NADPH) oxidase quantification using and ELISA assay. The gingival tissues surrounding the left maxillary second molars were collected for quantification of mRNA of measurement of OPG, RANKL, and Runx2. Blood was collected for quantification of CTX (carboxyterminal telopeptide cross-linked type I collagen) serum levels using an ELISA assay.

2.4 | Measurement of alveolar bone loss by micro-CT and morphometry

2.4.1 | Micro-CT acquisition

Micro-CT scans of the extracted maxillae were performed on a Skyscan 1174 micro-CT unit (Bruker, Kontich, Belgium) using the following settings: 50 kV, 800 μA, a 10.2-μm voxel size, a 0.5-mm aluminum filter, a rotation step 0.5°, 2 frames, and 180° rotation. All images were exported to the NRecon software (Bruker, Kontich, Belgium) and reconstructed using the tools: beam hardening correction (45%), smoothing filter (degree 2), and ring artifact correction (level 5). In addition, the histogram of all images was standardized for further analysis of bone mineral density (BMD).[32,33]

2.4.2 | Micro-CT evaluation

Bone Mineral Density analysis (BMD)

BMD was assessed using CT and software (Bruker, Kontich, Belgium), which was previously calibrated with the aid of a phantom composed of a solution of K2HPO4 at two different concentrations (600 e 1200 ml/mL) whose attenuation coefficients are equivalent, respectively, to the trabecular and cortical bone.[32,33] The phantom was scanned and reconstructed under the same parameters of the study sample, and a formula was generated in the software for BMD calculation (g/cm^3).

Subsequently, three regions of interest (ROI) (consisting of 50 slices each, 0.5mm in diameter) were established in the furcation

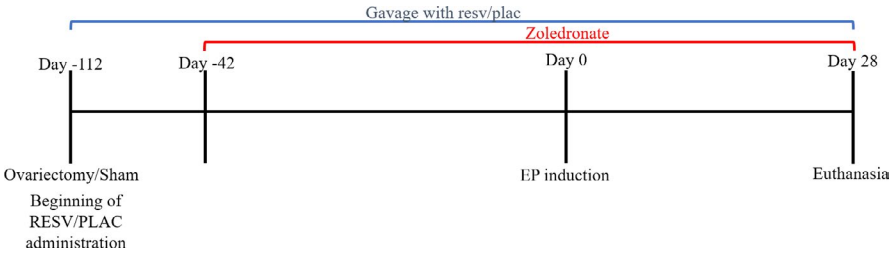

FIGURE 1 Schematic illustration of the experimental design

TABLE 1 Primer sequences for each gene, amplification profiles and the estimated length of qPCR product for each gene

Gene	Sequence (5´–3´)	Length of qPCR product (bp)	Amplification profile [temperature (°C)/time (s)]
RANKL	AGCGCTTCTCAGGAGTT	156	95/5, 55/4, 72/6
OPG	GCAGAGAAGCACCTAGC	168	95/10, 56/8, 72/7
Runx2	GCCACTTACCACAGAGC	157	95/10, 56/8, 72/7
GAPDH	TGAGTATGTCGTGGAGTCTACTG	159	95/10, 56/8, 72/7

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OPG, osteoprotegerin; RANKL, Receptor activator of the NF-KB ligand; Runx2, Runt-Related Transcription Factor 2.

region of the right upper second molar, at the level of cervical, middle and apical root thirds, totalizing 150 slices evaluated. Then, the BMD was obtained for each of these ROIs.

Bone Volume analysis

In order to assess the amount of bone remaining in the furcation region of the right upper second molar, another standardized ROI consisting of 150 slices and 0.5 mm in diameter was established at this location. The parameters bone volume fraction (BV) and total volume (TV) were measured, and the ratio of these numbers (BV/TV) was analysed.

