LEVELS OF RANKL AND OPG IN GINGIVAL CREVICULAR FLUID DURING ORTHODONTIC TOOTH MOVEMENT

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In partial fulfillment for the degree of

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BRANCH V

ORTHODONTICS AND DENTOFACIAL ORTHOPAEDICS

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CERTIFICATE

This is to certify that this dissertation titled **"LEVELS OF RANKL AND OPG IN GINGIVAL CREVICULAR FLUID DURING ORTHODONTIC TOOTH MOVEMENT"** is a bonafide record of work done by **Dr. RITIKA KAILEY** under my guidance during her postgraduate study period between 2008–2011.

This dissertation is submitted to **THE TAMIL NADU Dr. M.G.R. MEDICAL UNIVERSITY**, in partial fulfillment for the degree of **Master of Dental Surgery** in **BRANCH V – ORTHODONTICS AND DENTOFACIAL ORTHOPAEDICS.**

It has not been submitted (partially or fully) for the award of any other degree or diploma.

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CONTENTS

Introduction

INTRODUCTION

The rate of orthodontic tooth movement is affected by individual variations in tissue reactions, by the type of force applied, and by the mechanical principles involved. Practitioners have been using light force and frictionless appliances, as well as hormones, electricity, and magnetic force, to enhance the rate of orthodontic tooth movement.[60] Nonetheless, mechanical forces have been the most preferred and widely used choice by most contemporary clinicians. Forces exerted by mechanical means however, are exceedingly variable and the clinical response to its application remains unpredictable. This phenomenon has been demonstrated in laboratory animals,[45] as well as in patients, in whom teeth treated with similar orthodontic appliances have been shown to move at different rates.[57] Since the rate of tooth movement is intimately associated with bone remodeling activities, a better understanding of specific biochemical pathways in individual patients may provide the key to predicting how teeth will respond to mechanical force. This knowledge, in turn, can lead to the enhancement of tooth movement.

Orthodontic tooth movement results from remodeling of the periodontal ligament and alveolar bone. Mechanical stress from orthodontic appliances is considered to induce cells in the PDL to form biologically active

substances, such as prostaglandin E, interleukin (IL)-1b, IL-6, tumor necrosis factor (TNF)-a, and epidermal growth factor (EGF) which are responsible for connective tissue remodeling.[12] These factors contribute to bone remodeling, which is a dynamic interaction between bone-forming osteoblasts and bone-resorbing osteoclasts. The rate of remodeling is defined primarily by cells of the osteoblast lineage, which, in addition to bone formation, are also responsible for the activation and recruitment of osteoclast precursors.

Recently, an intermediary factor, receptor activator of nuclear factor κB ligand (RANKL), presenting on the surface of osteoblasts was found to be responsible for the induction of osteoclastogenesis.[87] Binding of RANKL to its cognate receptor, receptor activator of nuclear factor κB (RANK), expressed on the surface of osteoclast progenitor cells, induces osteoclastogenesis and activates osteoclasts, resulting in increased bone resorption. [47]

On the other hand, RANKL has a capability to bind to osteoprotegerin (OPG), which is also a member of the tumor necrosis factor (TNF) receptor family. OPG is a soluble decoy receptor protein that competitively binds to cell surface membrane-bound RANKL proteins and inhibits RANKL activation of osteoclastogenesis.[77] OPG is produced by human periodontal ligament cells, gingival fibroblasts, human pulp cells, and epithelial cells and has been found to be a key factor in the inhibition of osteoclast differentiation and activation.[28]

Although there some are contradictory data, in general when RANKL expression is up-regulated, OPG expression is down-regulated or not induced to the same degree as RANKL, such that the RANKL/OPG ratio changes in favor of osteoclastogenesis.[34,76] Osteoclast numbers and activity can increase if there is a change in the RANKL/OPG ratio due to either an increase in the former or a decrease in the latter or a change in both that leads to a change in the ratio in favor of RANKL.

