UNIVERSIDADE DE UBERABA

MÁRCIO YUKIO HASSUMI

AGONISTA PPAR-γ ROSIGLITAZONE PREVINE A PERDA ÓSSEA PERIODONTAL INFLAMATÓRIA PELA INIBIÇÃO DA OSTEOCLASTOGÊNESE

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Dissertação apresentada como parte dos requisitos para obtenção do título de Mestre em Odontologia, área de concentração Biopatologia na Universidade de Uberaba

Orientador: Profº Dr. Marcelo Henrique Napimoga

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DEDICATÓRIA

A Deus

Por trilhar e iluminar os meus caminhos, abençoando-me sempre e por me possibilitar dedicar esse trabalho as seguintes pessoas...

Aos meus pais Mitsuo Hassumi e Edina Shimizu Hassumi

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"Não há ensino sem pesquisa e pesquisa sem ensino. Esses quefazeres que se encontram um no corpo do outro. Enquanto ensino continuo buscando, reprocurando. Ensino porque busco, porque indaguei, porque indago e me indago. Pesquiso para constatar, constatando intervenho, intervindo educo e me educo. Pesquiso para conhecer o que ainda não conheço e comunicar ou anunciar a verdade ".

Paulo Freire

SUMÁRIO

RESUMO

Rosiglitazone (RGZ) é um agonista sintético de alta afinidade ao receptor ativado de proliferação de peroxissomos-γ (PPAR- γ) utilizado como agente anti-hiperglicemiante oral na terapia de Diabetes mellitus não-insulino dependente possuindo também propriedade antiinflamatória. No presente estudo, investigou-se os efeitos do RGZ na periodontite induzida por ligadura em ratos. A doença periodontal foi induzida por ligadura colocada ao redor do primeiro molar da mandíbula de cada animal. Ratos Wistar machos foram divididos em quatro grupos: sem colocação de ligaduras e administração de veículos (animais controle), animais com ligadura tratados com veículo, animais com ligadura tratados com RGZ via oral (10 mg/kg, diariamente) e animais com ligadura tratados com RGZ via subcutânea (10 mg/kg, diariamente). Trinta dias após a indução da doença periodontal os animais foram sacrificados e as mandíbulas e tecidos gengivais removidos para análises. Análises *in vitro* das células precursoras de osteoclastos também foram empregadas. As avaliações histométrica e histoquímica da perda óssea alveolar demonstraram que animais tratados com RGZ apresentaram diminuição da reabsorção óssea e redução da expressão de RANKL, respectivamente. Em adição, os tecidos gengivais dos animais tratados com RGZ mostraram menores níveis de migração de neutrófilos (teste de Mieloperoxidase). Confirmando esses resultados, foi demonstrado *in vitro* que RGZ diminuiu a diferenciação de osteoclastos na presença de RANKL, expressão de RANK em células MOCP-5 e que as células tratadas com RGZ (10μM) apresentaram regulação na expressão de RNAm de TRAP pelo PCR-RT. A análise desses dados indicou que RGZ exerceu papel antiinflamatório e pode ser utilizado para controlar a perda óssea durante a progressão da periodontite experimental em ratos.

Palavras-chave: PPARγ, Rosiglitazone, doença periodontal, perda óssea

ABSTRACT

Rosiglitazone (RGZ) is a high-affinity peroxisome proliferator-activated receptor-γ (PPAR-γ) synthetic agonist used as an oral anti-hyperglycemic agent in the therapy of non-insulin-dependent diabetes mellitus possessing potential anti-inflammatory properties. In the present study, we investigated the effects of RGZ in a ligature-induced periodontitis model in rats. Periodontal disease was induced by a ligature placed around the mandible first molars of each animal. Male Wistar rats were divided in four groups: no ligature placement and administration of vehicle (control animals), ligature-induced animals treated with vehicle, ligature-induced animals treated with oral RGZ (10mg/kg, daily) and ligature-induced animals treated with subcutaneous RGZ (10 mg/kg, daily). Thirty days after the induction of periodontal disease, the animals were sacrificed and mandibles and gingival tissues removed for further analysis. *In vitro* analysis with osteoclast precursor cells were also employed. Histometrical evaluation and immunohistochemical analysis of alveolar bone loss demonstrated that RGZ-treated animals presented a decreased bone resorption and reduced RANKL expression, respectively. In addition, gingival tissues from RGZ-treated animals showed lower levels of neutrophil migration (MPO assay). Confirming these results, we demonstrated *in vitro* that RGZ diminished osteoclast differentiation in the presence of RANKL, RANK expression in MOCP-5 cells, and that osteoclast cells treated with RGZ (10μM) down-regulated the expression of TRAP mRNA by RT-PCR. Analysis of these data indicates that RGZ exerted an anti-inflammatory role and may be useful to control bone loss during progression of experimental periodontitis in rats.

Keywords: PPARγ, Rosiglitazone, Periodontal disease, Bone resorption.

De acordo com o parágrafo § 42º do Regimento da Pró-reitoria de Pesquisa, Pós-graduação e Extensão da Universidade de Uberaba, a apresentação dessa Dissertação será realizada sob a forma de artigo científico. A organização do mesmo segue as normas da revista International Immunopharmacology.

PPAR-γ agonist rosiglitazone prevents inflammatory periodontal bone loss by inhibiting osteoclastogenesis

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Abstract

Rosiglitazone (RGZ), an oral anti-hyperglycemic agent used for non-insulin-dependent diabetes mellitus, is a high-affinity synthetic agonist for peroxisome proliferator-activated receptor-γ (PPAR-γ). Both *in vitro* and *in vivo* experiments have also revealed that RGZ possesses anti-inflammatory properties. Therefore, in the present study, we investigated the anti-inflammatory effects of RGZ in a rat model of periodontal disease induced by ligature placed around the mandible first molars of each animal. Male Wister rats were divided into four groups: 1) animals without ligature placement receiving administration of empty vehicle (control); 2) animals with ligature receiving administration of empty vehicle; 3) animals with ligature receiving administration with oral RGZ (10 mg/kg/day); and 4) animals with ligature receiving administration of subcutaneous RGZ (10 mg/kg/day). Thirty days after induction of periodontal disease, the animals were sacrificed, and mandibles and gingival tissues were removed for further analysis. An *in vitro* assay was also employed to test the inhibitory effects of RGZ on osteoclastogenesis. Histomorphological and immunohistochemical analyses of periodontal tissue demonstrated that RGZ-treated animals presented decreased bone resorption, along with reduced RANKL expression, compared to those animals with ligature, but treated with empty vehicle. Corresponding to such results obtained from *in vivo* experiments, RGZ also suppressed *in vitro* osteoclast differentiation in the presence of RANKL in MOCP-5 osteoclast precursor cells, along with the down-regulation of the expression of RANKL-induced TRAP mRNA. These data indicated that RGZ may suppress the bone resorption by inhibiting RANKL-mediated osteoclastogenesis elicited during the course of experimental periodontitis in rats.

Keywords: PPAR, Rosiglitazone, Periodontal disease, Bone resorption

1. Introduction

Periodontal disease (or periodontitis), which is known to be caused by pathogens resident in the oral cavity, such as *P. gingivalis*, leads to the development of inflammatory lesion accompanied by soft tissue destruction and bone resorption in the tooth-supporting structures [1]. Thus, the etiological role of microorganisms in the pathogenesis of periodontal disease is evident. However, it has also been shown that the immune response generated by the host can lead to protective and/or destructive effects on periodontal tissues [2]. Consequently, an unbalanced host response to periodontopathogens is an important determinant in the disease outcome [3].

