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RANKL From Osteocytes Contributes to Periodontal Bone Loss

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Abstract
Periodontal bone loss results from bacterial infection and the associated host response. The ligand for the receptor activator of NF-κB (RANKL) induces the differentiation of osteoclasts, resulting in periodontal bone loss. The role of osteocytes in periodontal bone loss was investigated in this study. Transgenic mice expressing RANKL under the control of dentin matrix protein 1 (DMP1) were infected with *Porphyromonas gingivalis-Fusobacterium nucleatum* bacteria (Pg-Fn) to induce periodontitis, and type 1 diabetes was induced in groups of mice. Control (DMP1Cre^{-}.RANKL$^{f/f}$) mice with periodontal infection showed increases in bone loss, osteoclast counts, eroded bone surfaces, and RANKL expression compared to experimental (DMP1-Cre^{+}.RANKL$^{f/f}$) mice. RANKL deletion from osteocytes resulted in less periodontal bone loss. Diabetes enhanced periodontal bone loss in the control mice. The diabetic control (DMP1Cre^{-}.RANKL$^{f/f}$) mice had more bone loss than the non-diabetic control (DMP1Cre^{-}.RANKL$^{f/f}$) mice. In the experimental (DMP1-Cre^{+}.RANKL$^{f/f}$) mice, diabetes had no influence. This study demonstrated, for the first time, the essential role of osteocytes in periodontal bone loss.

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ABSTRACT

Periodontal bone loss results from bacterial infection and the associated host response. The ligand for the receptor activator of NF-κB (RANKL) induces the differentiation of osteoclasts, resulting in periodontal bone loss. The role of osteocytes in periodontal bone loss was investigated in this study. Transgenic mice expressing RANKL under the control of dentin matrix protein 1 (DMP1) were infected with *Porphyromonas gingivalis- Fusobacterium nucleatum* bacteria (Pg-Fn) to induce periodontitis, and type 1 diabetes was induced in groups of mice. Control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice with periodontal infection showed increases in bone loss, osteoclast counts, eroded bone surfaces, and RANKL expression compared to experimental (DMP1-Cre^{+}.$RANKL^{f/f}$) mice. RANKL deletion from osteocytes resulted in less periodontal bone loss. Diabetes enhanced periodontal bone loss in the control mice. The diabetic control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice had more bone loss than the non-diabetic control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice. In the experimental (DMP1-Cre^{+}.$RANKL^{f/f}$) mice, diabetes had no influence. This study demonstrated, for the first time, the essential role of osteocytes in periodontal bone loss.

Literature review

Periodontal disease is a group of disorders that affect the periodontium. When inflammation is confined to gingiva without attachment loss, it is called gingivitis, and when it progresses to connective tissue and bone, resulting in attachment loss that manifests as pocket formation and/or gingival recession, it is referred to as periodontitis. Periodontitis is a biofilm-induced infectious disease that affects the periodontium; about 46% of US adults have periodontitis (Eke et al. 2015).

Dental plaque plays an essential role in the initiation of periodontal disease. However, it is the host response to this infection that propagates destruction in the periodontium. A biofilm is a multi-species community of microorganisms that adhere to each other and a surface and are embedded in an extracellular matrix (a complex polymeric substance that protects the microorganisms from environmental stresses). Bacteria living in a biofilm differ in physiology from free-living bacteria and are more difficult to eradicate with antibiotics. Dental plaque is the prototypical example of a biofilm (Glossary of Periodontal Terms, AAP, 2012). Up to 700 bacterial species are able to colonize the mouth, and any individual may typically harbor 200-300 species (Griffen et al. 2011).

Many bacteria species are involved in subgingival pockets and associated with periodontal diseases. They include *P. gingivalis, T. forsythia, T. denticola, F. nucleatum, P. intermedia*, and *Aggregatibacter actinomycetemcomitans* (Dzink et al. 1988, Lisgarten Max A. 1994, Socransky et al. 1998). As periodontal disease progresses, there is a shift from gram-positive microbiota to gram-negative anaerobic bacteria. These bacteria can produce factors that directly or indirectly cause the destruction of the periodontium, namely lipopolysaccharides (endotoxins), collagenase, immunoglobulin proteases, fibrinolysins, and hemolysins (Holt SC and Bramanti TE. 1991).

As bacteria invade the sulcus, an initial gingivitis lesion develops. An early lesion is next, followed by an established lesion. The subsequent stage is that of the advanced lesion (Page and Scheroder 1976). The advanced lesion marks the transition from gingivitis to periodontitis.

IL-1 and TNF from keratinocytes, endothelial cells, and other cells are capable to induce the production of IL-8, which is a major cytokine involved in the recruitment of neutrophils to the site of inflammation (Oppenheim et al. 1991). The vascular permeability increases and complement activation occurs, resulting in the increased recruitment of neutrophils and monocytes. Macrophages produce PE_2 (Offenbacher et al. 1993), which has the effect of bone resorption and produces other inflammatory mediators, including IL-lβ, 6, 10, and 12, tumor necrosis factor α (TNFα), matrix metalloproteinases (MMPs), interferon γ (INFγ), and a series of chemotactic substances (monocyte chemoattractant protein (MCP), macrophage inflammatory protein (MIP), and RANTES (regulated on activation, normal T-cell expressed and secreted)) (Korman et al. 1997, Page et al. 1997). As the inflammation progresses to deeper connective tissue, fibroblasts play a major role in periodontal breakdown by producing pro-inflammatory mediators such as PE_2 and MMPs.

Activated T cells produce a large number of inflammatory cytokines that induce the further destruction of the periodontium and facilitate the differentiation of B cells and the production of antibodies. Th1 is a type of $CD4^+$ T cell that produces pro-inflammatory cytokines such as INF γ (Vitetta et al. 1987, Mosmann and Coffman. 1989). There is evidence that Th17 cells are present in chronic inflammation in human periodontal disease and that IL-17 expression occurs in the alveolar bones of patients with chronic periodontitis (Cardoso et al. 2009).

Periodontal bone resorption due to osteoclastic activity is related to the proximity of the inflammatory infiltrate (Rowe and Bradley 1981). The receptor activator of nuclear factor kappa-B (RANK) and its ligand (RANKL) was recognized as a member of the family of tumor necrosis factor (TNF) receptors and ligands (Anderson et al. 1997, Wong et al. 1997). RANKL is a type II transmembrane protein of 316 amino acids with a predicted cytoplasmic domain of 48 amino acids and an extracellular domain of 247 amino acids (Anderson et al. 1997). Osteoprotegerin (OPG) is another member of the TNF receptor family (Simonet et al. 1997); it is a decoy RANKL receptor that prevents RANKL from binding to the receptor activator of nuclear factor kappa-B (RANK) and results in the inhibition of bone resorption. RANKL, RANK, and OPG are three key molecules that regulate osteoclast recruitment and function (Suda et al. 1999). Osteoclasts are multinucleated cells that originate from hematopoietic precursors and are responsible for bone resorption (Scott B.L. 1967, Marks S.C. 1983).

RANKL was identified as a major factor for oesteoclastogenesis (Kong et al. 1999): RANKL-deficient mice showed severe osteopetrosis with no osteoclast formation (Kim et al. 2000). TNFα and β, and IL-1α and β are involved in the stimulation of osteoclastic bone resorption (Pfeilschifter et al. 1989, Balga et al. 2006). IL-6 plays a role in the stimulation of osteoclast formation by inducing the release of IL-1β (Lowik et al. 1989, Kurihara et al. 1990). Another osteoclastogenic cytokine is IL-11 (Girasole et al. 1994). RANKL expression has been identified in various tissues, including T cells, dendritic cells (Wong et al. 1997), osteoblasts (V. Kartsogiannis et al. 1999), osteocytes (Tanaka et al. 1995), and fibroblasts (Kanzaki et al. 2001).