At the cellular and molecular levels, tooth movement is the result of the interaction between bone-forming osteoblasts and bone-resorbing osteoclasts. Given the critical role osteoclasts play in tooth movement, it follows that the RANK/RANKL/OPG pathway is likely to be critical in this process. Indeed, it has been shown that application of orthodontic forces results in the expression of RANKL protein in osteoblasts, osteocytes, fibroblasts, and osteoclasts.[88]

During orthodontic tooth movement, RANKL expression is induced on the compressed side of the tooth.[54,65] In contrast, on the tensile side of the tooth, there is an increase in OPG synthesis.[38,78] The relative expressions of OPG and RANKL on the tensile and compressed sides of the tooth during orthodontic tooth movement regulate bone remodeling. Orthodontic forces induce the movement of PDL fluids and, with them, any cellular biochemical product produced from prior mechanical perturbation. The direction of flow of the PDL fluid may be as follows: from an area of compression, to an area of

tension, both apically and coronally, towards the gingival sulcus, and/or into the alveolar marrow spaces. Therefore, compression of the PDL should cause cellular biochemical by-products to appear in the sulcus.[88]

As a result of the application of mechanical forces, PDL cells may produce sufficient amount of modulators during bone remodeling to diffuse into the gingival crevicular fluid (GCF). These substances can be monitored non-invasively in humans by following changes in the composition of the GCF during orthodontic tooth movement.[22] Clinical studies have confirmed that both RANKL and OPG can be detected in human gingival crevicular fluid (GCF) and indicate that RANKL is elevated whereas OPG is decreased in periodontitis. [42,72]

This phenomenon opens up an entire new vista, and with adequate scientific research could prove to be an important new biologic marker for tooth movement. Although it has been amply proven that the RANKL and OPG play critical roles in bone remodeling and during bone resorptive conditions like periodontitis, little information is available concerning the production of these modulators during orthodontic tooth movement in human subjects.[33,50] The available literature focuses primarily on an Oriental population (i.e. Japanese and Korean subjects).[33,50]

Keeping this in mind, the aim of this study was to assess the level of OPG and RANKL in the gingival crevicular fluid during orthodontic treatment at two different time points in an Indian population.

Results of this study could help us determine an optimum ratio of RANKL/OPG in the GCF of patients undergoing orthodontic tooth movement

The clinical implications of this study are far fetched at this stage, as the tests to evaluate the concentrations of these proteins are currently available only in a research setting. Further research in this field could see a refinement in these tests making them suitable for diagnostic use in a clinical setting. This would enable periodic monitoring of the levels of these cytokines in the GCF of orthodontic patients. This sort of assessment would give the orthodontist an insight into the epigenetic factors affecting tooth movement which cannot be ignored in a clinical setting.

Review of Literature

REVIEW OF LITERATURE

The role of RANK/RANKL/OPG in bone remodeling

Shalhoub V, Faust J, Boyle WJ, Dunstan CR, Kelley M, Kaufman S, Scully S, Van G, Lacey DL(1999)[63] demonstrated the effects of soluble OPGL and OPG on the developing human osteoclast phenotype, on bone slices, using peripheral blood mononuclear cells (PBMCs), cultured for 2 weeks, without stromal cells. They found that OPGL (2-50 ng/ml), increased the size of osteoclast-like cells on bone, as defined by the acquisition of osteoclast markers: vitronectin receptor (VR), tartrate-resistant acid phosphatase (TRAP), multinuclearity, and bone resorption. By 14 days, with 20 ng/ml OPGL, the largest cells/10x field had achieved an average diameter of 163+/-38 microm, but only approximately 10-20 microm in its absence and the number of osteoclast-like cells/mm2 bone surface was about 128. By scanning electron microscopy, OPGL-treated (20-ng/ml) cultures contained small osteoclast-like cells on bone with ruffled "apical" surfaces by day 7; by day 15, large osteoclast-like cells were spread over resorption lacunae. At 15 ng/ml OPGL, about 37% of the bone slice area was found to be covered by resorption lacunae. This study confirms a pivotal role for OPGL and OPG in the modulation of human osteoclast differentiation and function.