Under these conditions, the integrity of bone tissue depends on maintaining a delicate balance between bone resorption caused by osteoclasts and bone deposition caused by osteoblasts [1]. One mechanism regulating this activity is driven by two counteracting molecules, an osteoclast differentiation factor, receptor activator of NF-kB ligand (RANKL), and a soluble inhibitor for RANKL, osteoprotegerin (OPG), which belong to the tumor necrosis factor (TNF) and TNF receptor (TNFR) super families, respectively. The RANKL expressed as a cell surface transmembrane protein on osteoblasts can bind its receptor, RANK, expressed on osteoclast precursors and mature osteoclasts, thereby inducing homeostatic osteoclastogenesis [1]. It is also reported that RANKL-mediated osteoclastogenesis plays an important role in inflammatory bone resorption in periodontal disease [4].

Considerable interest has been focused on peroxisome proliferator-activated receptor-γ (PPAR-γ) ligands as potential therapeutic agents in the treatment of inflammatory disorders. PPAR-γ is a ligand-activated transcription factor belonging to the nuclear hormone receptors superfamily [5], which plays an important role in the regulation of adipocyte proliferation, glucose homeostasis, acceleration of leukotriene

degradation, cell cycle control, carcinogenesis, atherosclerosis and inflammation [6-10]. Recent findings indicate that activation of PPAR-γ can down-modulate inflammation, and this has prompted a number of groups to carry out *in vivo* studies to assess the potential use of PPAR-γ activators, such as thiazolidinediones and cyclopentenone prostaglandins, as anti-inflammatory drugs. For example, intraperitoneal administration of the PPAR-γ ligands troglitazone and 15d-PGJ_2 ameliorated adjuvant-induced arthritis with suppression of pannus formation and mononuclear cell infiltration in rats [11]. Also, it has been demonstrated that pharmacological activation of PPAR-γ by several ligands ameliorates the pathophysiological changes of cardiovascular shock secondary to polymicrobial sepsis [12]. While pathogenic bone resorption occurring in periodontal disease is known to be elicited by inflammation, it is still unclear if PPAR-γ activation can down-regulate the bone resorption mediated by RANKL-dependent osteoclastogenesis. Therefore, in the present study, we investigated the effect of rosiglitazone (RGZ), a high-affinity synthetic agonist for peroxisome proliferator-activated receptor-γ (PPAR-γ), on bone resorption induced in rats by experimental periodontitis caused by ligature placement around the tooth.

2. Materials and Methods

2.1. Animals: Forty male Wister rats (250-350g) were used in the study. The animals were kept in plastic cages with access to food and water *ad libitum*. Prior to the surgical procedures, all animals were allowed to acclimatize to the laboratory environment for a period of 5 days. All experiments were conducted in accordance with national health guidelines for the welfare of experimental animals and were approved by the Ethical Committee of the University of Uberaba, Minas Gerais, Brazil (protocol # 002/2008).

2.2. Experimental animal design: Experimental periodontitis was induced by a ligature placement. More specifically, under general anesthesia obtained by intramuscular administration of ketamine (1.0 ml/kg), ligature was placed and immobilized around both mandible first molars of each animal. The ligature was left in position for the whole experimental period so that inflammation could be constantly induced by the colonization of bacteria inside of it. One day following ligature placement, the animals were randomly assigned to one of the following groups: 1) animals without ligature placement receiving administration of empty vehicle (control) (n=10); 2) animals with ligature receiving administration of empty vehicle (n=10); 3) animals with ligature receiving administration with oral Rosiglitazone (10 mg/kg/day (n=10)); and 4) animals with ligature receiving administration of subcutaneous Rosiglitazone (10 mg/kg/day (n=10)). Rosiglitazone (RGZ; Avandia, Glaxo-Smith Kline, USA) or vehicle was administered daily for 30 days. Twenty-four hours after the last injection, the animals were sacrificed by anesthetic overdose. The current dose was chosen based on a previous study showing that it was effective to diminish the periodontal disease [13].

2.3. Histological and Immunohistochemical analyses: The right and left jaws were dissected, fixed in 10% buffered neutral formalin for 48h and decalcified in a decalcifying solution of EDTA 10% for 3 months. After that, briefly washed in running tap water, dehydrated and embedded in paraffin wax. Each sample was sliced into 6μm sections in sagital directions. Sections were mounted on glass slides and stained with hematoxylin and eosin (HE) for the evaluation of bone resorption. Using an image analysis system (ImageJ 1.41), the area of bone loss in the furcation region was histometrically determined as previously described [14].

Additional sections were mounted on glass slides pre-treated with 3-aminopropyltriethoxy-silane (Sigma Chemical Co., St. Louis, MO, USA) and used for immunohistochemical analysis. Sections were treated with 3% hydrogen peroxide (H_2O_2) for 30 minutes to block endogenous peroxidase. To block the nonspecific binding of antibody, the sections were treated with PBS-1% bovine serum albumin (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature before incubation with the primary antibody (polyclonal antibodies against RANKL (1:50) or OPG (1:10) Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 hours at 4°C. Biotinylated secondary antibody was used (1:100) for 60 minutes at room temperature. The slides were treated with Vectastain ABC-AP kit (diluted at 1:100; Vector Laboratories) for 60 minutes at room temperature, and the specific reaction by each antibody was visualized using 3, 3'diaminobenzidine (DAB). The slides were then counterstained with Mayer's hematoxylin, dehydrated through graded ethanol, cleared in xylene, and mounted in slides with the help of Permount mounting media (Fisher Scientific). Negative controls were obtained by omission of the primary antibodies.

At least ten representative sections of each specimen were analyzed under light microscope (Olympus BX50). Immunohistochemical analysis was performed individually by two examiners (Kappa index=0.95) that were blind to the treatment conditions. Relative staining intensity was assessed for each antibody. Samples were

scored as follows: 0 = no immunoreactivity; 1 = weak, but visible staining; 2 = moderate staining; and 3 = strong staining intensity.

2.4. Myeloperoxidase (MPO) activity measurement: Neutrophil infiltration to the gingival tissues of rats was evaluated by MPO kinetic-colorimetric assay as previously described [14]. Samples of gingival tissue were collected in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide (HTAB) and kept at –80°C until use. Samples were homogenized using a Polytron (PT3100) and centrifuged at 13,000 g for 4 min. The resulting supernatant devoid of debris was subjected to MPO activity assay determined by spectrophotometer at 450 nm (Spectra max®) with three readings within 1 min. Briefly, 10 µL of sample was mixed with 200 µL of 50 mM phosphate buffer pH 6.0, containing 0.167 mg/ml o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The MPO activity of samples was compared with a standard curve of neutrophils. The results were presented as MPO activity (number of neutrophils X mg gingival tissue).

2.5. MOCP-5 culture: Mouse monocyte/macrophage cell line was kindly provided by Dr. Yi-Ping Li of The Forsyth Institute. Cells were cultured in a 25 cm^2 tissue culture plate at a density of 5×10^3 cells/plate and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal bovine serum (FBS).

2.6. Osteoclastogenesis *in vitro*: MOCP-5 cells were plated at a density of 5×10^3 cells/well for 6 days in a 96-well tissue culture plate. Cells were stimulated with 50 ng/ml of RANKL, 10 ng/ml of M-CSF and 10 µM of Vitamin D and treated in a final concentration of 10 µM with RGZ. The culture medium was replaced with fresh medium containing these reagents every 3 days. After the culture, the cells were subjected to tartrate-resistant acid phosphatase (TRAP) staining.