2.4.3 | Linear measurement of cementum enamel junction—alveolar bone (CEJ-AB) distance

After gingival dissection, the maxillae were de-fleshed by immersing the specimens in 8% sodium hypochlorite for 4 hours. The samples were washed in running water and dried with compressed air. To discriminate the CEJ, 1% aqueous methylene blue solution was applied for 1 minutes followed by a wash in running water.[25,27,28] 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 the minimal focal distance thereby insuring 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. Photographs of the palatal aspects were taken, and representative linear alveolar bone loss was assessed on the buccal surface of the molars by measuring the distance of the CEJ from the alveolar bone crest at three equally distant sites using an image analysis system (Image-Pro; Media Cybernetics, Silver Spring, MD, USA). The average CEJ–ABC distance of each tooth was calculated. To validate measurement conversions, all specimens were photographed alongside a millimeter ruler.[25,27,28] The measurements were performed after intra-examiner calibration by evaluating 10 images not taken for this study. The single examiner (AMM) was blinded to the experimental group identities when performing morphometric measurements. The examiner took the linear measurements of all photographs twice within 24 hours. The intra-class correlation was 95.2%.

2.5 | Immunoenzymatic assay

The collected tissues were placed into sterile tubes containing 400 μL phosphate-buffered saline (PBS) with 0.05% Tween-20.[25,26] Briefly, the tissue was weighed, then cut into small pieces (1-2 mm^3) using scissors, and solubilized in PBS to a final concentration of 100 mg tissue/mL. After extraction on a Vortex mixer for 10 min, each sample was centrifuged at 370 g for 5 minutes, and the supernatant was collected, divided into small portions, and stored at −70°C until use. To avoid protease activity, the entire procedure was carried out at 4°C. The levels of IL-1β, IL-4, IL-6, IL-17, and INF-γ were determined by Luminex/MAGpix assay using commercially available kits (RCYTOMAG-80K; Millipore, Billerica, MA, USA) and following the manufacturers' instructions. The levels of SOD1 and NDPH oxidase were measured by using a rat ELISA Kit (MBS2514900; MBS2602768—Mybiosorce; respectively). Samples and standards were prepared following the manufacturer's instructions and analysed on a fluorescence multi-well plate spectrophotometer.

For CTX serum analysis, 100 μL of blood was collected from the orbital plexus under general anesthesia using an ELISA kit (MBS2602646; Mybiosorce). Serum samples and standards were prepared following the manufacturer's instructions and analysed by spectrophotometer.

2.6 | Gene expression analysis of bone-related markers in the gingival tissue

The tissues from the second maxillary molars were stored in a stabilization solution (RNAlater®) at −70°C. Evaluation of the mRNA levels for SIRT1 was performed using qRT-PCR, as described previously.[25]

Total RNA from the biopsies was isolated using the Trizol method (Gibco BRL, Life Technologies). Total RNA treated with DNase treated to reduce the likelihood of false-positive readings (Turbo DNA-frees, Ambion Inc), and 1 μg was used for complementary DNA (cDNA) synthesis. The RNA concentration was determined with optical density using a micro-volume spectrophotometer (Nanodrop 1000, Nanodrop Technologies LLC). The reaction was carried out using a cDNA synthesis (kit Roche Diagnostic Co) following the manufacturer's recommendations. Primers were designed using probe-design software (Light-Cycler Roche probe design software, Diagnostics

GmbH). The amplification profiles, primer sequences, and lengths of PCR products are demonstrated in Table 1. The qPCR reactions were performed in a real-time PCR apparatus (LightCycler® 96 Instrument, Roche Diagnostics GmbH) using a Syber Green kit (FastStart DNA Masterplus Syber Green, Roche Diagnostic Co). The outcomes were expressed as relative amounts of the target gene using GAPDH as the internal reference gene by using the delta-cycle threshold (CT) method. For example, CT values obtained for target genes were normalized against CT values of the reference gene within the same sample. Water was used as a negative control in each run.

2.7 | Statistical analysis

Statistical analysis was performed using statistical program (SAS software Program Release 9.3). The data were examined for normality using the Kolmogorov-Smirnov test and homogeneity of variance by Levene test. The data that achieved normality were analysed using parametric methods. The data were then analysed by Kruskal-Wallis or ANOVA/Tukey tests for comparison between the experimental groups. A significance level of 5% was adopted for all evaluations. The primary outcome was defined as linear alveolar bone loss.