Takahashi N, Udagawa N, Suda T. (1999)[70] showed that osteoblasts/stromal cells express a member of the TNF-ligand family "osteoclast differentiation factor(ODF)/osteoprotegerin ligand (OPGL)/TNF-related activation-induced cytokine (TRANCE)/receptor activator of NF-kB ligand (RANKL)" as a membrane associated factor. Osteoclast precursors which possess RANK, a TNF receptor family member, recognize ODF/OPGL/TRANCE/RANKL through cell-to-cell interaction with osteoblasts/stromal cells, and differentiate into osteoclasts in the presence of macrophage colony-stimulating factor. Mature osteoclasts also express RANK, and their bone-resorbing activity is also induced by ODF/OPGL/TRANCE/RANKL which osteoblasts/stromal cells possess. Osteoprotegerin (OPG)/osteoclastogenesis inhibitory factor (OCIF)/TNF receptor-like molecule 1 (TR1) is a soluble decoy receptor for ODF/OPGL/TRANCE/RANKL. They found that activation of NF-kB through the RANK-mediated signaling system appears to be involved in differentiation and activation of osteoclasts.

Hailing Hsu, David Lacey, Colin r. Dunstan, Irina Solovyev, Anne Colombero, Emma Timms, Hong-lin Tan, Gary Elliott, Michael J. Kelley, Ildiko Sarosi, Ling wang, Xing-zhong Xia, Robin Elliott, Laura Chiu, Tabitha Black, Sheila Scully, Casey Capparelli, Sean Morony, Grant Shimamoto, Michael Bass, and William Boyle (1999)[25] took the genomic approach to examine genes expressed in murine osteoclast precursors. They

described the identification and characterization of the osteoclast differentiation and activation receptor that is present on normal mouse osteoclast progenitors and which mediates OPGL-induced osteoclast differentiation and activation. The identified receptor is indeed identical to the TNFR family member RANK. Like several known TNFR family members, the signaling pathway of RANK involves the interaction with cytoplasmic TNFR-associated factor (TRAF) proteins. Cumulatively, their findings reveal that OPGL–RANK–OPG comprise key regulatory proteins that govern osteoclast development.

Teresa L. Burgess, Yi-xin Qian, Stephen Kaufman, Brian D. Ring, Gwyneth Van, Charles Capparelli, Michael Kelley, Hailing Hsu, William J. Boyle, Colin R. Dunstan, Sylvia Hu, and David L. Lacey (1999)[75] using primary cultures of rat osteoclasts on bone slices, found that OPGL causes approximately sevenfold increase in total bone surface erosion. By scanning electron microscopy, it was found that OPGL-treated osteoclasts generate more clusters of lacunae on bone suggesting that multiple, spatially associated cycles of resorption have occurred. However, the size of the individual resorption events are unchanged by OPGL treatment. Mechanistically, OPGL binds specifically to mature OCs and rapidly (within 30 min) induces actin ring formation; a marked cytoskeletal rearrangement that necessarily precedes bone resorption. Furthermore, they also showed that antibodies raised against the OPGL receptor, RANK, also induce actin ring formation. OPGL treated

mice exhibit increases in blood ionized Ca^{++} within 1 h after injections, consistent with immediate OC activation in vivo.

Suda T, Takahashi N, Udagawa N, Jimi E, Gillespie MT, Martin TJ. (1999)[68] showed that osteoclast precursors express RANK, a TNF receptor family member, recognize RANKL through cell-to-cell interaction with osteoblasts/stromal cells, and differentiate into osteoclasts in the presence of M-CSF.