2.7. TRAP staining: The cultured adherent cells were fixed with 5% formalin for 1.5 h, then washed with PBS and incubated for 1.5h with 150 mM of Tartrate solution, followed by incubation for 30 min at room temperature with the TRAP-staining solution (Sigma-Aldrich). TRAP-positive cells appeared dark red, and TRAP-positive multinucleated cells containing three or more nuclei were counted as mature osteoclasts.

2.8. RNA isolation for RT-PCR analysis: Total RNA was extracted from cell cultures which were treated with or without RGZ, in the presence or absence of RANKL, for 24 h, using the RNAbee RNA Isolation kit (Tel-Test, Friendswood, TX). cDNA was synthesized from 10 µg of the total RNA by using reverse transcriptase superscript II and amplified with respective primer set by 30 cycles (94ºC for 1 min, 58ºC for 1 min, 72℃ for 1 min, and final extension time for 10 min at 72ºC). Resulting PCR products were separated in 1.5% agarose gel and stained with Sybr Safe.

2.9. Western blot: Immunoblotting analysis was performed with proteins isolated from MOCP-5 cells treated with or without RGZ, in the presence or absence of RANKL, for 24 h. Proteins were separated by 4-12% gradient SDS/polyacrylamide gel electrophoresis (Invitrogen) and then transferred to nitrocellulose (NC) membrane (Bio-Rad). After incubation of the membrane in blocking solution (TBST: 5% non-fat milk and 0.1% Tween 20 in Tris buffered saline [TBS: 150 mM NaCl, 10 mM Tris Base, pH 7.4]) for 2 h at room temperature, the NC membrane was reacted with primary antibody specific to RANK or α-tubulin (Santa Cruz Biotechnology), respectively, followed by peroxidase-conjugated secondary antibody. The proteins of interest recognized by each primary antibody were visualized on the NC membrane using ECL solution (Pierce) for 60 sec, then exposed to x-ray film (Kodak).

2.10 Statistical analysis

Data were expressed as mean ± SD (standard deviation). Statistical comparisons between groups were made using ANOVA analysis of variance followed by Bonferroni test. Significance was accepted when the *p* value was ≤ 0.05.

3. Results

3.1. Effect of Rosiglitazone (RGZ) on the inflammatory bone loss induced at furcation of first molar: To assess the effects of RGZ on the periodontal bone resorption induced by ligature, the histomorphological evaluation at the furcation of first molar region was conducted by the staining of decalcified section with HE. Experimental periodontitis induced by ligature significantly increased the bone loss compared to the control group (Fig 1A. vs. 1B; $p<0.05$). Systemic administration of RGZ, either by the oral or subcutaneous route, to the animals that received ligature showed significantly reduced bone resorption ($p < 0.05$). More specifically, oral RGZ administration showed better results in the inhibition of bone loss (Fig. 1D) compared to animals receiving subcutaneous RGZ administration (Fig. 1C) ($p < 0.05$). Values of the resorption area of the four groups are shown in Fig. 1E. These results indicated that RGZ can indeed suppress the periodontal bone resorption caused by inflammatory reactions induced by ligature.

3.2. Rosiglitazone administration reduces neutrophil migration: We next investigated the infiltration of neutrophils into the gingival tissues of rats, using an MPO activity assay. Oral and subcutaneous administrations of RGZ did reduce the numbers of infiltrating neutrophils. However, only oral administration showed a significant decrease in the migration of neutrophils compared to control vehicle ($p < 0.05$), while subcutaneous administration showed lower values, but no statistically significant difference (Figure 2). When comparing both treatments, no statistical significance was observed ($p > 0.05$). Since it is considered that neutrophil migration is induced by the local inflammatory stimuli, the reduced number of neutrophils in the gingival tissue of rats receiving systemic administrations of RGZ indicates that RGZ suppresses the inflammation induced by ligature at the periodontal tissues.

3.3. Effect of RGZ on the RANKL and OPG expression: Current consensus supports the idea that bone resorption is regulated by the balance between RANKL and OPG expressed in the micro-environment where osteoclast-mediated bone resorption occurs. As described in the introduction, one mechanism regulating this activity is driven by two counteracting molecules, an osteoclast differentiation factor, receptor activator of NF-kB ligand (RANKL), and a soluble inhibitor for RANKL, osteoprotegerin (OPG). Consequently, we analyzed whether RGZ administration changes the expression of these two molecules, RANKL and OPG, during the progression of experimental periodontitis, using an immunohistological method (Figure 3). As anticipated, RANKL expression levels were statistically higher in the group that received ligature alone, compared to the control group which did not receive either ligature or RGZ administration (Fig 3A and Fig. 3B). However, both groups receiving subcutaneous and oral RGZ (Figure 3C and 3D) in addition to ligature placement showed a significantly lower level of RANKL expression compared to the group receiving ligature and delivery vehicle without RGZ (Fig. 3B). The difference in the routes of RGZ administration did not alter the ability of RGZ to suppress the expression of RANKL. In contrast to the expression pattern of RANKL, OPG expression levels were not affected among all 4 groups tested (Fig. 4A-D). These data demonstrated that systemic administration of RGZ can suppress the RANKL expression induced by inflammatory stimulus, while the level of OPG expression was not affected by either the presence of RGZ or in the context of inflammation, indicating that RGZ reduces the ration of RANK to OPG in the inflammatory lesion induced by ligature.

3.4. Rosiglitazone inhibits osteoclast differentiation: In order to determine if PPAR-γ agonist RGZ can affect RANKL-mediated osteoclastogenesis, we examined the effects of RGZ on *in vitro* osteoclast differentiation induced in MOCP-5 osteoclast precursor cells by stimulation with recombinant RANKL. The formation of TRAP-

positive multinucleated cells induced in the culture of RANKL-stimulated MOCP-5 cells was inhibited by the addition of RGZ (10 μM). The number of TRAP+ positive cells counted was expressed in the Figure 5. The induction of TRAP mRNA expression in the RANKL-stimulated MOCP-5 cells was also examined using RT-PCR (Figure 6A and B). The up-regulation of TRAP mRNA induced in the RANKL-stimulated MOCP-5 cells was remarkably suppressed by the addition of RGZ (10μM) (Figure 6A and B). These data indicate that RGZ can directly act on osteoclast precursors and suppress their RANKL-mediated differentiation to mature TRAP+ osteoclast cells.

3.5. Rosiglitazone decreases the expression of RANK: In an attempt to examine whether RGZ can also suppress the expression of RANK, the receptor for RANKL, RANK protein expressed in MOCP-5 cells was monitored using Western blotting analysis. The addition of RGZ suppressed the expression of RANK in the MOCP-5 cells treated with RANKL (Figure 7A. Figure 7B shows the relative expression level of RANK detected in the Western blot assay which was quantified by densitometry as standardized based on the expression level of internal control protein, α-tubulin.

4. Discussion

The thiazolidinedione (TZD) class of synthetic PPAR-γ agonists and anti-diabetic agents, including troglitazone, pioglitazone, and rosiglitazone, have been reported to inhibit *in vitro* osteoclast differentiation and bone resorption activity mediated by such *in vitro*-induced osteoclasts [15]. In the present study we specifically demonstrated that the administration of Rosiglitazone (RGZ) inhibited the bone loss induced in a rat model of experimental periodontitis which was elicited by ligature placement. Our results indicated that this effect of RGZ in suppressing bone loss may, at least in part, be derived from a reduced expression of the bone resorption promotion molecule (RANKL) and suppression of RANKL-mediated osteoclastogenesis.