3 | RESULTS

3.1 | Clinical analysis

As described above, the number of animals included in the present study was based on previous studies that had found significant differences in the levels of bone loss, inflammatory markers, and gene expression.[25-28] The animals did not show any signs of systemic illness throughout the study. The rats also did not lose significant weight throughout the experimental period. During the euthanasia, clinical examination revealed signs of gingival inflammation, including color/volume changes and bleeding around the ligated teeth of all groups. The study followed the ARRIVE guidelines.[34]

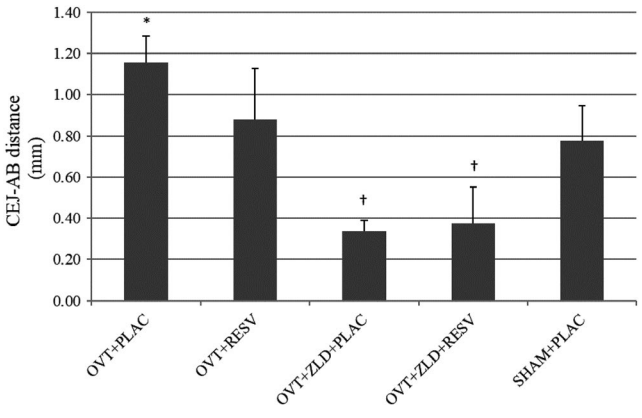

FIGURE 2 Mean ± SD of alveolar bone loss (millimeters) for ligated teeth. * Represents significant difference compared with the other groups (ANOVA/Tukey; $P < .05$). †Represents significant difference compared with OVT + RESV and SHAM + PLAC (ANOVA/Tukey; $P < .05$)

3.2 | Alveolar bone loss analysis

3.2.1 | Morphometric results—linear bone loss measurement

The OVT + PLAC group presented higher bone loss compared to the other groups ($P < .05$). In addition, OVT + ZLD +PLAC and OVT + ZLD +RESV presented lower bone loss when compared to OVT + RESV and SHAM + PLAC ($P < .05$) groups. The morphometric results are shown in Figure 2.

3.3 | Micro-CT results

3.3.1 | Bone mineral density

Lower bone density was observed in the OVT + PLAC group compared with the other groups ($P < .05$). Treatment with zoledronate (OVT + ZLD +PLAC and OVT + ZLD +RESV) promoted higher bone density when compared to OVT + RESV and SHAM + PLAC ($P < .05$). The BMD result can be observed in Figures 3 and 4.

3.3.2 | Bone volume fraction

Lower percentage of bone volume was observed in the OVT + PLAC group compared with OVT + ZLD +PLAC, OVT + ZLD +RESV, and SHAM + PLAC ($P < .05$) (Figure 4A). Similarly to bone density, treatment with zoledronate (OVT + ZLD +PLAC and OVT + ZLD +RESV) promoted higher percentage of bone volume when compared to OVT + RESV and SHAM + PLAC ($P < .05$) (Figure 4B). The BV/TV result can be observed in Figure 5B. A lower percentage of bone volume was observed in the OVT + PLAC group compared with OVT + ZLD +PLAC, OVT + ZLD +RESV and SHAM + PLAC ($P < .05$) (Figure 4B). The combination resveratrol/zoledronate did not bring additional benefit in this parameter (Figure 4).

3.4 | Inflammatory markers levels

IL-4 levels were significantly lower in the OVT + PLAC group versus OVT + ZLD +RESV and SHAM + PLAC ($P < .05$) (Figure 5A). When IL-1β and IL-17 were analysed, higher levels of this markers were observed in OVT + PLAC group compared to SHAM + PLAC ($P < .05$) (Figure 5 B and C, respectively). Considering the concentration of IL-6, IFN-γ, and TNF-α, OVT + ZLD +PLAC group presented lower values when compared to the OVT + PLAC group ($P < .05$) (Figure 5D, E, and F, respectively).