Kong YY, Feige U, Sarosi I, Bolon B, Tafuri A, Morony S, Capparelli C, Li J, Elliott R, McCabe S, Wong T, Campagnuolo G, Moran E, Bogoch ER, Van G, Nguyen LT, Ohashi PS, Lacey DL, Fish E, Boyle WJ, Penninger JM. (1999)[36] showed that bone remodelling and bone loss are controlled by a balance between the tumour necrosis factor family molecule osteoprotegerin ligand (OPGL) and its decoy receptor osteoprotegerin (OPG). They reported that activated T cells can directly trigger osteoclastogenesis through OPGL. Systemic activation of T cells in vivo leads to an OPGL-mediated increase in osteoclastogenesis and bone loss. They demonstrated that in a T-cell-dependent model of rat adjuvant arthritis characterized by severe joint inflammation, bone and cartilage destruction and crippling, blocking of OPGL through osteoprotegerin treatment at the onset of disease prevents bone and cartilage destruction but not inflammation. These results show that both systemic and local T-cell activation can lead to OPGL production and subsequent bone loss.

H. Kanzaki, M. Chiba, Y. Shimizu, and H. Mitani (2001)[23] examined the consequences of cell-to cell interactions between peripheral blood mononuclear cells (PBMCs) and PDL cells during osteoclastogenesis. indirectly with PDL cells for two to four weeks. PBMCs that were directly co-cultured with PDL cells formed significantly more resorption pits on dentin slices than did PBMCs that were cultured alone. PDL cells expressed both RANKL and OPG mRNA. They concluded that PDL cells support osteoclastogenesis through cell to- cell contact. PDL cells might regulate osteoclastogenesis by opposing mechanisms: stimulation of resorptive activity by RANKL and inhibition by OPG thus affecting processes such as periodontitis and orthodontic tooth movement.

Shimizu-Ishiura M, Kawana F, Sasaki T (2002)[64] examined the effects of OPG administration on the distribution, ultrastructure and vacuolar-type H+-ATPase expression of osteoclasts and resulting trabecular bone loss in the femurs of ovariectomized (OVX) mice. Two-month-old female mice were allocated to the following groups: (1) pretreatment base-line controls; (2) untreated sham-operated controls; (3) untreated OVX; and (4) OPG-administered OVX mice. Postoperatively, OPG (0.3 mg kg(-1) day(-1)) was intraperitoneally administered daily to OVX mice for 7 days. On postoperative day 7, all mice were sacrificed, and the dissected femurs were examined by means of light and immunoelectron microscopy and quantitative backscattered-electron image analysis. Backscattered-electron examination

revealed that trabecular bone area/unit medullary area in untreated OVX mice was significantly lower than that of base-line control and sham-operated control mice. Compared with untreated OVX mice, OPG administration to OVX mice significantly increased trabecular bone area, which was similar to that of sham-operated control mice. Surprisingly, the number of TRAP-positive osteoclasts along the trabecular bone surfaces in OPG-administered OVX mice was not significantly decreased compared with that of sham-operated control and untreated OVX mice. Ultrastructurally, OPG administration caused disappearance of ruffled borders in most osteoclasts, but induced neither necrotic nor apoptotic changes. In addition, the expression of vacuolar-type H+-ATPase in osteoclasts was decreased by OPG administration. Their results suggest that low-dose OPG administration significantly reduces trabecular bone loss in OVX mice via impairment of the structure and bone resorbing activity of osteoclasts.

Sasaki T (2003)[62] in an examination of preosteoclast (pOC) culture demonstrated that RANKL and OPG are important regulators of not only the terminal differentiation of OC but also their resorptive function. The study showed pOCs formed without any additives expressed tartrate-resistant acid phosphatase (TRAP), but showed little resorptive activity. pOC treated with RANKL became TRAP-positive OC, which expressed intense vacuolar-type H(+)-ATPase and exhibited prominent resorptive activity. Such effects of RANKL on pOC were completely inhibited by addition of OPG. OPG