Finding a strategy to inhibit bone resorption triggered by inflammation is a particular focus for researchers seeking to establish a therapeutic approach to prevent the bone destruction occurring in inflammatory bone loss lesions, such as periodontal disease and Rheumatoid arthritis. Several different drugs, particularly bisphosphonates, have been developed and are currently being used in clinical settings. However, because of the side effects of such anti-bone loss drugs, e.g., bisphosphonate-induced osteonecrosis of jaw (ONJ) [16], there is still a demand for the development of therapeutic regimens to prevent inflammatory bone resorption. Although TZD drugs were initially developed to treat diabetic insulin resistance [5,8], this class of drugs has also demonstrated a novel efficacy in the control of inflammatory conditions [5,6,9,17-19]. Based on such background evidence and the fact that bone resorption in periodontal diseases is elicited by ongoing inflammation, the use of this class of drug on periodontal inflammatory bone resorption was addressed in this study.

Previous studies have found that the receptor activator NF-κB ligand (RANKL) plays an important role in osteoclast formation. RANKL/RANK interaction is

needed for differentiation and maturation of osteoclast precursor cells to activate osteoclasts and for the survival of mature osteoclasts [20,21]. Conversely, however, osteoprotegerin (OPG), a decoy receptor, binds to RANKL with high affinity and blocks RANKL from interacting with RANK, inhibiting osteoclastogenesis [22]. In fact, it is this activity between counteracting molecules that maintains the delicate balance between bone resorption caused by osteoclasts and bone deposition caused by osteoblasts [1]. Very importantly, we found that the key osteoclast differentiation molecule, RANKL, was down-regulated in RGZ-treated animals (Figure 3), while OPG expression levels were essentially unaffected by RGZ treatment (Figure 4). Crotti et al. [23] showed that there is an elevation of RANKL and a reduction of OPG expression in human periodontal granulation tissue adjacent to the site of alveolar bone loss. Kawai et al. [4] also demonstrated that T and B cells are the major sources of elevated RANKL expression in the gingival tissue of periodontitis patients with bone loss lesion. Therefore, suppression of RANKL expression in the inflammatory periodontal bone loss lesion, which was mediated by systemic RGZ treatment, appeared to be the underlying mechanism of RGZ-mediated suppression of periodontal bone resorption in the present rat periodontitis model.

The infiltration of neutrophils into the rat periodontal lesion induced by ligature was also suppressed by treatment with RGZ. While the infiltration of neutrophils is indicative of the level of inflammation induced by the placement of ligature, it is plausible that RGZ may directly act on neutrophils and, consequently, down-regulate their infiltration into the inflammatory lesion. Our results, therefore, corroborate a previous report showing that RGZ administration can reduce the number of neutrophils infiltrating the muco-gingival tissue [13]. In general, immune responses to bacteria are considered to be a host protective mechanism to pathogenic bacteria. However, while neutrophils do exert a protective role in combating against infectious microorganisms, collateral tissue damage caused by intense neutrophil migration is considered to be a deleterious consequence of infectious diseases [24]. While it is

unclear if collateral tissue damage caused by an intense infiltration can lead to bone resorption, the suppression of neutrophil migration by RGZ would be of benefit for the host in the context of periodontal inflammation. Several previous studies used the commercial available drug (not only RGZ) instead of the active principle alone, and the most common administration route used was subcutaneous. However, the commercial drugs are formulated to be used orally, thus, we tested if there is any difference on the biological result using both routes. Despite the fact that neutrophil migration was not statistically different between groups, when administered subcutaneously, an important decreased neutrophil infiltration was observed. Also, it is noteworthy that both subcutaneous and oral administration reduced bone resorption (statically significant) evoked by ligature in the present study.

In the present study, we have suggested that the mechanism underlying the suppression of bone resorption mediated by osteoclasts involves the inhibitory effect of RGZ on the RANKL molecule as well as suppression of following RANKL-mediated osteoclastogenesis. Other studies, though controversial have hypothesized that RGZ may inhibit bone formation and reduce bone mineral density by its effects on osteoblasts [25-28]. For example, Ali et al. [26] demonstrated that bone loss caused by RGZ administration is caused, at least in part, by a reduction in osteoblast differentiation from early progenitors. In addition, PPAR-γ activation led to changes in marrow structure and function, such as a decrease in osteoblast number, an increase in marrow fat cells, an increase in osteoclast number and a loss of the multipotential character of marrow mesenchymal stem cells [27]. In a review article, Grey [28] showed that activation of PPAR-γ inhibits bone formation by diverting mesenchymal stem cells from the osteogenic to the adipocytic lineage. An *in vivo* study showed that the administration of RGZ to ovariectomized rats can promote bone loss by increasing bone resorption, but not by decreasing bone formation [29]. Although the latter study [29] indicates that PPAR-γ agonists may stimulate osteoclasts in the

ovariectomized osteoporotic rats, it is noteworthy that periodontal bone resorption demonstrated in the present study is elicited by inflammation caused by mechanical ligature placement, not by the depletion of estrogen, which is a result of ovariectomization. These studies have all presented findings critical to the understanding of the effects of RGZ on the bone regeneration process after quenching the ligature-induced periodontal inflammatory bone resorption. However, they are, for the most part, based on the RGZ's effects on static or non-inflammatory bone regeneration processes. Our work, on the other hand, aimed to confirm that the PPAR-γ agonist inhibits bone resorption by decreasing osteoclast activity in the context of inflammation. Important to mention that a previous work from Di Paola [13] demonstrated that RGZ exert an anti-inflammatory action in a rat model of ligature-induced periodontitis by reducing neutrophil migration, iNOS, nitrotyrosine formation, and poly (ADP-ribose) expression. In our study, we provided new evidences of how RGZ may exert its anti-inflammatory action, decreasing RANK at the membrane of the osteoclast as well RANKL expression consequently inhibiting the osteoclastogenesis.

As such, we demonstrated *in vitro* that RGZ diminished osteoclast differentiation in the presence of RANKL, and that the addition of RGZ reduced the formation of TRAP-positive multinucleated osteoclast cells. This finding is consistent with previous reports that both 15d-PGJ_2 and Ciglitazone act as natural ligands and synthetic agonists for PPAR-γ, respectively, inhibiting TNF-α-induced osteoclast differentiation determined by TRAP staining [30]. Recently, Park et al. [31] found that a novel PPAR-γ agonist, KR62776, suppressed the *in vitro* activation of TRAP expression in the M-CSF and RANKL-stimulated bone marrow mononuclear (BMM) cells. In the same study, RGZ was able to reduce about 50% osteoclast formation in BMM. Furthermore, in a study conducted by Tomita et al. [32], the authors showed that THR0921, another novel PPAR-γ agonist with potent anti-diabetic properties,

reduces *in vivo* the severity of collagen-induced arthritis as well as inhibits *in vitro* osteoclast differentiation in BMM cells induced by M-CSF and RANKL. Because these studies show the efficacy of RGZ and PPAR-γ agonists on the inhibition of *in vitro* osteoclastogenesis, they support our findings which strongly indicated that RGZ can suppress periodontal bone loss induced by ligature-mediated, mechanically induced inflammation.

In conclusion, we demonstrated that Rosiglitazone may be able to reduce alveolar bone loss in ligature-induced periodontitis by decreasing RANKL expression and inhibiting osteoclast differentiation. Further studies are necessary to establish the potential use of this drug on periodontal therapy.