Several risk factors that affect periodontal diseases have been identified. One of the most important ones is diabetes mellitus (AAP academy report 1999, Mealy and Oates 2006). The term “diabetes mellitus” describes a metabolic disorder of multiple etiologies that is

characterized by chronic hyperglycemia with disturbances of carbohydrate, fat, and protein metabolism and results from defects in insulin secretion, insulin action, or both (Zimmet and Alberti 1998). Type 1 diabetes (previously known as insulin-dependent, juvenile, or childhood-onset diabetes) is characterized by deficient insulin production and requires the daily administration of insulin. The cause of type 1 diabetes is not known, and it is not preventable with current knowledge. Symptoms include the excessive excretion of urine (polyuria), excessive thirst (polydipsia), constant hunger, weight loss, changes in vision, and fatigue. These symptoms may occur suddenly. Type 2 diabetes results from the body's lack of response to insulin combined with inadequate production of insulin to compensate for the insulin resistance. Type 2 diabetes accounts for 90% of the cases of diabetes around the world (Zimmet and Alberti 1998) and is largely the result of excess body weight and physical inactivity. The associated symptoms may be similar to those of type 1 diabetes, but they are often less marked. As a result, the disease may be diagnosed several years after onset, once complications have already arisen. Until recently, this type of diabetes was evident in adults, but it now also occurs in children (WHO Diabetes Fact Sheet, 2015). According to recent statistics, 9.3% of (29.1 million) Americans have diabetes, and 25.9% of the population aged 65 years or more has diabetes (CDC. National Diabetes Statistics Report, 2014). Periodontitis is considered to be the sixth complication of diabetes mellitus; diabetes mellitus is associated with increased periodontal attachment and bone loss (Löe H. 1993). As early as 1862, there were reports linking diabetes mellitus to severe periodontal destruction; diabetic patients with periodontal diseases experienced more gingival inflammation and attachment loss than non-diabetic periodontitis patients (Cohen et al. 1970). Diabetes is associated with elevated levels of inflammatory mediators. Advanced glycation end

products (AGEs) are associated with increased oxidative stresses and upregulate inflammatory mediators such as IL-1β, TNFα, and IL-6 (Preshaw et al. 2012).

Diabetes enhances monocytic TNFα activity in diabetic patients with periodontitis 4.6 times (Salvi et al. 1997). Other studies show elevated levels of pro-inflammatory cytokines, including IL-1β, TNFα and PGE_2, in diabetic patients with periodontal disease (Salvi et al. 1997). Diabetes associated with an altered inflammatory response to bacteria impacts periodontitis, wound healing, and apoptosis (Graves et al. 2006).

In normal bone remodeling, bone resorption and bone formation are coupled. However, in periodontitis, this coupling is impaired (Baylink and Liu CC. 1979). In diabetes, cytokines such as IL-1β and TNFα can up-regulate RANKL expression in periodontal cells and increase osteoclast formation (Pacios et al. 2012). Due to persistent bacterial inflammation, diabetes results in the aggravation of the condition through enhanced bone loss and diminished bone formation (Liu et al. 2006).

Since it is unethical to investigate disease processes in humans, animal models have been introduced, allowing the investigation of disease processes and cause-effect relationships. Animal models, including those involving mice (Baer and Newton. 1960), rats (Rovin et al. 1966), squirrel monkeys (Kennedy and Polson 1973), and beagle dogs (Saxes et al. 1967) have been described to investigate periodontitis. Different animal models for studying periodontitis have been established. They include the rat ligature model, the rat *A. actinomycetemcomitans* infection model, the oral gavage model, the lipopolysaccharide injection model, the calvarial model, and the rat-mandibular critical size defect model (Graves et al. 2008, Graves et al. 2012). Polak et al. (2009) showed that oral inoculation with *Porphyromonas gingivalis-Fusobacterium nucleatum* bacteria (Pg-Fn) was an effective model for studying periodontitis.

Mouse models are considered to be among the best animal models of disease: Mice and humans share 95-99% of their DNA (Gregory et al. 2002, Waterston et al. 2002). The ability to knock out a specific mouse gene induced a huge advance in understanding the nature of the disease and offered promising insights into possible treatments. More than 4,000 out of ~35,000 mouse genes have been knocked out, and more than 500 mouse models of human disease have been created (Goldstein J. 2001, Austin et al. 2004).

The expression of RANKL in B and T lymphocytes in periodontal tissues was investigated. RANKL from B and T lymphocytes reported to be higher in patients with chronic periodontitis than in patients with healthy periodontium (Kawai et al. 2006). RANKL mRNA was higher in advanced periodontitis, and the level of OPG mRNA associated with both advanced and moderate periodontitis was lower than that in the healthy group. Moreover, RANKL mRNA was expressed in inflammatory cells, mainly lymphocytes and macrophages. In addition, the proliferating epithelium in the vicinity of inflammatory cells expressed high levels of RANKL mRNA (Liu et al. 2003).

IL-1a stimulated both the upregulation of RANKL expression and the downregulation of OPG expression via PGE2 production, depending on ERK activation of human periodontal ligament fibroblasts (Fukushima et al. 2005). By contrast, another study showed that PDL fibroblasts played a protective role in inflammation and LPS stimulated the expression of OPG rather than that of RANKL in periodontal ligament fibroblasts through the induction of IL-1β and TNFα (Wada et al. 2004). Human PDL fibroblasts from patients with chronic periodontitis expressed a RANKL level 2.5 times higher than that of healthy gingival fibroblasts and higher levels of IL-6 and TNFα (Matarese et al. 2015).

Osteoblast lineage cells express RANKL for differentiation and the activation of osteoclasts (Lacey et al. 1998, Hofbauer et al. 2000). Mice with RANKL deficiency in osteoblast lineage were protected from bone loss induced by ovariectomy and from joint destruction associated with arthritis, whereas the loss of RANKL in T cells did not confer such protection (Fumoto et al. 2014). Another study demonstrated the important role of osteoblast lineage cells in inflammation-induced periodontal bone loss (Pacios et al. 2015). *P. gingivalis*-infected mouse osteoblastic cells induced RANKL expression in osteoblasts through activator protein1 (AP-1) signaling pathways (Okahashi et al. 2004).

For many years, osteocytes were thought to be inactive cells that resided inside lacunae. Recent studies have revealed these cells' major role in controlling bone hemostasis (Dallas et al. 2013, Al-Dujaili et al. 2011). RANKL expression by osteocytes was first identified in vitro in chick cells (Tanaka et al. 1995). Later, MLO-Y4 osteocyte-like cells (from mice) expressed RANKL on their surfaces (Zhao et al. 2002). There is recent evidence of the regulation of bone remodeling by osteocytes through RANKL expression; the deletion of RANKL from osteocytes results in an increase in bone mass (Xiong et al. 2011, Xiong et al. 2015). RANKL expression in osteocytes is essential for osteoclast formation and for controlling bone remodeling (O'Brien and Xiong 2012, Nakashima et al. 2011) as well as controlling bone loss in unloaded conditions (Xiong et al. 2011, Tatsumi et al. 2007).

The role of osteocytes in periodontitis-associated bone loss has not been fully studied. RANKL expression by those cells has been established, but the magnitude of the effect of those cell type in periodontal bone loss is still to be investigated.

Dentin matrix acidic phosphoprotein 1 (DMP1) is a major constituent of the extracellular matrix of bone and dentin; it was first isolated from dentin (Georg et al. 1993) then from enamel, bone,

and cementum (D'Souza et al. 1997, MacDougall et al. 1998). Recent studies demonstrate that osteocytes express high levels of DMP1 (Toyosawa et al. 2001, Feng et al. 2002, Lu et al. 2007, Canalis et al. 2013). Transgenic mice expressing the diphtheria toxin receptor under the control of dentin matrix protein 1 (DMP1) promoter serve as a mouse model for the study of osteocytes (Komori T. 2014).

Cre (cyclization recombination) is a site-specific recombinase that allows for deletions, insertions, or modification at specific sites in the DNA of cells utilizing the Cre/loxP system (where "loxP" refers to the locus of crossing [x-ing]-over of bacteriophage P1). The Cre/loxP system functions by delivering Cre to specific cells or tissues using a particular promoter. The Cre recombinase excises the essential exon of the gene of interest, which is floxed (sandwiched between two loxP sites), thus generating a null allele in all cells where Cre is active.

Cre activity under the control of the 9.6-kb DMP1 promoter gene was established (Lu et al. 2007). Using Cre recombinase, it is possible to delete the floxed RANKL genes (encoded by the Tnfsf11 gene) in cells expressing the DMP1 gene in transgenic mice (Nakashima et al. 2011).

To confirm the selective deletion of RANKL in osteocytes, a reporter mouse R26R line that expressed ROSA26 reporter genes was crossed with (DMP1- Cre.RANKL$^{f/f}$) transgenic mice. By detecting b-galactosidase activity with either X-gal staining or immunofluorescence staining that targets b-galactosidase in the tissues, we can examine the effectiveness of Cre activation and identify the tissue-specific distribution of Cre recombinase with a specific promoter (Soriano P. 1999).

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Introduction

Periodontitis is an inflammatory disease that results in clinical attachment loss, alveolar bone loss, and periodontal pocketing. It is initiated by a bacterial biofilm and progresses due to a host response that leads to soft and hard tissue destruction (Graves and Cochran 2003). Up to 700 bacterial species can colonize the mouth; any individual may typically harbor 200-300 such species (Griffen et al. 2011). Many bacterial species are involved in subgingival pockets and associated periodontal diseases. They include *P. gingivalis, T. forsythia, T. denticola, F. nucleatum, P. intermedia*, and *Aggregatibacter actinomycetemcomitans* (Dzink et al. 1988, Lisgarten Max A. 1994, Socransky et al. 1998).