inhibited ruffled border formation in mature OC and reduced their resorptive activity, and also induced apoptosis of some OC. Although OPG administration significantly reduced trabecular bone loss in the femurs of ovariectomized (OVX) mice, the number of TRAP-positive OC in OPG-administered OVX mice was not significantly decreased. Rather, OPG administration caused the disappearance of ruffled borders and decreased H(+)-ATPase expression in most OC. OPG deficiency causes severe osteoporosis. RANKL localization and OC induction in periodontal ligament (PDL) during experimental movement of incisors in OPG-deficient mice was also examined. Compared to wild-type OPG (+/+) littermates, after force application, TRAP-positive OC were markedly increased in the PDL and alveolar bone was severely destroyed in OPG-deficient mice. In both wild-type and OPG-deficient mice, RANKL expression in osteoblasts and fibroblasts became stronger by force application. These in vitro and in vivo studies suggest that RANKL and OPG are important regulators of not only the terminal differentiation of OC but also their resorptive function. Lastly, he examined the expression of H(+)-ATPase, cathepsin K, and matrix metalloproteinase-9 in odontoclasts (OdC) during physiological root resorption in human deciduous teeth, and found that there were no differences in the expression of these molecules between OC and OdC. RANKL was also detected in stromal cells located on resorbing dentine surfaces. This suggests that there is a common mechanism in cellular resorption of mineralized tissues such as bone and teeth.

Yamazaki H, Sasaki T (2005)[86] using OPG-deficient mice, attempted to clarify the differentiation and ultrastructure of osteoclasts located on the destroyed growth plate cartilage and trabecular bone matrix in long bones. In (-/-) homozygous OPG knockout mice, adjacent to the growth plate cartilage, the formation of bone trabeculae without a calcified cartilaginous core resulted in an irregular chondrocyte distribution in the growth plate cartilage. At the metaphyseal ossification center, TRAP-positive osteoclasts showed unusual localization on both type-II collagen-positive cartilage and type-I collagen-positive bone matrix. Osteoclasts located on cartilage matrix lacked a typical ruffled border structure, but formed resorption lacunae. During growth plate cartilage destruction, osteoclasts formed ruffled border structures on bone matrix deposited on the remaining cartilage surfaces. These findings suggest that, in OPG (-/-) mice, osteoclast structure differs, depending on the matrix of either cartilage or bone. Then, they examined the effects of OPG administration on the internal trabecular bone structure and osteoclast differentiation in OPG (-/-) mice. OPG administration to OPG (-/-) mice significantly inhibited trabecular bone loss and maintained the internal trabecular bone structure, but did not reduce the osteoclast number on bone trabeculae. For most osteoclasts, OPG administration caused disappearance or reduction of the ruffled border, but induced neither necrotic nor apoptotic damages. These results suggest that OPG administration is an effective means of maintaining the internal structure and volume of trabecular bone in metabolic bone diseases by inhibition of osteoclastic bone resorption.

Baud'huin M, Duplomb L, Ruiz Velasco C, Fortun Y, Heymann D, Padrines M (2007)[6] showed that any dysregulation of their respective expression of RANKL/OPG leads to pathological conditions. Furthermore, they also demonstrated that the OPG-RANK-RANKL system modulates cancer cell migration, thus controlling the development of bone metastases.

Ominsky MS, Kostenuik PJ, Cranmer P, Smith SY, Atkinson JE (2007)[52] investigated the safety and pharmacology of the RANKL inhibitor OPG-Fc in gonad-intact cynomolgus monkeys. Cortical and trabecular volumetric BMD and BMC, cortical thickness, and cross-sectional moment of inertia were significantly increased by OPG-Fc treatment at the proximal tibia and distal radius metaphyses. Increases in cortical thickness were associated with significantly greater periosteal circumference. They inferred that OPG-Fc increased cortical and trabecular BMD and BMC in young gonad-intact cynomolgus monkeys.