5. Legends

Fig. 1. RGZ decreases alveolar bone resorption. Histology at the furcation of first molars sampled from the rats sacrificed after 30 days of experiments is shown (staining with HE); (A) control animals, (B) ligature-induced periodontitis treated with vehicle for 30 days, (C) ligature-induced periodontitis treated with subcutaneous RGZ administration (10mg/kg/day for 30 consecutive days), (D) ligature-induced periodontitis treated with oral RGZ administration (10mg/kg/day for 30 consecutive days). (E) The bone resorption area measured at the furcation of first molars is expressed in a histogram. Results are expressed as mean area (mm^2) ±SD of 10 animals in each group. # $p<0.05$ compared to control animals; * $p<0.05$ compared to ligature-induced periodontitis treated with vehicle; $^{+}$ $p<0.05$ indicates statistical significance between subcutaneous or oral administration (ANOVA followed by Bonferroni's test).

Fig. 2. Effect of RGZ on neutrophil migration to the gingival tissue of rat after ligature. In order to estimate the relative numbers of infiltrating neutrophils in the gingival tissue, myeloperoxidase (MPO) activity present in the gingival tissue homogenates was measured. Results are shown as mean MPO activity ± SD. # $p<0.05$ compared to control animals; * $p<0.05$ compared to ligature-induced periodontitis treated with vehicle (ANOVA followed by Bonferroni's test).

Fig. 3. **Immunohistochemical staining for RANKL.** Expression patterns of RANKL in the periodontal tissue at the furcation of first molar sampled from the rats sacrificed at 30 days are shown. (A) Weak positive staining for RANKL was observed in the gingival tissues of control animals. (B) Moderate staining for RANKL was observed on ligated teeth after 30 days. (C) In the gingival tissues of rats receiving subcutaneous or oral (Fig. 3C or D, respectively) RGZ administration (10mg/kg/day

for 30 consecutive days), a weak positive staining was observed, respectively. (E) Data are presented as median ± SD of the average RANKL staining of all sections analyzed per area. Negative control staining was carried out by the incubation with secondary antibody alone which showed lack of immunostaining pattern in all experimental groups (data not shown). # $p < 0.05$ compared to control animals; * $p < 0.05$ compared to ligature-induced periodontitis treated with vehicle (ANOVA followed by Bonferroni's test). The figure is representative of at least 4 different sections obtained from each group. Scale bar at lower (40×) magnification=100 μm, and scale bar at higher (400×) magnification=20 μm.

Fig.4. Immunohistochemical staining for OPG. Expression patterns of OPG in the periodontal tissue at the furcation of first molar sampled from the rats sacrificed at 30 days are shown. (A) Moderate staining for OPG was observed in the gingival tissues of control animals. (B) Moderate staining for OPG was observed in the ligated-teeth group after 30 days. In the gingival tissues of subcutaneous and oral RGZ- treated rats (10mg/kg/day for 30 consecutive days), a moderate positive staining was observed (C and D, respectively). (E) Data are presented as median ±SD of the average staining of all sections analyzed per area. # $p < 0.05$ compared to control animals; * $p < 0.05$ compared to ligature-induced periodontitis treated with vehicle (ANOVA followed by Bonferroni's test). The figure is representative of at least 4 different slides obtained from each group. Scale bar at lower (40×) magnification=100 μm, and scale bar at higher (400×) magnification=20 μm).

Fig. 5: Effects of RGZ on *in vitro* osteoclastogenesis using mouse monocyte/macrophage cell line MOCP-5. RGZ inhibited the formation of TRAP-positive mononucleated cells compared to culture medium stimulated with RANKL. # $p < 0.05$ compared to MOCP-5 cells with medium alone; * $p < 0.05$ compared to MOCP-5 cells in the presence of RANKL (ANOVA followed by Bonferroni's test).

Fig. 6: TRAP mRNA expression in MOCP-5 cells. (A) RT-PCR was performed to detect the expression of TRAP mRNA in the total RNA extracted from the MOCP-5 cells incubated with or without RGZ in the presence or absence of RANKL (50 ng/ml), M-CSF (10 ng/ml) and Vitamin D (10 μM). Expression of TRAP mRNA was strongly detected in cells stimulated with RANKL, whereas little or no expression of TRAP mRNA was observed in the cells cultured with RGZ or in the control culture. (B) The relative intensity of each PCR product in the agarose gel was scanned by densitometer using the UVP Image analysis system and expressed as relative intensity of PCR product normalized based on the expression level of internal control β-actin. The results are expressed as mean ± SD of three culture wells. # $P<0.05$ compared to cells incubated in the medium alone; *$P<0.05$ compared to cells stimulated with RANKL.

Fig. 7: RANK protein expression in the MOCP-5 cells in the presence or absence of RGZ. (A) Cells were collected 24h after incubation, and RANK protein expression was analyzed by Western blot. (B) Intensity of optical density of bands measured from Western blots. Density of the RANK bands were normalized to α-tubulin expression. Protein band intensity is represented as arbitrary units. The results are expressed as mean ± SD of three culture wells. # $P<0.05$ compared to cells incubated in the medium alone; *$P<0.05$ compared to cells stimulated with RANKL.

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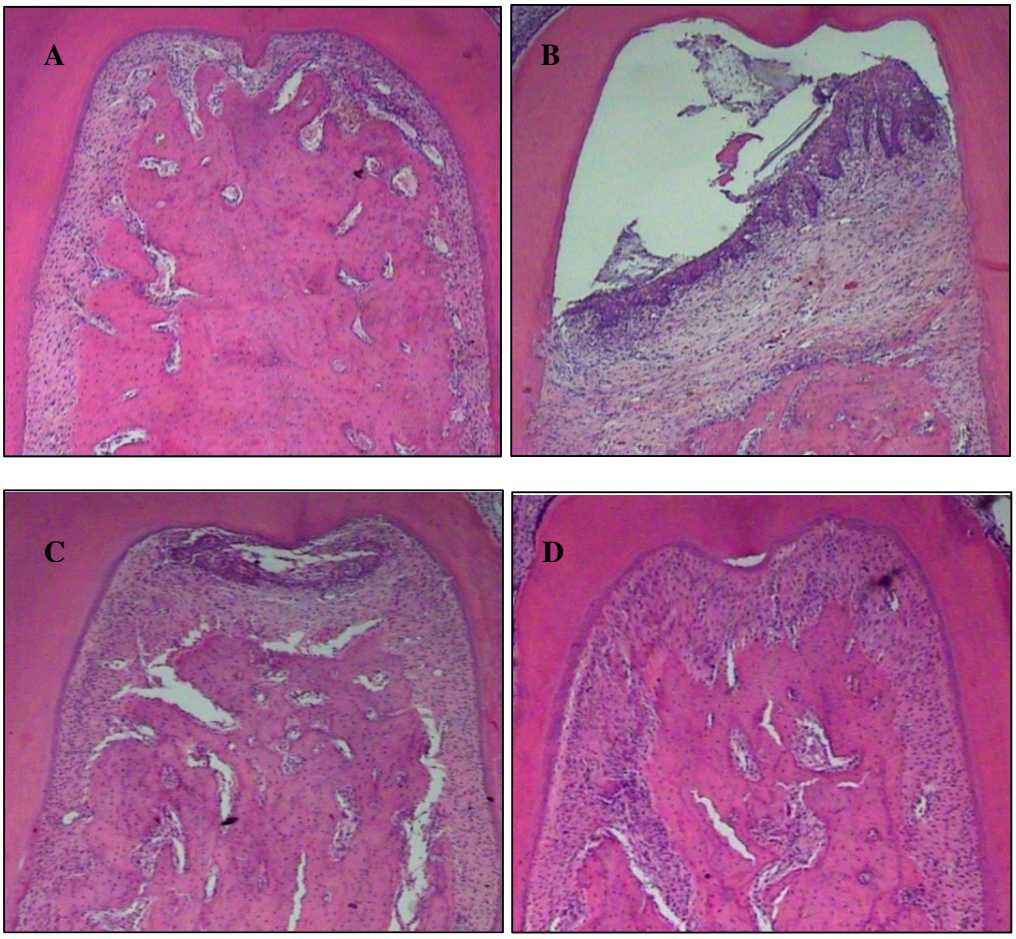
7. Figuras e Gráficos