Tissue destruction is mediated by the production of cytokines by many types of cells. Examples of these cytokines are IL-1β, TNFα (Graves 2008), and the ligand for the receptor activator of NF-κB (RANKL), which induce the differentiation of osteoclasts (Yamashita et al. 2007). RANKL-deficient mice showed severe osteopetrosis with no osteoclast formation (Kim et al. 2000).

RANKL from lymphocytes was considered the primary cause of osteoclast-induced periodontal bone loss (Kawai et al. 2006). However, a recent study (Pacios, S. et al. 2015) demonstrated the important role of osteoblast-lineage cells in inflammation-induced periodontal bone loss. RANKL expressed in osteocytes is essential for osteoclast formation and controlling bone remodeling (O'Brien and Xiong 2012, Nakashima et al. 2011) as well as controlling bone loss in unloaded conditions (Xiong et al. 2011, Tatsumi et al. 2007).

Dentin matrix acidic phosphoprotein 1(DMP1) is a major constituent of the extracelluar matrix of bone and dentin. It was first isolated from dentin (Georg et al. 1993) then from enamel, bone,

and cementum (D'Souza et al. 1997, MacDougall et al. 1998), Recent studies demonstrate that DMP1 is expressed at high levels in osteocytes (Toyosawa et al. 2001, Feng et al. 2002, Lu et al. 2007, Canalis et al. 2013).

Oral inoculation with Pg-Fn bacteria is an effective model for studying periodontitis (Graves et al. 2008, Graves et al. 2012, Polak et al. 2009). Transgenic mice expressing the diphtheria toxin receptor under the control of dentin matrix protein 1 (DMP1) promoter serve as a mouse model for studying osteocytes (Tatsumi et al. 2007, Komori T. 2014).

Several risk factors that affect periodontal diseases have been identified; one of the most important factors is diabetes mellitus (AAP academy report 1999, Mealy and Oates 2006). According to recent statistics, 9.3% of (29.1 million) of Americans have diabetes, and 25.9% of the population aged 65 years or more have diabetes (CDC. National Diabetes Statistics Report, 2014). Periodontitis is considered to be the sixth complication of diabetes mellitus; diabetes mellitus is associated with increased periodontal attachment and bone loss (Löe H. 1993). As early as 1862, there were reports linking diabetes mellitus to severe periodontal destruction; diabetic patients with periodontal diseases had more gingival inflammation and attachment loss than non-diabetic periodontitis patients (Cohen et al. 1970). Diabetes is associated with elevated levels of inflammatory mediators. Moreover, advanced glycation end products (AGEs) are associated with increased oxidative stress and upregulate inflammatory mediators such as IL-1β, TNFα, and IL-6 (Preshaw et al. 2012).

Diabetes enhances monocytic TNFα activity in diabetic patients with periodontitis 4.6 times (Salvi et al. 1997). Other studies show elevated levels of pro-inflammatory cytokines, including IL- 1β, TNFα, and $PGE_{2,}$ in diabetic patients with periodontal disease (Salvi et al. 1997).

Diabetes associated with altered inflammatory responses to bacteria has an impact on periodontitis, wound healing, and apoptosis (Graves et al. 2006).

In normal bone remodeling, bone resorption and bone formation are coupled. However, in periodontitis, this coupling is impaired (Baylink and Liu CC. 1979). In diabetes, cytokines such as IL-1β and TNFα can up-regulate RANKL expression in periodontal cells and increase osteoclast formation (Pacios et al. 2012). Due to persistent bacterial inflammation, diabetes results in the aggravation of the condition through enhanced bone loss and diminished bone formation (Graves et al. 2006).

Cre (cyclization recombination) is a site-specific recombinase that allows for deletions, insertions, or modification at specific sites in the DNA of the cells utilizing the Cre/loxP system (where "loxP" is the locus of crossing [x-ing]-over of bacteriophage P1). The Cre/loxP system works by delivering Cre to specific cells or tissues using a particular promoter. The Cre recombinase excises the essential exon of the gene of interest, which is floxed (sandwiched between) two loxP sites, thus generating a null allele in all cells where Cre is active.

Cre activity under control of the 9.6-kb Dmp1 promoter gene was established (Lu et al. 2007). Using Cre recombinase, it is possible to delete the floxed RANKL genes (encoded by the Tnfsf11 gene) in cells expressing the DMP1 gene in transgenic mice (Nakashima et al. 2011).

To confirm the selective deletion of RANKL in osteocytes, a reporter mouse R26R line that expressed ROSA26 reporter genes was crossed with (DMP1- Cre.RANKL$^{f/f}$) transgenic mice. By detecting b-galactosidase activity with either X-gal staining or immunofluorescence staining that targeted b-galactosidase in the tissues, it was possible to examine the effectiveness of Cre activation and identify the tissue-specific distribution of Cre recombinase with a specific promoter (Soriano P. 1999).

Since no published studies have evaluated the role of osteocytes in periodontitis, the aim of this study is to investigate the role of RANKL from osteocytes in periodontitis-induced bone loss and to determine whether diabetes has an influence on it.

The first null hypothesis (H_0) follows: RANKL deletion from osteocytes will have no effect on periodontal bone loss. Its alternative hypothesis (H_1) follows: RANKL deletion from osteocytes will result in less periodontal bone loss.

The second null hypothesis (H_0) follows: RANKL deletion from osteocytes in diabetic subjects will have no effect on periodontal bone loss. Its alternative hypothesis (H_1) follows: RANKL deletion from osteocytes in diabetic subjects will result in less periodontal bone loss.

Materials and method

All methods were carried out in accordance with the approved guidelines of the Institutional Animal Care and Use Committee at the University of Pennsylvania. All mice were housed in the Levy Animal Facility for Oral Health Research at the University of Pennsylvania School of Dental Medicine. Later, for the experiment design stage (bacterial inoculation required a Biosafety Level 2 lab), they were transferred to John Morgan Building's animal facility at the University of Pennsylvania's Perelman School of Medicine.

Transgenic mice (DMP1- Cre.RANKL$^{f/f}$) were obtained from Dr. Jerry Feng of the Baylor College of Dentistry in Dallas, Texas. They expressed Cre recombinase under the control of the Dmp1 promoter in osteocytes as described in the literature (Lu et al. 2007). DMP1-Cre.ROSA26 transgenic mice for monitoring the Cre recombinase expression site were obtained from Jackson Laboratories (in Bar Harbor, Maine).

All experiments were carried out when the mice were 8-10 weeks old. Two of them were conducted at the same time. The first experiment compared the effect of RANKL deletion from osteocytes on periodontal bone loss in the normoglycemic experimental (DMP1- Cre^{+}.RANKL$^{f/f}$) group to that in the normoglycemic control (DMP1- Cre^{-}.RANKL$^{f/f}$) group. The second experiment compared the effect of RANKL deletion from osteocytes on periodontal bone loss in the hyperglycemic experimental (DMP1- Cre^{+}.RANKL$^{f/f}$) group to that in the hyperglycemic control (DMP1- Cre^{-}.RANKL$^{f/f}$) group.

Induction of diabetes

The weights of the mice were taken 2 times before the induction of diabetes. When the mice were approximately 8-10 weeks old, type 1 diabetes was induced in them through the intraperitoneal injection of streptozotocin (STZ) (50 mg/kg; 5mg/mL in 100 mM sodium citrate pH 4.5 buffer; Sigma, St. Louis, Missouri) for 5 consecutive days. Control animals were treated with citrate buffer alone (1.47 gr of sodium citrate to 500 ml distilled water (DW), adjusted pH 4.5). Ten days after the last STZ injection, blood glucose was taken by tail prick to determine whether hyperglycemia had developed. All glucose measurements were taken in the morning using blade#11. The weights of the mice were obtained every week after the STZ injection.

The mice were considered to be hyperglycemic when their glucose values exceeded 225 mg/dl. If the mice were not diabetic, up to 4 more injections was given as follows:

200-220 mg/dl - 1 STZ injection

175-200 mg/dl - 2 STZ injections

150-175 mg/dl - 3 STZ injections

125-150 mg/dl - 4 STZ injections

<125 mg/dl - 5 STZ injections

Diabetic mice are vulnerable to dehydration and weight loss. The mice that lost more than 10% of weight compared to the control mice were given a dietary supplement (Love Mash™, Bio.Serv; Flemington, New Jersey). The mice were checked for dehydration; if a mouse was dehydrated, a subcutaneous injection of 1 mL of 0.9% saline solution (9 g of NaCl to 100 ml DW) was administered daily. Mice were maintained in the diabetic (hyperglycemic) state for at least one week before the next step. The final blood glucose measurement was obtained immediately after euthanasia.

Antibiotic treatment

A 5-ml preparation of trimethoprim (1.6 mg/ml) and sulfadimethoxine (8 mg/ml) was added to the drinking water (150 ml of DW) of each cage and changed every other day in the morning for 7 consecutive days. These broad spectrum antibiotics were administered to all mice to reduce the endogenous oral flora.