Brendan F. Boyce, and Lianping Xing (2008)[9] demonstrated that hypertrophic and to a lesser extent prehypertrophic chondrocytes express RANKL, OPG and RANK. Mice deficient in RANKL, RANK and NF-κB p50 and p52 develop osteopetrosis because they do not form osteoclasts and have thickened hypertrophic cartilage zones in their growth plates. This defect is rectified spontaneously between 2 and 3 weeks of age in the RANKL−/− and RANK−/− mice and 2–3 weeks later in the NF-κB p50 and p52 double

knockout mice. The precise role of RANKL/RANK/NF-κB signaling in chondrocytes during endochondral ossification remains poorly understood, but these findings suggest that it may regulate the lifespan of hypertrophic chondrocytes through NF-κB p50 and p52-regulated genes at least temporarily, similar to MMP-9 (11), which is regulated by NF-κB. All of these knockout mice are dwarfed, suggesting that RANKL/RANK/NF-κB signaling during the first 2–3 weeks of life is essential for attainment of full skeletal growth

Ominsky MS, Li X, Asuncion FJ, Barrero M, Warmington KS, Dwyer D, Stolina M, Geng Z, Grisanti M, Tan HL, Corbin T, McCabe J, Simonet WS, Ke HZ, Kostenuik PJ. (2008)[53] examined whether the RANKL inhibitor osteoprotegerin (OPG) would preserve bone volume, density, and strength in ovariectomized (OVX) rats. OVX was associated with significantly greater serum RANKL. They found that OPG markedly reduced osteoclast surface and serum TRACP5b while completely preventing OVX-associated bone loss in the lumbar vertebrae, distal femur, and femur neck. Vertebrae from OPG-treated rats had increased dry and ash weight, with no significant differences in tissue mineralization versus OVX controls. Bone strength was also significantly increased by OPG.

Stolina M, Schett G, Dwyer D, Vonderfecht S, Middleton S, Duryea D, Pacheco E, Van G, Bolon B, Feige U, Zack D, Kostenuik P (2009)[67] demonstrated that RANKL inhibition prevented local and systemic bone loss without significantly inhibiting local or systemic inflammatory parameters. Lewis rats with established rat adjuvant-induced arthritis (AIA) or collagen-induced arthritis (CIA) were treated for 10 days (from day 4 post onset) with RANKL inhibitor (osteoprotegerin (OPG)-Fc). Local inflammation was evaluated by monitoring hind paw swelling. Bone mineral density (BMD) of paws and lumbar vertebrae was assessed by dual X-ray absorptiometry. OPG-Fc reduced BMD loss in ankles and vertebrae in both models, but had no effect on paw swelling. They found that RANKL inhibition by OPG-Fc did not lessen systemic cytokine levels in either model but prevented local and systemic bone loss.

Bastos MF, Brilhante FV, Gonçalves TE, Pires AG, Napimoga MH, Marques MR, Duarte PM.(2010)[4] evaluated the ligature-induced bone loss (BL) and quality of tooth-supporting alveolar bone in spontaneously hypertensive rats (SHRs) by histometric, histochemical, and immunohistochemical analyses. They found a decreased expression of RANKL in the treated SHR group. They concluded that SHRs present harmful alterations in the quality of tooth-supporting bone and attributed this to the decreased the expression of bone-resorption markers.

The relation between RANK/RANKL/OPG and Periodontal disease development

T Nagasawa, H Kobayashi, M Kiji, M Aramaki, R Mahanonda, T Kojima, Y Murakami, M Saito, Y Morotome, and I Ishikawa (2002)[69] examined the expression of RANKL and its inhibitor, osteoprotegerin (OPG), in inflamed gingival tissue and attempted to clarify the role of human gingival fibroblasts (HGFs) in osteoclastogenesis regulated by RANKL. HGFs and gingival mononuclear cells (GMCs) were obtained from chronic periodontitis patients during routine periodontal surgery. Expression of OPG and RANKL mRNA in gingival tissue and HGFs was examined with RT-PCR. OPG production was measured using ELISA. Expression of RANKL, CD4, CD8 and CD69 on GMCs was determined by flow-cytometry using RANK-Fc fusion protein and the respective monoclonal antibodies. Osteoclastogenesis by RANKL was assayed by counting the number of tartarate-resistant acid phosphatase (TRAP)-positive cells after culturing human peripheral blood monocytes with recombinant human RANKL and macrophage-colony stimulating factor (M-CSF) for 10 days. OPG and RANKL mRNA were expressed in 80% (16/20) and 25% (5/20) of periodontitis lesions, respectively. OPG, but not RANKL, mRNA was expressed within HGFs. OPG mRNA expression and production by HGFs was augmented by LPS stimulation. All GMC samples expressed CD69, and two of five GMC samples expressed RANKL. The culture supernatant of LPS-stimulated