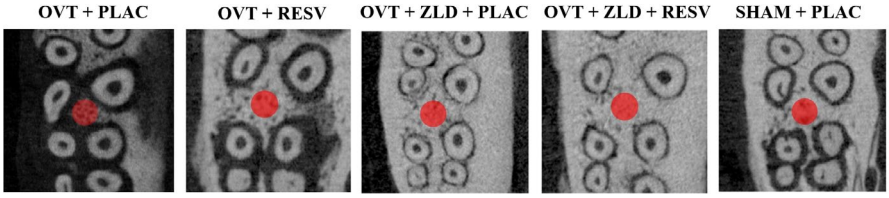

FIGURE 3 Axial view of representative micro-CT images illustrating bone density at interradicular area of all groups. Also, it is observed the region of interest highlighted in red (0.5 mm diameter and 100 slices) placed among the roots of the right upper second molar. Original magnification x10

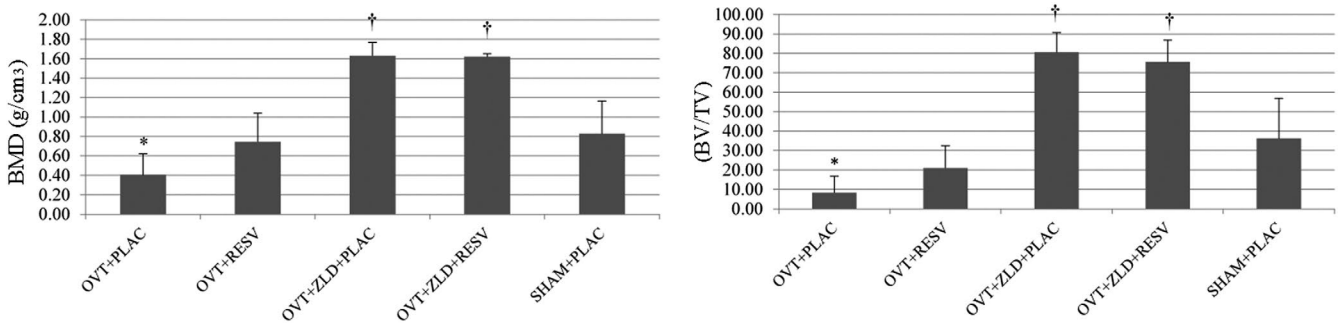

FIGURE 4 Mean ± SD of bone density at the interradicular area (g/cm^3) and of total bone volume at furcation area (%). A, BMD: * Represents significant difference compared with the other groups (ANOVA/Tukey; $P < .05$), †Represents significant difference compared with OVT + RESV and SHAM + PLAC (ANOVA/Tukey; $P < .05$); B, BV/TV: * Represents significant difference compared with OVT + ZLD+PLAC, OVT + ZLD+RESV, and SHAMP + PLAC (ANOVA/Tukey; $P < .05$), †Represents significant difference compared with OVT + RESV and SHAM + PLAC (ANOVA/Tukey; $P < .05$)

3.5 | Oxidative stress-related markers

NADPH oxidase levels were significantly lower in OVT + RESV group when compared to OVT + PLAC ($P < .05$) (Figure S1A). When analysed the levels of SOD1, it was observed that OVT + PLAC group had lower concentration of this protein versus OVT + ZLD +PLAC and SHAM + PLAC ($P < .05$) (Figure S1B).

The OVT + PLAC group had higher levels of CTX compared with OVT + ZLD +PLAC, OVT + ZLD +RESV and SHAM + PLAC ($P < .05$). It was also observed a significant difference in the concentration of this protein in the OVT + RESV group versus SHAM + PLAC ($P < .05$) (Appendix S1).

3.6 | Gene expression of bone markers levels

The analysis of gene expression revealed significant differences only in OPG mRNA levels, with lower concentration in OVT + PLAC when compared to the SHAM + PLAC group ($P < .05$). No differences were observed for RANKL and RUNX-2 mRNA levels ($P > .05$) (Figure S2).