Induction of periodontitis

Bacteria preparation and inoculation

For this study, the anaerobic bacteria, *Porphyromonas gingivalis* (Pg) strain (ATCC 33277) and *Fusobacterium nucleatum* (Fn) strain (ATCC 25586), were prepared in an anaerobic chamber from frozen culture. The bacteria were tested for aerobic contamination. The details of the bacteria inoculation follow: We inoculated 100 μl (50 μl on each side of the molars) with a gavage needle in each mouse's mouth two times, between the mucosal tissue of cheek and the molar teeth. Overall, *P. gingivalis* and F. nucleatum (2x10^9 colony forming unit CFU in 200 ul of 2% methylcelluolose) were given to the mice; the non-infected group was inoculated with the vehicle alone (100 μl of 2% methylcellulose in sterile PBS). Bacterial inoculation was performed three times every week over a 2-week period. The mice were monitored for a period of 6 weeks, and their weights were checked weekly.

Mouse euthanasia and tissue harvesting

The mice were all euthanized at the same time: during the 6th week. They were decapitated under 150-200 mg/kg of ketamine and 15-20 mg/kg of xylazine and 1-3 mg/kg of Acepromazine. Specimens of their tails were obtained for PCR genotyping to confirm the initial genotyping for RANKL and Cre expression (Figure1).

Tissue harvesting

The specimens were fixed in 5 ml/specimen of 4% paraformaldehyde at 4°C for 24 hours. Then they were taken for microCT and, subsequently, decalcified in 10% EDTA (Fisher Scientific; Hampton, New Hampshire) for 4–5 weeks. The specimens were embedded in paraffin and sectioned in sagittal sections of 4-μm thickness that included the region of the 1st-2nd and 2nd-3rd molars, including the teeth, PDL, alveolar bone, and gingiva.

MicroCT analysis

Specimens were scanned using an in vivo MicroCT machine (viva CT40 SCANCO Medical, Brüttisellen, Switzerland) and analyzed using OssiriX MD imaging software (Pixmeo; Geneva, Switzerland). The bone area between the first and second molar was measured. Measurement was performed on the sagittal section of the MicroCT, considering that the root canal of the distobuccal root of the first molar and the mesiobuccal root of the second molar was visible in the sagittal section. The first cement-enamel junctions (CEJs) of the first and second molars had to be identified by increasing the WL contrast to around 8000. A line was drawn from the CEJ of the first molar to the CEJ of the second molar, another perpendicular line was drawn extending 700 um downwards from each CEJ to the roots, and the last line connected the two vertical lines

(a box with height of 700 um and oriented on the CEJs of the teeth helped with measurements). The contrast was decreased to around 3000 for bone area measurement. The area between the roots, the CEJ, and the height limit line was calculated as the total area. Then the area of the alveolar bone inside the total area was calculated as the remaining bone area (Figure2a,b). Afterwards, the percentage of the remaining bone area was calculated using the formula, % of Remaining Bone Area = (Bone Area/ Total Area)*100.

Histomorphometric analysis

Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) staining were performed on the first slide (the slide that showed the 1st and 2nd molar crowns, roots, and root canals) of each specimen to ensure consistency. Bone area measurement was performed using Nikon NIS-elements D image analysis software (Nikon, Tokyo, Japan) at 20X magnification, as follows: A horizontal line connected the CEJ of the 1st molar to the 2nd molar, then a vertical line was extended 0.08 mm downwards for the epithelium and part of the connective tissue. Afterwards, a horizontal line was produced at that level, and a third vertical 0.32-mm line was made (two verticals, one for each root). The last line was a horizontal one connecting the two vertical lines. The area between the roots and the two horizontal lines was measured as the total area, and the bone area was measured the same way between the two roots and the two horizontal lines: The percentage of the remaining bone area was calculated using the formula, % Remaining Bone Area = (Bone Area/ Total Area)*100 (Figure3).

Trap Staining

A trap staining kit (Sigma Aldrich, St. Louis, Missouri), was used for osteoclast staining, osteoclast counts, and eroded bone measurement that employed Nikon NIS-elements D image analysis software (Nikon, Tokyo, Japan) at 20X magnification on the second slide of each specimen to ensure consistency: A horizontal line was made on the top of the interproximal bone (between the first and second molars). Then a 0.35-mm vertical line was made downwards from the first horizontal line (one for each root). The last line was a horizontal one connecting those two vertical lines. The osteoclasts on the distal side of the interproximal bone (mesial to second molar) were used for the osteoclast count. The number of osteoclasts was then divided by the bone length (distal bone); resorption lacunae on the distal side were also measured and divided by the total bone length (distal bone) then multiplied by 100 to get the eroded bone surface (Figure4).

RANKL Immunofluorescence Staining

Third-slide specimens were selected for the analysis of RANKL expression in order to confirm that RANKL had been knocked out from the osteocytes in the DMP1- Cre^{+}.$RANKL^{f/f}$ mice.

Antigen retrieval was undertaken using Proteinase K (1:10 dilution). Nonspecific binding blocking was performed using donkey serum blocking buffer. The primary antibody was RANKL Antibody (N-19) (sc-7628) goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, California) at a concentration of 2 ug (1:100). For control, we used normal goat IgG (5 mg/ml, 1-5000, Vector) at a concentration of 2 ug (1:100). The specimens were treated with the secondary antibody of donkey anti-goat biotinylated antibody (1:1000 dilution) (Jackson Immuno Research; West Grove, Pennsylvania) then subjected to ABC treatment (ABC Vector Elite; Vector Labs)

and TSA (1:500 dilution). Afterwards, the specimens were subjected to streptavidin-Alexa 546 (invitrogen S1225) (1:400) then mounted in DAPI solution. Measurements were done using MFI at a maximum intensity of 3000. Six to 7 pictures were taken around the alveolar bone. One picture was taken of connective tissue right above the bone crest, and another one was taken of the epithelium. Measurements for the bone (osteocytes), PDL, CT, and epithelium were determined. All images were captured using a fluorescence microscope (Eclipse 90i; Nikon) and a Cool-Snap EZ camera. Image analysis was performed using Nikon AR image analysis software (Nikon).

B-Gal Immunofluorescence Staining

The β-galactosidase (lacZ) gene is a reporter gene that is used to confirm the specificity of other genes.

Antigen retrieval was performed using citric acid (pH 6.0, 10 mM), and nonspecific binding blocking was undertaken with donkey serum blocking buffer. The primary antibody was rabbit anti-beta galactosidase polycolonal antibody (Bioss Antibodies; Woburn, Massachusetts) at a concentration of 1:200. For control, we used rabbit IgG (1:2500). Secondary antibody treatment entailed the use of biotinylated donkey anti-rabbit (1:200 conc.) then ABC treatment (ABC Vector Elite; Vector Labs) followed by streptavidin-Alexa 546 (invitrogen S-11225) (1:400). The specimens were mounted in DAPI solution. All images were captured using a fluorescence microscope (Eclipse 90i; Nikon) and a Cool-Snap EZ camera. Image analysis was performed using Nikon AR image analysis software (Nikon). Since the staining was performed to confirm tissue specificity, no measurements were obtained.

Statistical analysis

Statistical analyses were performed using GraphPad Prism6 Software (La Jolla, California). One-way ANOVA was used to compare the differences between groups; post-hoc tests were used. The significance level was set at $P<0.05$, and each group had 5-7 mice.

Results

Tissue specificity of Dmp1-Cre mice

Beta-galactosidase immunofluorescence of ROSA26 reporter mice revealed the tissue specificity of DMP1 mice. The test group showed the expression of beta-gal on about 80% of osteocytes and about 20% of bone lining cells (Figure5). The control group of C57BL/6J mice showed no expression (Figure5).

Bone area:

Micro CT Analysis

Effect of infection: In the normoglycemic (NG) group, oral infection induced 16% periodontal bone loss in control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice compared to the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P<0.05$). Infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice showed no difference in periodontal bone loss from the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P>0.05$), (Figure6b).

In the hyperglycemic (HG) group; oral infection induced 22% periodontal bone loss in the control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice compared to the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P<0.05$). The infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice showed no difference in periodontal bone loss from the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P>0.05$), (Figure7b).

Effect of RANKL: In the normoglycemic (NG) infected group; RANKL deletion played a significant role. Control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice had 16% more bone loss than experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P<0.05$). In the normoglycemic non-infected group, RANKL deletion resulted in no significant difference ($P>0.05$) (Figure6b).

In the hyperglycemic (HG) infected group, the control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice had 33% more bone loss than the experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P<0.05$). In the hyperglycemic (HG) non-infected group, the (DMP1- Cre^{-}.$RANKL^{f/f}$) mice had no significant bone loss compared to the experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P>0.05$), (Figure7b).