Figure 1



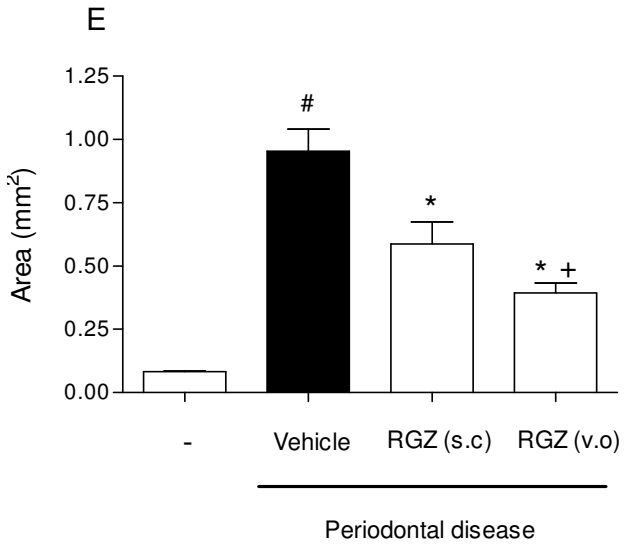


Figure 2

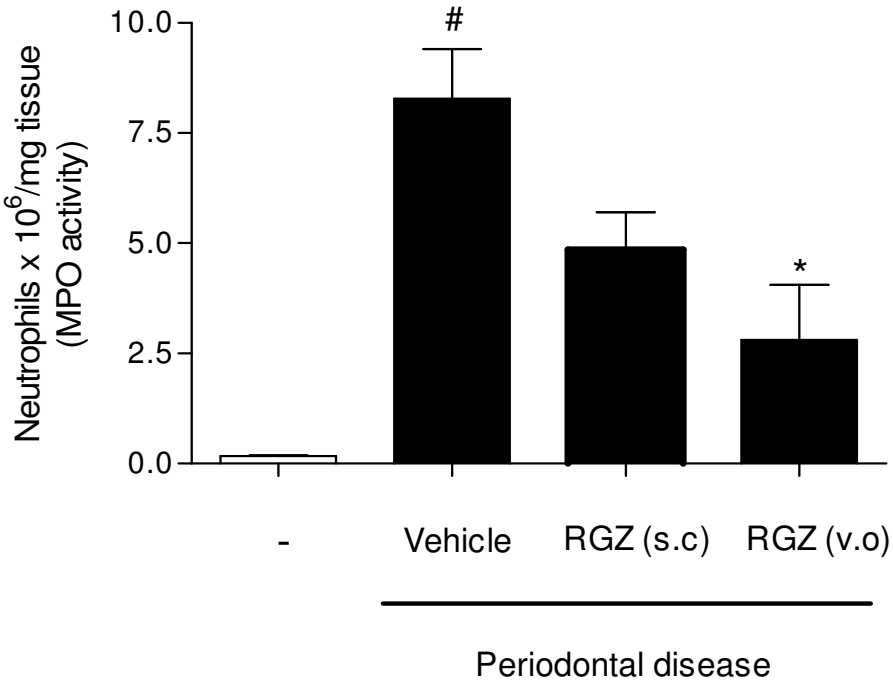
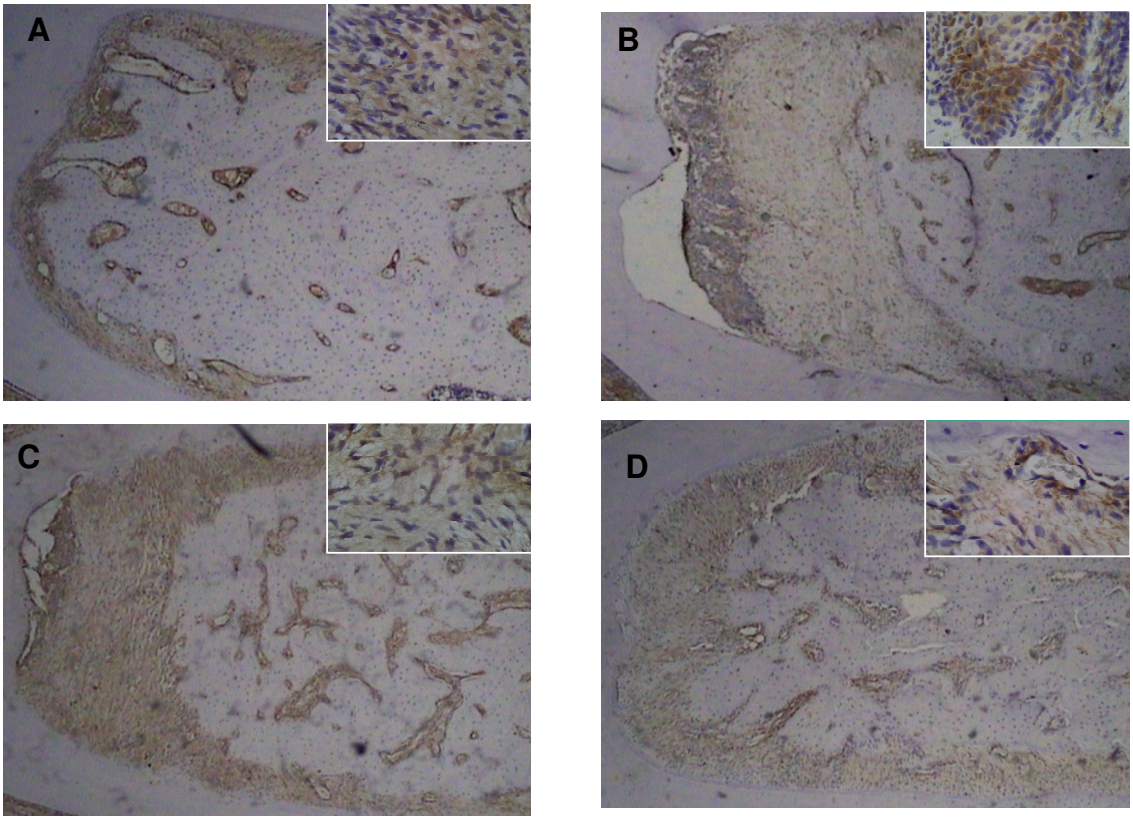

10.0
7.5
5.0
2.5
0.0
Neutrophils x 10^6/mg tissue
(MPO activity)
#
*
-
Vehicle
RGZ (s.c)
RGZ (v.o)
Periodontal disease