4 | DISCUSSION

Osteoporotic patients present greater severity of periodontitis, as well as a significant reduction of bone crest.[5-7] Antiresorptive drugs, such as zoledronate, act to reduce reabsorption and increase bone formation. However, these drugs can generate important adverse effects, such as osteonecrosis of the jaw, whose etiology is also related to the presence of periodontitis.[17,18] Resveratrol has shown anti-inflammatory, antioxidant, and anti-resorptive effect.[25-28] The present study evaluated the effect of resveratrol on the progression of periodontitis in the presence of osteoporosis induced by estrogen deficiency. The results showed lower bone loss and reduction of pro-inflammatory markers in animals treated with zoledronate, regardless of treatment with resveratrol. Resveratrol administered alone reduced alveolar bone loss in relation to the ovariectomized group that received only placebo and the levels of important inflammatory markers did not present statistically significant difference when compared to the groups treated with zoledronate. Besides, when resveratrol was given alone, the levels of NADPH oxidase were downregulated.

Resveratrol treatment isolated reduced alveolar bone loss in the presence of estrogen deficiency when compared to the ovariectomized group that received placebo and was similar to the non-ovariectomized group. This is the first study to evaluate the effect of resveratrol in the progression of EP in ovariectomized animal. Other ovariectomized rodent models summarized in a review, show the beneficial effect of resveratrol on bone mass and trabecular bone.[24] It was observed that resveratrol treatment in high doses had similar effects to estrogen replacement therapy, but without side effects. In line, the present study agrees to previous studies where resveratrol was able to reduce alveolar bone loss both in healthy [27,28] and systemic compromised conditions.[25,26] In