Effect of diabetes: In the non-infected groups, the hyperglycemic (HG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice experienced no significant bone loss compared to the normoglycemic (NG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P>0.05$). In addition, the hyperglycemic (HG) experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice experienced no significant bone loss compared to the normoglycemic (NG) experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P>0.05$).

In the infected groups, the hyperglycemic (HG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice experienced 19% more bone loss than the normoglycemic (NG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P<0.05$). The hyperglycemic (HG) experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice experienced no significant bone loss compared to the normoglycemic (NG) experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P>0.05$) (Figure8a).

Histology Analysis:

A. Bone area

Effect of infection: In the normoglycemic (NG) group, oral infection induced 19% periodontal bone loss in the control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice compared to the group of non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P<0.05$). Infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice experienced no significant bone loss compared to non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P>0.05$), (Figure6c).

In the hyperglycemic (HG) group, oral infection induced 31% periodontal bone loss in the control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice compared to the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P<0.05$). The infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice experienced no significant bone loss compared to the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P>0.05$), (Figure7c).

Effect of RANKL: In the normoglycemic (NG) infected group, RANKL deletion played a significant role. The control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice experienced 20% more bone loss than the experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P<0.05$), while, in the normoglycemic (NG) non-infected group, there was no significant difference ($P>0.05$ (Figure6c).

In the hyperglycemic (HG) infected group, the infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice experienced 38% more bone loss than the experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P<0.05$), while, in the non-infected group, the control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice experienced no significant bone loss compared to the experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P>0.05$), (Figure7c).

Effect of diabetes: In the non-infected groups, the hyperglycemic (HG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice experienced no significant bone loss compared to the

normoglycemic (NG) control (DMP1□Cre^{-}.RANKL$^{f/f}$) mice (P>0.05). Moreover, the hyperglycemic (HG) experimental (DMP1□Cre^{+}.RANKL$^{f/f}$) mice experienced no significant bone loss compared to normoglycemic (NG) experimental (DMP1□Cre^{+}.RANKL$^{f/f}$) mice (P>0.05).

In the infected groups, the hyperglycemic (HG) control (DMP1□Cre^{-}.RANKL$^{f/f}$) mice experienced 28% more bone loss than the normoglycemic (NG) control (DMP1□Cre^{-}.RANKL$^{f/f}$) mice (P<0.05). In addition, the hyperglycemic (HG) experimental (DMP1□Cre^{+}.RANKL$^{f/f}$) mice experienced no significant bone loss compared to the normoglycemic (NG) (DMP1- Cre^{+}.RANKL$^{f/f}$) mice, (P>0.05), (Figure8b).

B. Osteoclast counts

Effects of infection:

In the normoglycemic (NG) group, oral infection resulted in 4 times more osteoclasts in the control (DMP1- Cre^{-}.RANKL$^{f/f}$) mice than in the non-infected control (DMP1- Cre^{-}.RANKL$^{f/f}$) mice (P<0.05). The infected experimental (DMP1- Cre^{+}.RANKL$^{f/f}$) mice showed no significant difference from the non-infected control (DMP1□Cre^{-}.RANKL$^{f/f}$) mice (P>0.05), (Figure6d).

In the hyperglycemic (HG) group, oral infection resulted in 3 times more osteoclasts in the control (DMP1- Cre^{-}.RANKL$^{f/f}$) mice than in the non-infected control (DMP1□Cre^{-}.RANKL$^{f/f}$) mice (P<0.05). The infected experimental (DMP1- Cre^{+}.RANKL$^{f/f}$) mice showed no significant difference from the non-infected control (DMP1- Cre^{-}.RANKL$^{f/f}$) mice (P>0.05), (Figure7d).

Effect of RANKL: In the normoglycemic (NG) groups, the infected control (DMP1- Cre^{-}.RANKL$^{f/f}$) mice had 5 times more osteoclasts than the infected experimental

(DMP1- Cre^{+}.$RANKL^{f/f}$) mice (P<0.05). RANKL deletion had no significant effect on the non-infected normoglycemic (NG) mice (P>0.05), (Figure6d).

In the hyperglycemic (HG) groups, the infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice had 5 times more osteoclasts than the infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice (P<0.05). RANKL deletion had no significant effect on the non-infected normoglycemic (NG) mice (P>0.05) (Figure7d).

Effect of diabetes: In the non-infected group, hyperglycemic (HG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice showed no difference in comparison to the normoglycemic (NG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice (P>0.05). In the infected group, the hyperglycemic (HG) control (DMP1□Cre^{-}.$RANKL^{f/f}$) mice had 5 times more osteoclasts than the normoglycemic (NG) control (DMP1□Cre^{-}.$RANKL^{f/f}$) mice (P<0.05). Diabetes had no significant effect on the experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice in the infected and non-infected groups (P>0.05), (Figure8c).

C. Eroded Bone Surface

Effect of infection: In the normoglycemic (NG) group, the infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice had 5.7 times more eroded bone surfaces than the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice (P<0.05). The infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice showed no difference from the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice (P>0.05) (Figure6e).

In the hyperglycemic (HG) group, the infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice had 2.7 times more eroded bone surfaces than the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice

(P<0.05). Furthermore, the infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice showed no difference from the non-infected control (DMP1-Cre^{-}.$RANKL^{f/f}$) mice (P>0.05) (Figure7e).

Effect of RANKL: In the normoglycemic (NG) groups, the infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice had 5.3 times more eroded bone surfaces than the infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice (P<0.05). RANKL deletion had no significant effect on the non-infected groups (Figure6e).

In the hyperglycemic (HG) groups, the infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice had 3.5 times more eroded bone surfaces than infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice, (P<0.05). RANKL deletion had no significant effect on the non-infected groups (Figure7e).

Effect of diabetes: In the non-infected group, the hyperglycemic(HG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice showed no difference from the normoglycemic (NG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice (P>0.05). In the infected group, the hyperglycemic (HG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice had more eroded bone surfaces than the normoglycemic (NG) control (DMP1-Cre^{-}.$RANKL^{f/f}$) mice (P<0.05). In the non-infected group, the hyperglycemic (HG) control (DMP1- Cre^{+}.$RANKL^{f/f}$) mice showed no difference from the normoglycemic (NG) control (DMP1□Cre^{+}.$RANKL^{f/f}$) mice (P>0.05). In the infected group, the hyperglycemic(HG) control (DMP1- Cre^{+}.$RANKL^{f/f}$) mice showed no difference from the normoglycemic (NG) control (DMP1- Cre^{+}.$RANKL^{f/f}$) mice (P>0.05), (Figure8d).

D. RANKL Immunofluorescence

Effect of infection: In the normoglycemic (NG) group, oral infection induced 1.5 times more RANKL expression in the infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice than in the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P<0.05$). The infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice showed no difference from the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice (Figure7f).

In the hyperglycemic (HG) group, oral infection induced 2.2 times more RANKL expression in the infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice than in the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P<0.05$). The infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice showed no difference from the non-infected control (DMP1□Cre^{-}.$RANKL^{f/f}$) mice (Figure8f).

Effect of RANKL: In the normoglycemic (NG) groups, the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice showed no difference from the non-infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P>0.05$). The infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice showed 1.6 times more RANKL expression than the infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P<0.05$). (Figure7f).

In the hyperglycemic (HG) groups, the non-infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice showed no difference from the non-infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P>0.05$). The infected control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice showed 1.6 times more RANKL expression than the infected experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P<0.05$) (Figure8f).

Effect of diabetes: In the non-infected group, the hyperglycemic (HG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice showed no difference in RANKL expression in the bone from the normoglycemic (NG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P>0.05$). In the infected group, the

hyperglycemic (HG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice showed 1.7 times more RANKL expression in the bone than the normoglycemic (NG) control (DMP1- Cre^{-}.$RANKL^{f/f}$) mice ($P<0.05$). In the non-infected group, the hyperglycemic (HG) experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice showed no difference in RANKL expression in the bone from the normoglycemic (NG) experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P>0.05$). In the infected group, the hyperglycemic (HG) experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice showed no difference in RANKL expression in the bone from the normoglycemic (NG) experimental (DMP1- Cre^{+}.$RANKL^{f/f}$) mice ($P>0.05$) (Figure8e).

Discussion

We confirmed the tissue specificity of DMP1 in osteocytes. About 80% of periodontal bone osteocytes in the (DMP1-Cre.ROSA26) mice (about 20% of the bone lining cells) showed DMP1 expression. These lining cells could be osteocytes that have started to form on the bone's surface (Dallas et al. 2013; Dallas et al. 2015) or osteoblasts that form bone lining cells (Xiong et al. 2015).

Toyosawa et al. (2001) showed that DMP1 is expressed in the osteocytes of chicken and rat tibia. Feng et al. (2002) demonstrated DMP1 expression in the osteocytes of mice. RANKL expression in osteocytes was first identified in vitro from chick cells (Tanaka et al. 1995) and later identified in MLO-Y4 osteocyte-like cells (from mice) (Zhao et al. 2002).