Figure 3


A
B
C
D

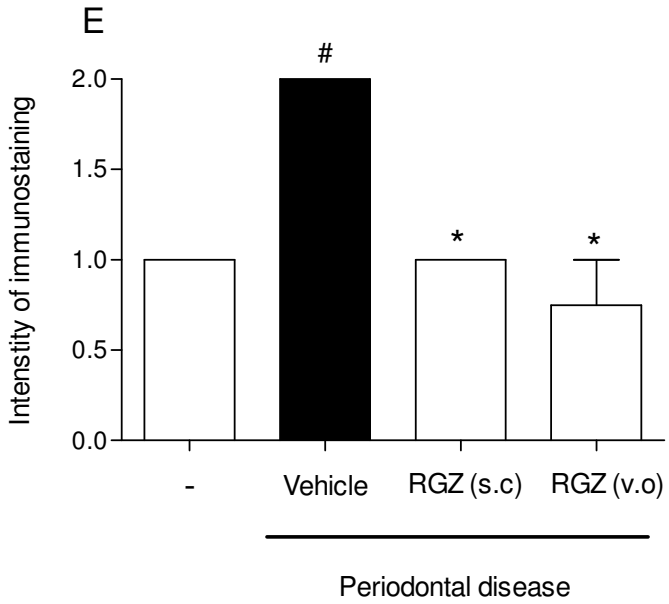
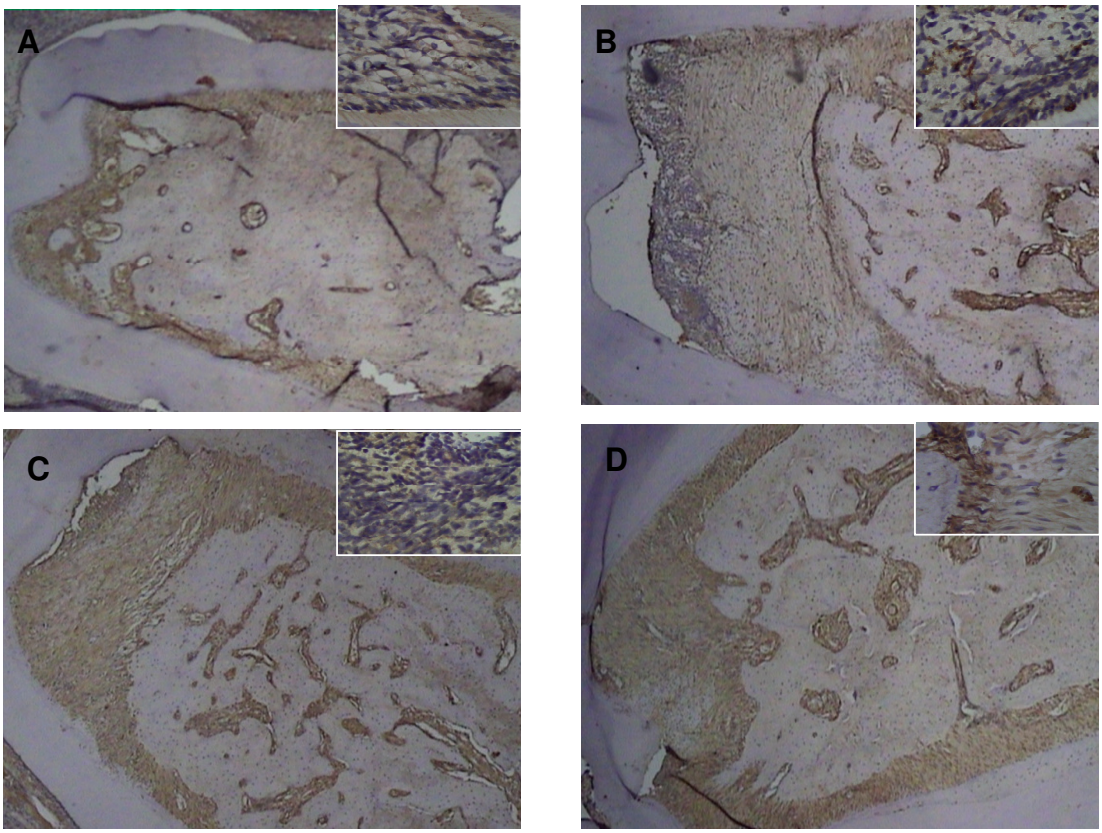

E
#
2.0
1.5
1.0
0.5
0.0
Intenstity of immunostaining
*
*
-
Vehicle
RGZ (s.c)
RGZ (v.o)
Periodontal disease

Figure 4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B
C
D

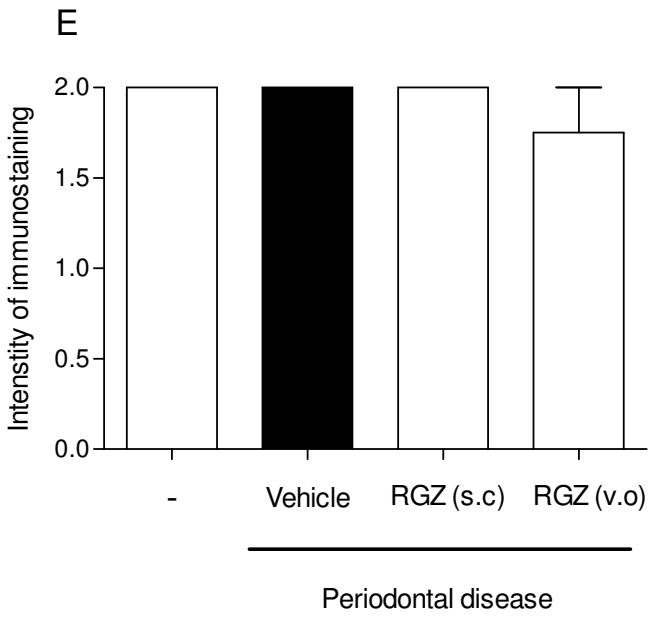

E
Intenstity of immunostaining
2.0
1.5
1.0
0.5
0.0
-
Vehicle
RGZ (s.c)
RGZ (v.o)
Periodontal disease

Figure 5

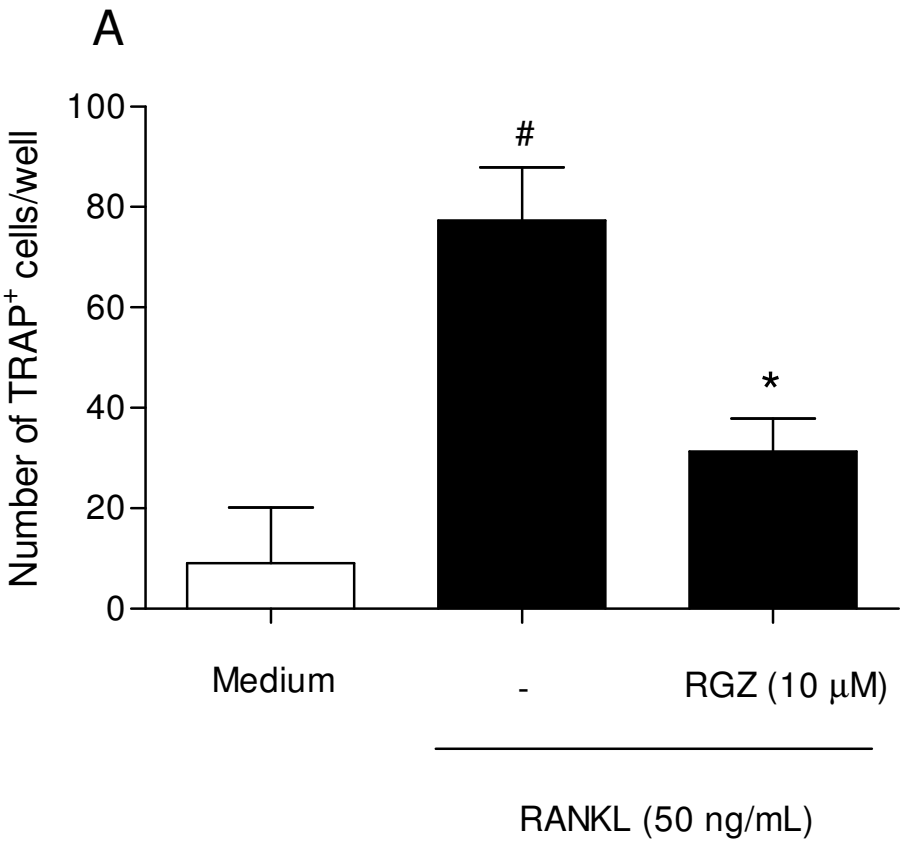

A
Number of TRAP+ cells/well
100
80
60
40
20
0
#
*
Medium
-
RGZ (10 μM)
RANKL (50 ng/mL)