gingival fibroblasts significantly reduced the number of TRAP positive cells generated by culturing monocytes with RANKL and M-CSF. The present study suggests that LPS-stimulated HGFs inhibit monocyte differentiation into osteoclasts through the production of OPG.

Liu D, Xu JK, Figliomeni L, Huang L, Pavlos NJ, Rogers M, Tan A, Price P, Zheng MH (2003)[40] used semi-quantitative RT-PCR to compare the gene expression of RANKL and OPG, between moderate and advanced periodontitis, and healthy subjects. The level of RANKL mRNA was highest in advanced periodontitis. In contrast, the level of OPG mRNA in both advanced and moderate periodontitis was lower than that in the healthy group. They demonstrated an increased ratio of RANKL to OPG mRNA in periodontitis. In situ hybridization was performed to localize RANKL mRNA and it was found to be expressed in inflammatory cells, mainly lymphocyte and macrophages. In addition, proliferating epithelium in the vicinity of inflammatory cells expressed high levels of RANKL mRNA.Their data suggest that up regulation of RANKL mRNA in both inflammatory cells and epithelium may be associated with the activation of osteoclastic bone destruction in periodontitis.

Tania Crotti, Malcolm D. Smith, Robert Hirsch, Steven Soukoulis, Helen Weedon, Maria Capone, Michael J. Ahern, David Haynes (2003)[72] compared the levels of the RANKL and OPG in the granulomatous tissue adjacent to areas of alveolar bone loss from patients with periodontitis

to that present in tissue from patients without periodontitis. Semiquantitative image analysis demonstrated that significantly higher levels of RANKL protein were expressed in the periodontitis tissue. Conversely, OPG protein was significantly lower in the periodontitis tissues. RANKL protein was associated with lymphocytes and macrophages. OPG protein was associated with endothelial cells in both tissues.

M. Mogi, J. Otogoto, N. Ota, and A. Togari (2004)[42] reported the *in vivo* concentrations of RANKL and the RANKL decoy receptor osteoprotegerin (OPG) in the gingival crevicular fluid (GCF) of periodontal subjects with severe, moderate, and mild forms of the disease. An increased concentration of RANKL and a decreased concentration of OPG were detected in GCF from patients with periodontitis ($p < 0.05$ *vs.* control subjects). The ratio of the concentration of RANKL to that of OPG in the GCF was significantly higher for periodontal disease patients than for healthy subjects ($p < 0.01$). These data suggest that RANKL and OPG contribute to osteoclastic bone destruction in periodontal disease.

Garlet GP, Martins W Jr, Fonseca BA, Ferreira BR, Silva JS (2004)[19] investigated the expression of matrix metalloproteinases (MMPs) and the osteoclastogenic factor receptor activator of nuclear factor-kappaB ligand (RANKL), their respective tissue inhibitors of metalloproteinases (TIMPs) and osteoprotegerin (OPG) in different forms of human periodontal diseases (PDs), and the possible correlation with the expression of inflammatory and

regulatory cytokines. Quantitative polymerase chain reaction (real-time PCR) was performed with gingival biopsies mRNA from aggressive (AP) and chronic periodontitis (CP) patients. They found that patients with periodontitis exhibited higher expression of all analyzed factors when compared with healthy tissues. The expression of MMPs and RANKL were similar in AP and CP, as well as the expression of TNF-alpha. On the other hand, the expression of TIMPs and OPG was higher in CP, and was associated with lower IFN-gamma and higher IL-10 expression, compared with AP. They thus concluded that the pattern of cytokines expressed determines the stable or progressive nature of the lesions and regulates the severity of PD, driving the balance between MMPs and TIMPs, RANKL and OPG expression in the gingival tissues controlling the breakdown of soft and bone tissues and, consequently, the disease severity.