The oral inoculation model of *Porphyromonas gingivalis–Fusobacterium nucleatum* bacteria (*Pg-Fn*) has shown their ability to induce periodontal bone loss. This is consistent with the findings of (Polak et al. 2009). These bacteria produce several pathogenic factors that induce tissue destruction as well as propagate the host response to inflammation. Periodontal tissue destructions occur as a result of bacterial infection and host response. Several cellular events happen during infection with these bacteria, including innate and adaptive immune response.

RANKL plays a major role in the differentiation and activation of osteoclasts. Activated osteoclasts resorb the bone. The role of RANKL from the osteocytes in the regulation of physiological bone remodeling has been well established (Nakashima et al. 2011; O'Brien and Xiong 2012; Xiong et al. 2015). However, the role of osteocytes in inflammation-induced bone loss is not well understood.

Periodontal bone loss was observed in infected control (DMP1-Cre^{-}.RANKL$^{f/f}$) mice as evident from the remaining bone area in the results of both microCT and histology. This bone loss

resulted from increased osteoclast activity, which was proven through the significantly increased number of osteoclasts in the infected control (DMP1-Cre^{-}.RANKL$^{f/f}$) mice. The same pattern was observed for the eroded bone surface and RANKL expression. On the other hand, there was lack of periodontal bone loss in infected experimental (DMP1-Cre^{+}.RANKL$^{f/f}$) mice.

Although RANKL is described as a membrane-bound protein (Anderson et al. 1997), Lum et al. (1999) showed that it can also be expressed in soluble form. Nakashima et al. (2011) suggested that the membrane-bound form of RANKL is more potent. Osteocytes have dendritic processes that connect them to each other. These dendritic processes can reach the bone surface and come into direct contact with osteoblasts and osteoclasts (Dallas et al. 2015). Through these possible pathways, osteocytes play a major role in controlling bone remodeling.

In this study, we found that diabetes enhances periodontal bone loss, which is consistent with earlier studies (Graves et al. 2006; Pacios et al. 2012). Diabetes can enhance inflammatory bone loss through the upregulation of the inflammatory response and the downregulation of the healing response. The findings in hyperglycemic (HG) infected control (DMP1-Cre^{-}.RANKL$^{f/f}$) mice were as expected: more osteoclasts and RANKL expression as results of higher inflammatory response to the periodontal infection as well as of the impaired reparative capacity. However, the new finding in this study is the prevention of periodontal bone loss through the blocking of RANKL from osteocytes.

It was thought that RANKL-induced periodontal bone loss is mainly from lymphocytes (Kawai et al. 2006). Recent findings from Pacios et al. (2015) revealed the important role of osteoblast lineage cells in RANKL-induced periodontal bone loss. This study revealed the critical role of osteocytes in inflammation-induced periodontal bone loss. However, further investigations are

needed, such as measuring the mRNA expression of RANKL and investigating the transcription level of the cellular mechanism of osteocytes in inflammatory bone loss.

The findings of this study open doors for new possible areas to study the role of other cells in controlling inflammatory bone loss. One of these is periodontal ligament fibroblast.

Based on our results we reject both of the null hypothesis and we accept both of the alternative hypothesis. We demonstrated for the first time the important role of osteocytes in periodontal bone loss through the expression of RANKL. This finding may contribute to future studies that target cells of this type, thus preventing inflammatory bone loss.

Annexes

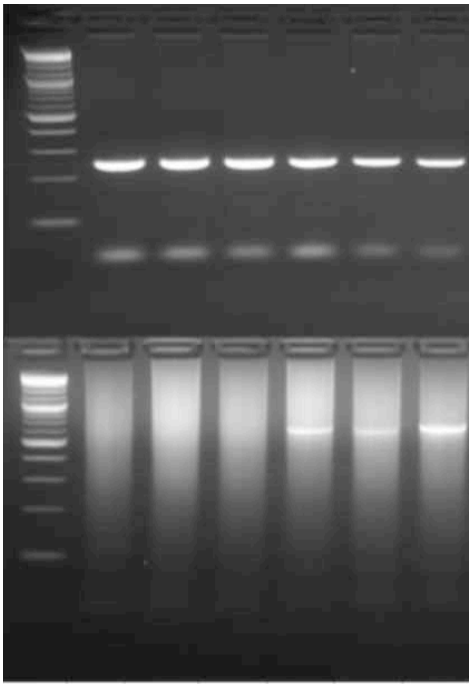

Figure1: PCR to confirm genotyping of RANKL and Cre.

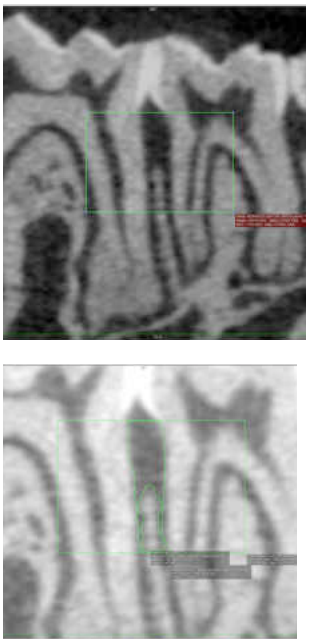

Figure2, A: measurement box at the level of the CEJs of the first and second molars, B: Total area and bone area measured between the distal and mesial roots of the first and second molars respectively.

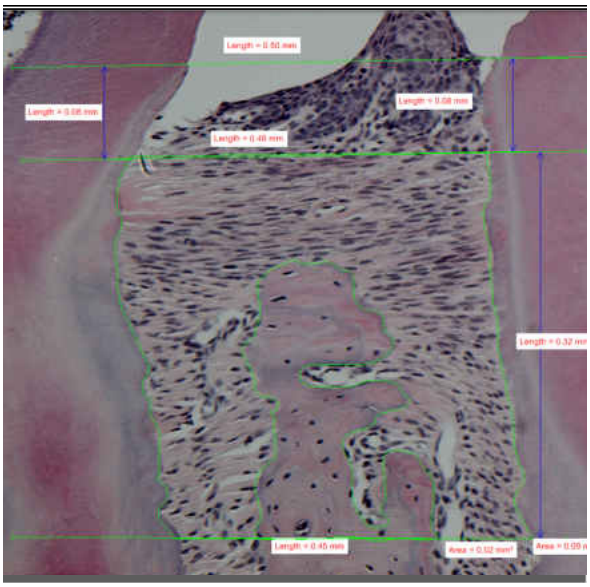

Figure3: Bone area measurements.

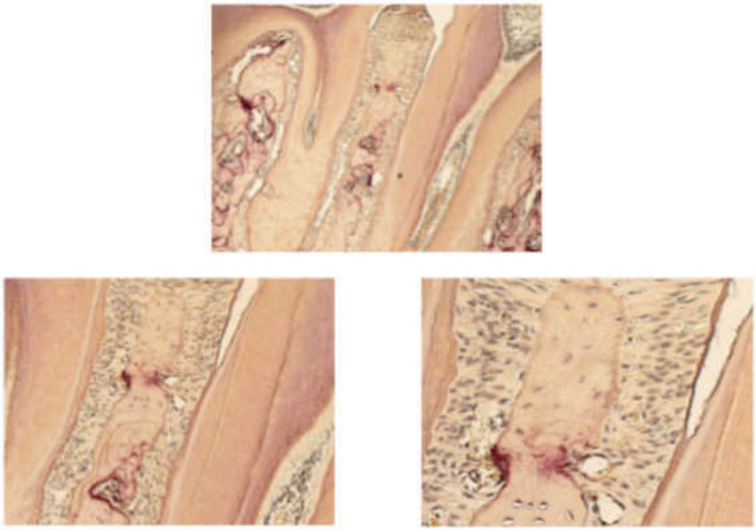

Figure4: Trap staining for osteoclasts and eroded bone.