Figure 6

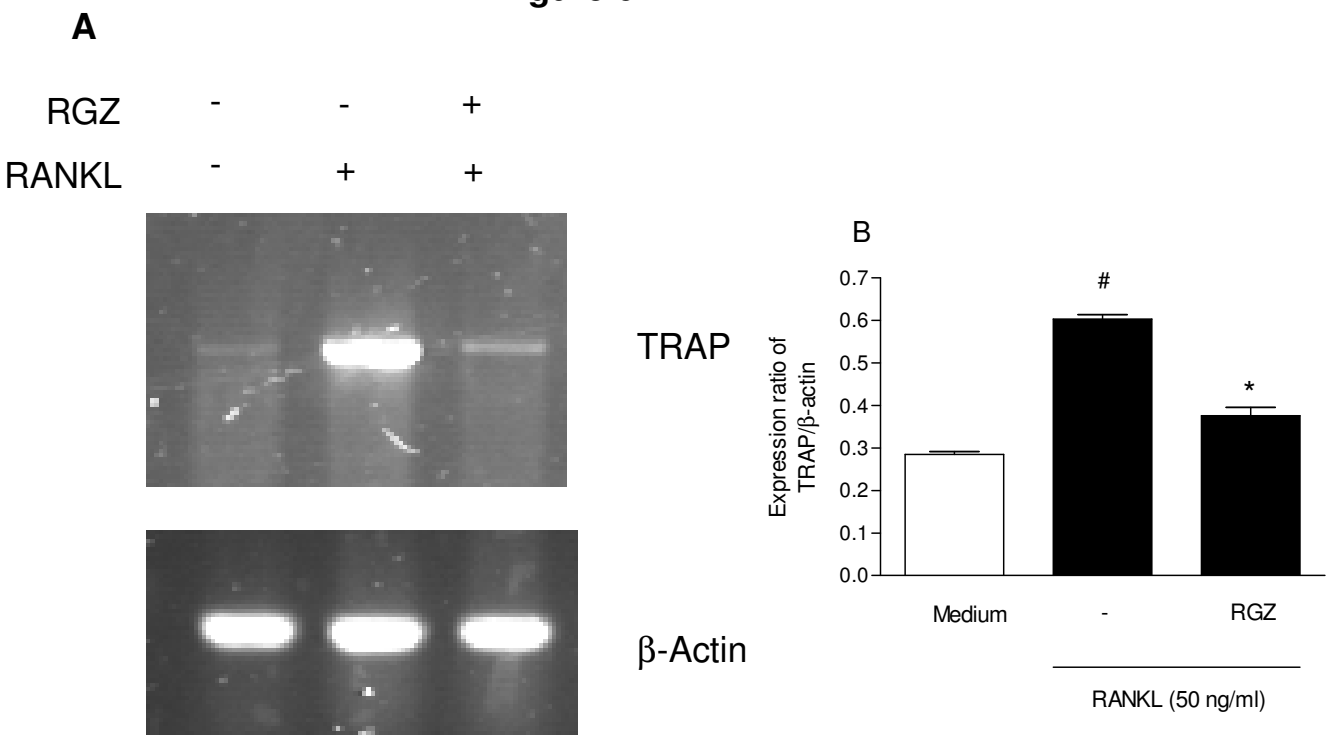
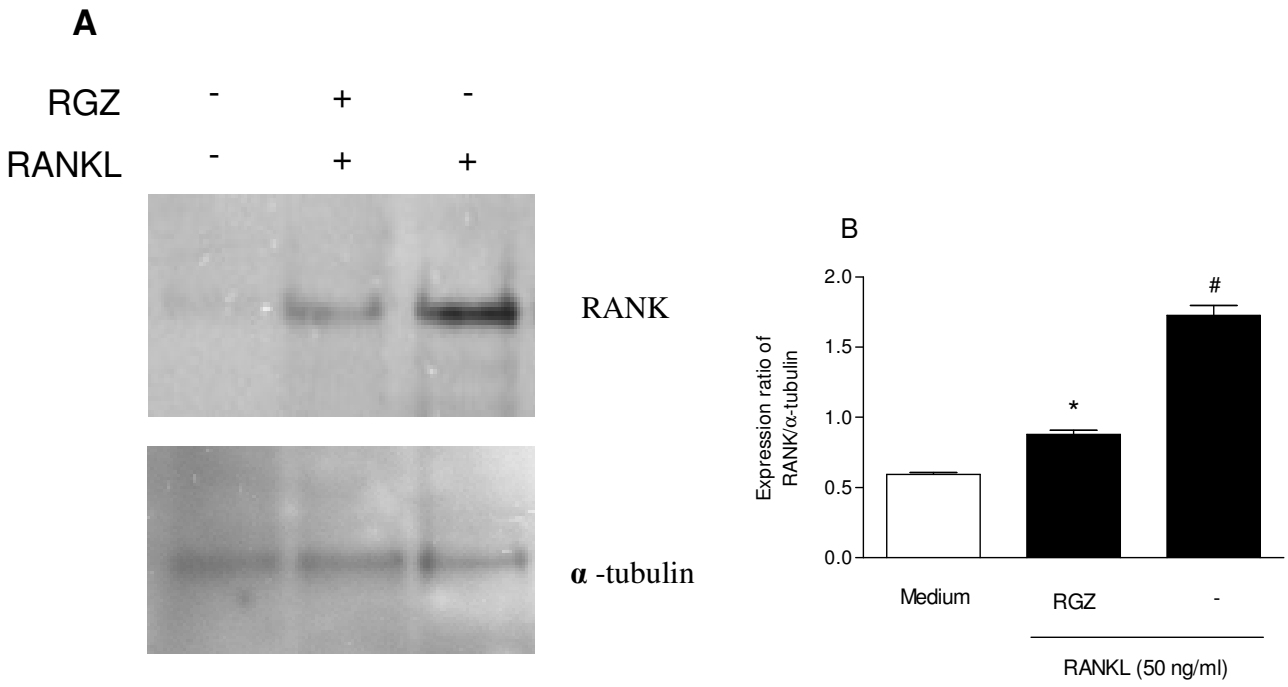

A
RGZ - - +
RANKL - + +
TRAP
β-Actin
B
Expression ratio of TRAP/β-actin
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
#
*
Medium
-
RGZ
RANKL (50 ng/ml)

Figure 7


A
RGZ - + -
RANKL - + +
RANK
α -tubulin
B
Expression ratio of RANK/α-tubulin
2.0
1.5
1.0
0.5
0.0
*
#
Medium
RGZ
-
RANKL (50 ng/ml)