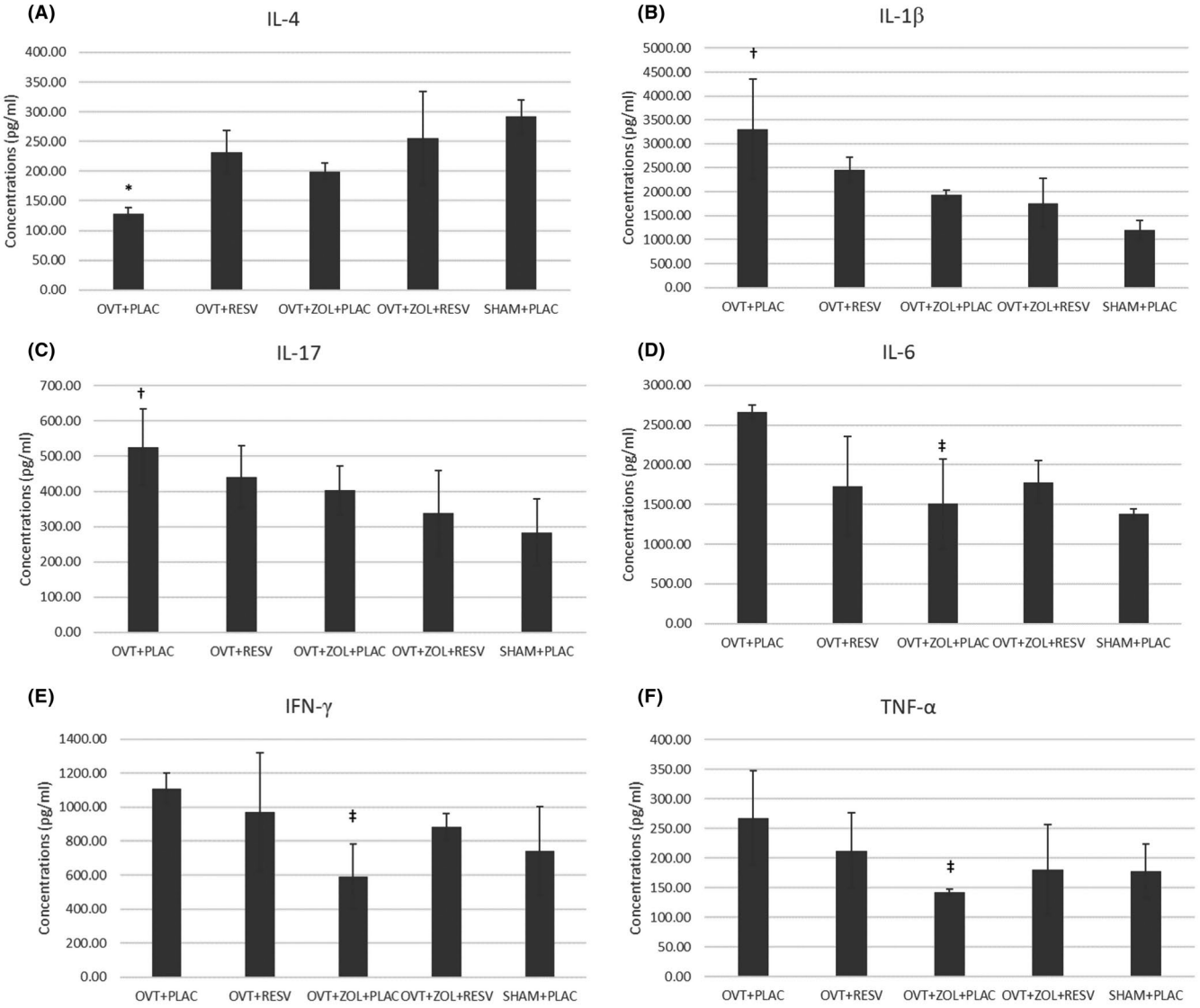

FIGURE 5 Mean ± SD of IL-4, IL-1β, IL-17, IL-6, IFN-γ, and TNF-α concentrations (pg/mL) measured by Luminex/MAGpix. A, IL4: * Represents significant difference compared with OVT + ZLD+RESV and SHAMP + PLAC (Kruskal-Wallis/Dunn, $P < .05$); B, IL-1β, C, IL-17: † Represents significant difference compared with SHAM + PLAC ($P < .05$); (D) IL-6, (E) IFN-γ, (F) TNF-α: ‡ Represents significant difference compared with OVT + PLAC (Kruskal-Wallis/Dunn, $P < .05$)

this study, resveratrol alone did not reduce alveolar bone loss as much as zoledronate treatment did. Nevertheless, zoledronate is related to osteonecrosis of the jaw, especially in the presence of periodontitis,[17,18] while resveratrol is a natural substance with few or any adverse effect.[35,36]

Although resveratrol did not modulate most of the studied cytokines, there was an increase in IL-4 levels in the group in which the two treatments (zoledronate and resveratrol) were associated. IL-4 is an anti-inflammatory cytokine whose levels are reduced in periodontitis.[37] This protein acts as an inhibitor in the production of proinflammatory factors such as TNF-α, IL-1α, IL-1β, IL-6, and IL-8.[38] Consequently, low IL-4 levels favor periodontal destruction, while high levels protect against destruction. Interestingly, IL-4 levels were not positively modulated when zoledronate and resveratrol were given alone. It can be suggested that zoledronate and resveratrol had synergic effect on the levels of IL-4 and higher doses of resveratrol associated with lower doses of zoledronate could modulate other cytokines.

This study presents a confirmatory result related to the systemic treatment with zoledronate reducing alveolar bone loss due to experimental periodontitis in the presence of estrogen deficiency. The morphometric results showed less bone loss in the groups that received the treatment with this substance. Additionally, the micro-CT analysis revealed both higher bone density and higher total bone volume in these same groups. These results agree with previous studies in which treatment with zoledronate attenuated alveolar bone loss, increased total bone volume and density.[16,18] Additionally, other authors have shown lower alveolar bone loss with other types of bisphosphonates, both locally and systemically applied, in animals.[8,39,40]