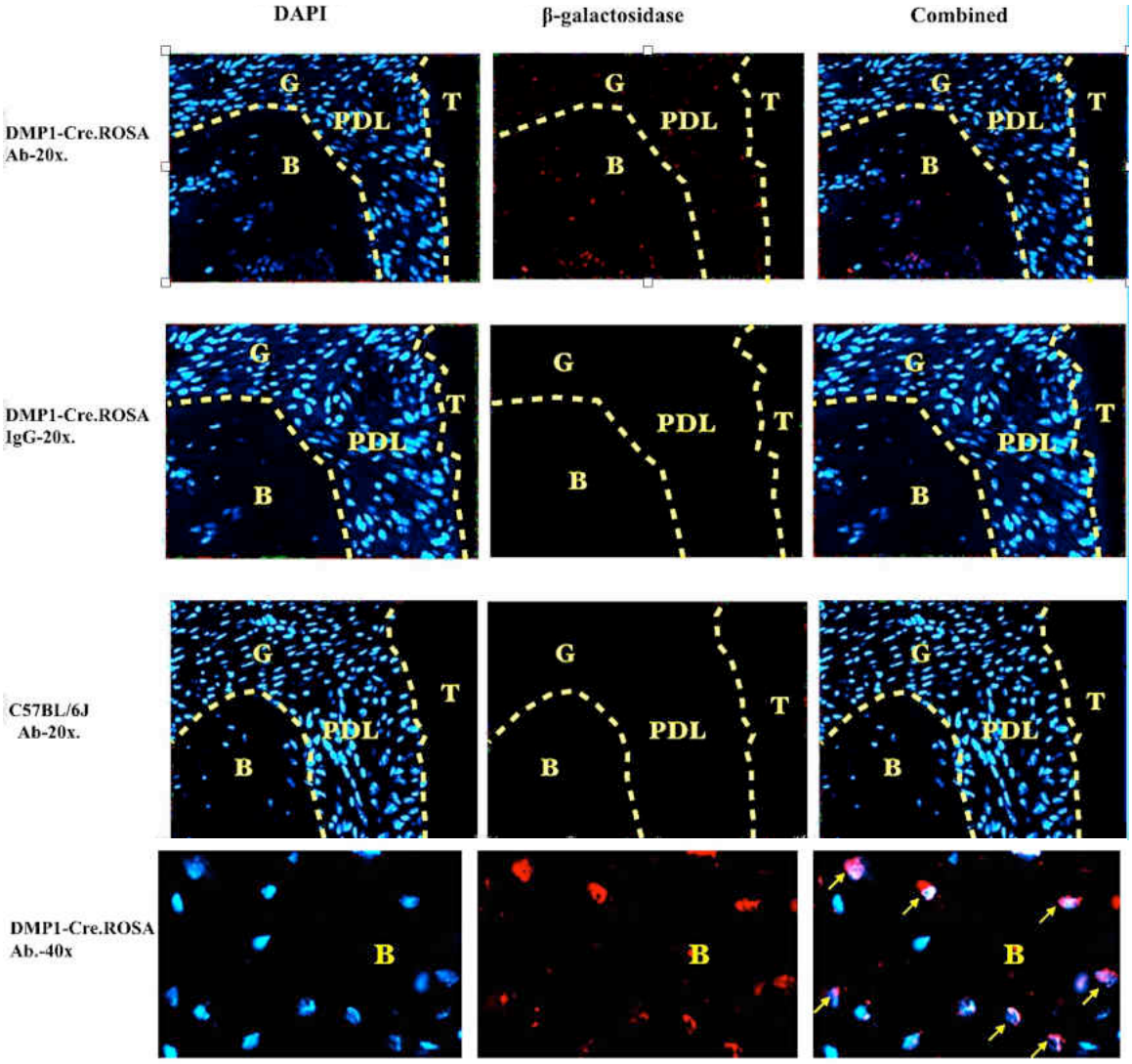

Figure5: Expression of DMP1 in (DMP1-Cre.ROSA26) mice and in control (C57BL/6J) mice.

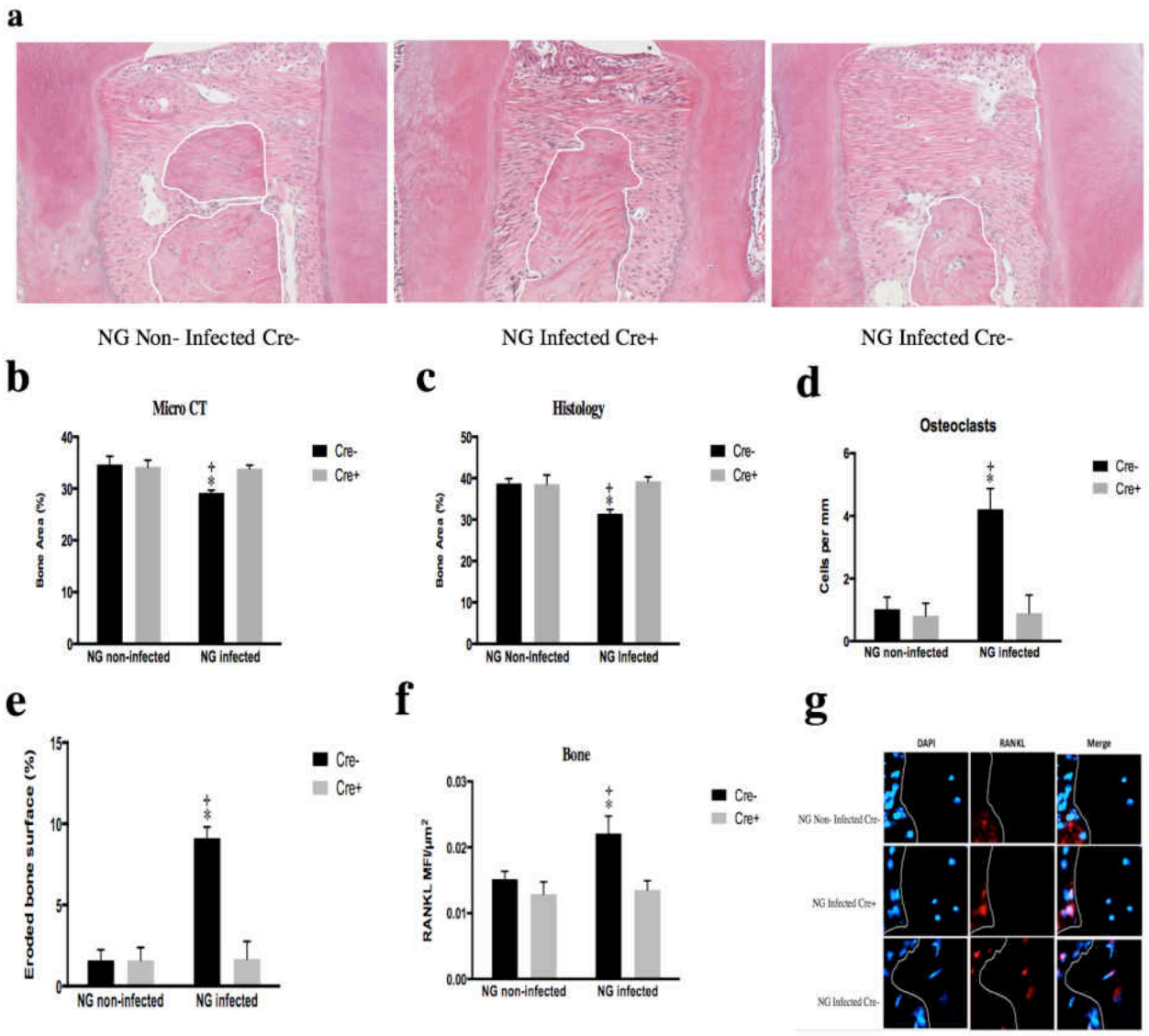

Figure 6. RANKL deletion from osteocyte block periodontitis-induced periodontal bone loss in normoglycemic mice. *P. gingivalis* and *F. nucleatum*, or vehicle alone, were orally inoculated into control (DMP1- Cre^{-}.RANKL$^{f/f}$) mice (Cre-) and experimental (DMP1-Cre^{+}.RANKL$^{f/f}$) mice (Cre+). The mice were euthanized 6 weeks after oral inoculation. (**a**) Hematoxylin and eosin (HE) stained mice periodontal tissues in a longitudinal plan (original magnification 200x). Solid line marks the interproximal bone area (between the first and the second maxillary molar) in sections from normoglycemic (NG) noninfected or infected control (Cre-) mice and infected experimental (Cre+) mice. (**b**) MiroCT analysis of the bone area between the first and the second maxillary molar. (**c**) HE histology analysis of the bone area between the first and the second maxillary molar. (**d, e**) Osteoclasts were counted as multinucleated cells lining the bone surface in sections stained by tartrate-resistant acid phosphatase (TRAP). Eroded bone surface was measured as the length of resorption lacunae divided by the total bone length in TRAP-stained sections. (**f, g**) The RANKL expression from osteocytes were analyzed using the quantification of the mean fluorescence intensity (MFI) of the immunofluorescence images of the interproximal bone. Cells were stained with RANKL (red) and counterstained with DAPI (blue). For each group, n = 5–7. Data represent the mean + SEM. ✻ Significantly different in the infected compared with the matched noninfected group; ✢ significantly different in the infected experimental compared with the infected control. $P < 0.05$.

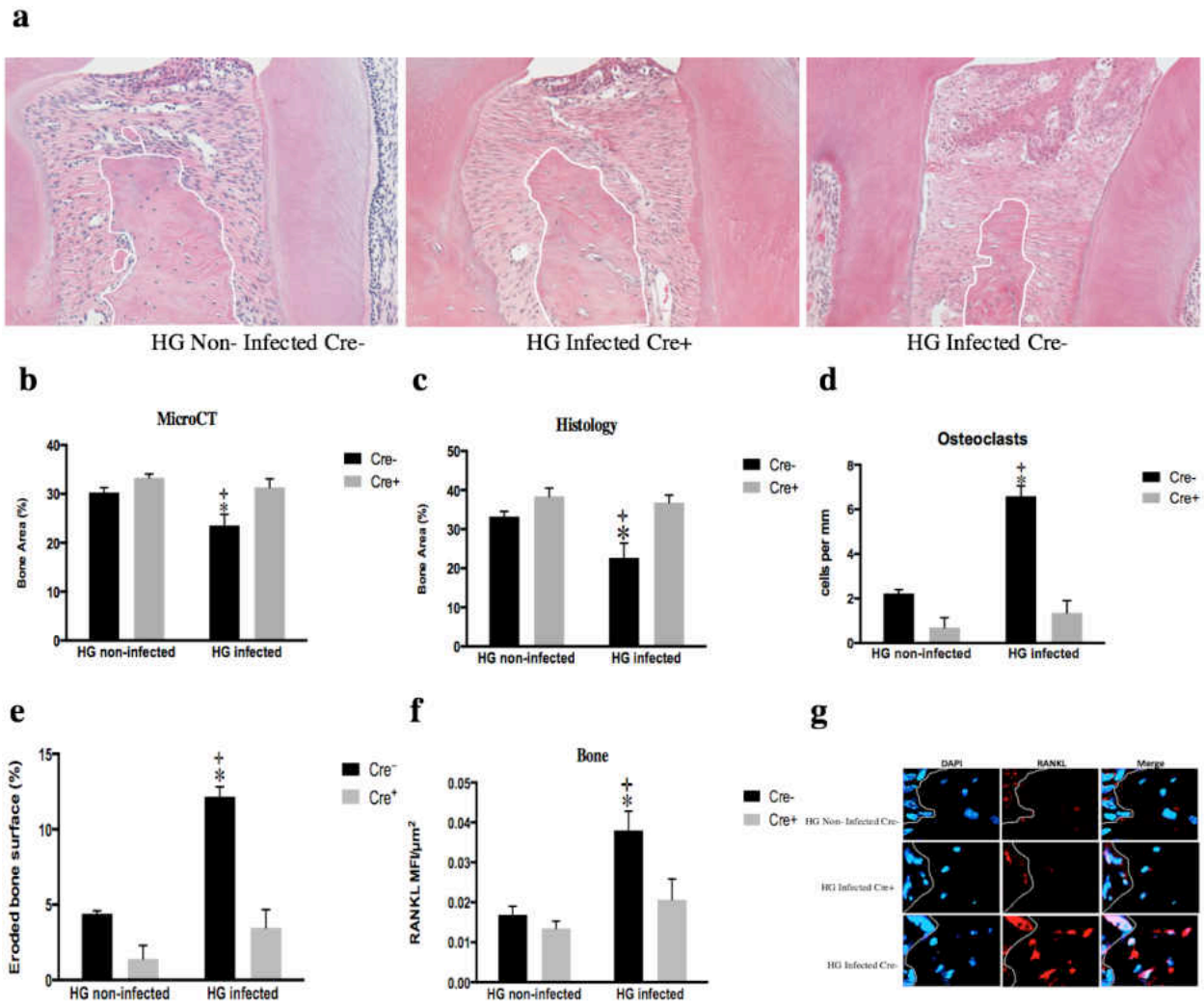

Figure 7. RANKL deletion from osteocyte block periodontitis-induced periodontal bone loss in hyperglycemic mice. *P. gingivalis* and *F. nucleatum*, or their vehicle alone, were orally inoculated into diabetic control (DMP1□Cre^{-}.RANKL$^{f/f}$) mice (Cre-) and experimental (DMP1-Cre^{+}.RANKL$^{f/f}$) mice (Cre+). The mice were euthanized 6 weeks after oral inoculation. (**a**) Hematoxylin and eosin (HE) stained mice periodontal tissues in a longitudinal plan (original magnification 200x). Solid line marks the interproximal bone area (between the first and the second maxillary molar) in sections from hyperglycemic (HG) noninfected or infected control (Cre-) mice and infected experimental (Cre+) mice. (**b**) MiroCT analysis of the bone area between the first and the second maxillary molar. (**c**) HE histology analysis of the bone area between the first and the second maxillary molar. (**d, e**) Osteoclasts were counted as multinucleated cells lining the bone surface in sections stained by tartrate-resistant acid phosphatase (TRAP). Eroded bone surface was measured as the length of resorption lacunae divided by the total bone length in TRAP-stained sections. (**f, g**) The RANKL expression from osteocytes were analyzed using the quantification of the mean fluorescence intensity (MFI) of the immunofluorescence images of the interproximal bone. Cells were stained with RANKL (red) and counterstained with DAPI (blue). For each group, n = 5–7. Data represent the mean + SEM. ✻ Significantly different in the infected compared with the matched noninfected group; ✢ significantly different in the infected experimental compared with the infected control. $P < 0.05$.

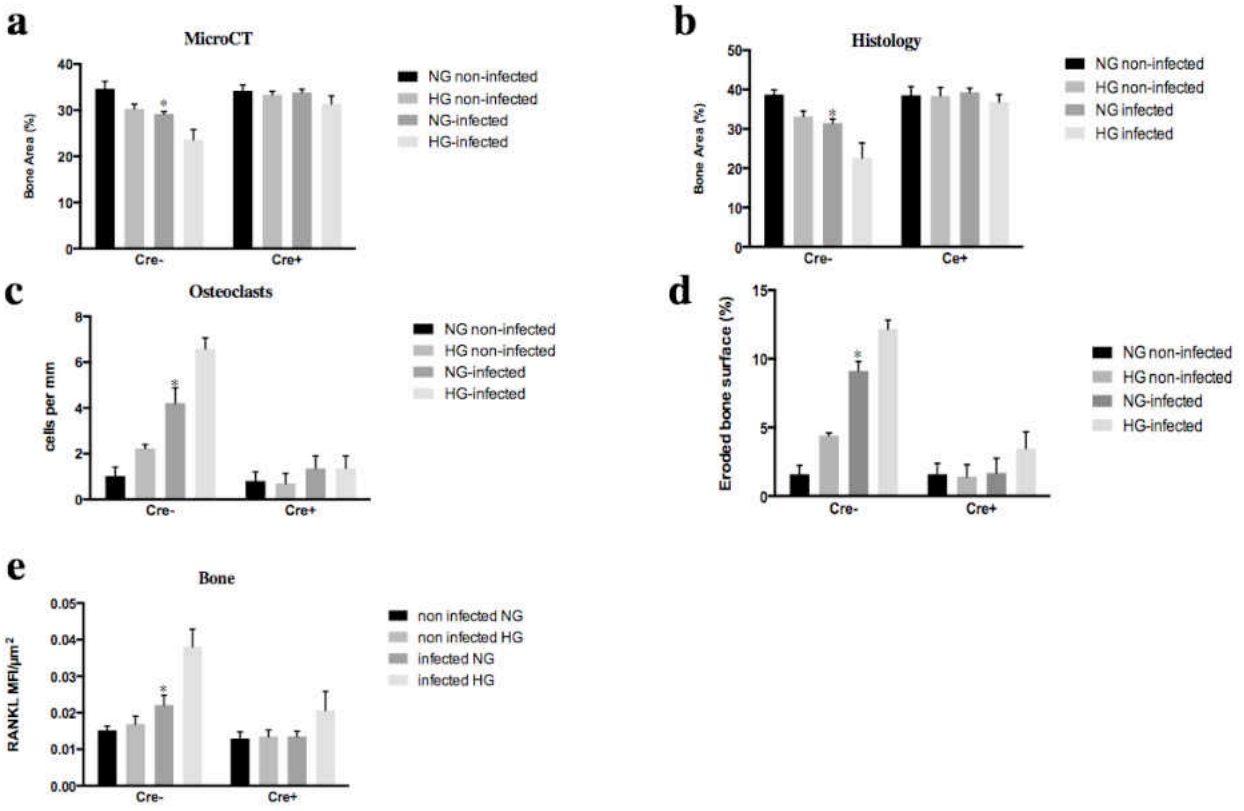

Figure 8. Diabetes-enhanced periodontitis-induced periodontal bone loss. The combined results of both the normoglycemic (NG) and the hyperglycemic (HG) group. (**a**) MiroCT analysis. (**b**) HE histology analysis. (**c, d**) Osteoclast counts. Eroded bone in TRAP-stained sections. (**e**) RANKL expression from osteocytes. For each group, n = 5–7. Data represent the mean + SEM. ✻ Significantly different in the normoglycemic(NG) compared with the matched hyperglycemic (HG) group. $P < 0.05$.

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