Interestingly, zoledronate promoted modulation of important pro-inflammatory cytokines (IL-6, IFN-γ, and TNF-α), reducing their levels. This result may explain the reduction of alveolar bone loss observed in the morphometric findings, as well as, justify the higher density and total volume of bone found in micro-CT analysis. The reduction in IL-6 and TNF-α levels could suppress the formation of osteoclasts and, consequently, reduce alveolar bone loss. In addition, IFN-γ is related to the severity of periodontitis.[41] These molecular findings are elucidative, since the production of IL-6, IFN-γ, and TNF-α is involved in the pathogenesis of osteoporosis. Recent studies have suggested the effects of reducing this hormone on T-cell function. Estrogen deficiency results in increased production of IL-17, leading to T-cell activation, accompanied by increased production of interferon (IFN)-γ and TNF-α by these cells,[42] heightening the osteoclastogenic activity. It is also known that estrogen treatment increases the production of insulin-like growth factor (IGF-1) and transforming growth factor (TGF) -β by osteoblasts,[43] zoledronate was able to attenuate the negative effects of estrogen deficiency on the immunoinflammatory response related to the progression of periodontitis.

Another important finding relates to the levels of SOD1, which increased with zoledronate treatment. This is an important protein that acts on the removal of reactive oxygen species (ROS).[44] Dysfunctions in estrogen levels may cause oxidative stress, reducing antioxidant defense mechanisms and elevating the formation of reactive oxygen species (ROS).[45] Inflammatory infiltrate in periodontitis is composed of T lymphocytes, macrophages, and polymorphonuclear neutrophils (PMNs). PMNs are responsible for the production of ROS, which in excess contribute to tissue destructive process.[4,46,47] ROS formation may occur as a sub-product of biological cellular reactions, as well as by enzymatic systems that generate these molecules, such as the NADPH oxidases family.[44] In addition, the association between increased oxidative stress and decreased antioxidant defense plays an important role in the immunoregulatory response of bone tissue under conditions of osteopenia/osteoporosis.[45] Studies also indicate that ROS formation stimulates the formation activity of osteoclasts, contributing to TNF-α increase [46.] Therefore, the reduction of TNF-α levels observed with zoledronate treatment may be related to increased levels of SOD1. Therefore, zoledronate positively regulated SOD levels, even with estrogen deficiency, resulting in decreased alveolar bone loss. However, only when resveratrol was administered alone, it was observed downregulation of NADPH oxidase levels, which is an enzyme responsible to produce the first reactive oxygen species (ROS).[48,49] Resveratrol has been shown to be a potent antioxidant.[26,50] Accordingly, Corrêa et al (2018)[25] showed reduction in NADPH oxidase levels with resveratrol treatment, even in the presence of cigarette smoke inhalation. In addition, other authors also demonstrated regulation of NADPH oxidase expression with resveratrol treatment.[51] Thus, the modulation of the levels of NADPH oxidase by resveratrol in the present study is consistent with the reduction in alveolar bone loss with resveratrol treatment in comparison with the placebo group. It is important to emphasize that the levels of alveolar bone loss in the group treated with resveratrol reached the levels of health (SHAM + PLAC).

This ovariectomized rat model is largely used in preclinical studies [52] and is the accepted model by the Food and Drug Administration regarding the investigation postmenopausal osteoporosis.[53] Elevated serum CTX levels in the group of ovariectomized animals treated with placebo show that the model leads to bone resorption activity, since this marker is a carboxy-terminal fraction of type I collagen formed during bone resorption and is used as a biochemical marker for the verification and monitoring of the metabolic activity of bone tissue.[54] In addition, zoledronate-treated groups of the present study had CTX levels reduced, confirming the anti-resorptive effect of this substance.

In view of the presented results, it would be interesting to conduct further studies to investigate the effect of different doses of resveratrol in the condition of estrogen deficiency, specially related to the inflammatory markers. The results related to zoledronate treatment confirm the findings of the literature. Considering the limits of the present study, it can be concluded that resveratrol modulated alveolar bone loss in the presence of estrogen deficiency by downregulating the levels of NADPH oxidase and led to levels of bone loss similar to that observed in health condition.

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CONFLICT OF INTEREST

The authors report no conflicts of interest related to this study.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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