Vernal R, Chaparro A, Graumann R, Puente J, Valenzuela MA, Gamonal J.(2004)[81] demontrated that RANKL was found in a higher proportion (85%) of samples from patients than from controls (46%). The total amount of RANKL was significantly higher in patients (115.53 +/- 78.18 picograms/ml) than in healthy subjects (63.08 +/- 55.08 pg/ml). Active sites, presumably associated with tissue destruction, had significantly higher levels of RANKL than their inactive counterparts (125.95 pg/ml versus 91.80 pg/ml)

Garlet, G. P., Cardoso, C. R., Silva, T. A., Ferreira, B. R., Ávila-Campos, M. J., Cunha, F. Q. and Silva, J. S. (2006)[20], investigated the expression of matrix metalloproteinases (MMPs) and the osteoclastogenic factor RANKL (receptor activator of nuclear factor-κB ligand), their respective inhibitors TIMPs (tissue inhibitors of metalloproteinases) and OPG (osteoprotegerin) and their possible correlation with the expression of inflammatory and regulatory cytokines in the course of experimental periodontal disease in mice. Their data demonstrates two distinct patterns of MMP/TIMP and RANKL/OPG expression in the course of experimental periodontal disease. The expression of MMPs (MMP-1, 2 and 9) and RANKL was correlated with the expression of interleukin-1β, tumor necrosis factor-α and interferon-ϒ, in a time period characterized by the intense increase of inflammatory reaction and alveolar bone loss. On the other hand, interleukin-4 and interleukin-10 were associated with higher expression of TIMPs (TIMP 1, 2 and 3) and OPG, with a lower expression of MMPs and RANKL, and with reduced rates of increase of cellular infiltration in periodontal tissues and alveolar bone loss. They inferred that the pattern of cytokines produced in periodontal tissues determines the progression and the severity of experimental periodontal disease, controlling the breakdown of soft and bone tissues through the balance between MMPs/TIMP and RANKL/OPG expression in gingival tissues.

Lu HK, Chen YL, Chang HC, Li CL, Kuo MY (2006)[41] carried out a study to quantify OPG/RANKL in the gingival crevicular fluid (GCF) and connective tissue of patients with periodontitis, and to clarify possible correlations with disease severity. They found that GCF RANKL, but not OPG, was elevated in diseased sites of patients with periodontitis. However, the expressions of OPG and RANKL showed no correlation with disease severity. Immunohistochemical staining showed that RANKL-positive cells were significantly distributed in the inflammatory connective tissue zone of diseased gingiva, compared with those of samples from non-diseased persons.

Kawai T, Matsuyama T, Hosokawa Y, Makihira S, Seki M, Karimbux NY, Goncalves RB, Valverde P, Dibart S, Li YP, Miranda LA, Ernst CW, Izumi Y, Taubman MA. (2006)[32] conducted a studyto identify the cellular source of RANKL in the bone resorptive lesions of periodontal disease. The concentrations of soluble RANKL, but not its decoy receptor osteoprotegerin, measured in diseased tissue homogenates were found to be significantly higher in diseased gingival tissues than in healthy tissues. Double-color confocal microscopic analyses demonstrated less than 20% of both B cells and T cells expressing RANKL in healthy gingival tissues. By contrast, in the abundant mononuclear cells composed of 45% T cells, 50% B cells, and 5% monocytes in diseased gingival tissues, more than 50 and 90% of T cells and B cells, respectively, expressed RANKL. RANKL production by nonlymphoid cells was not distinctly identified. Lymphocytes isolated from

gingival tissues of patients induced differentiation of mature osteoclast cells in a RANKL-dependent manner in vitro. However, similarly isolated peripheral blood B and T cells did not induce osteoclast differentiation, unless they were activated in vitro to express RANKL; emphasizing the osteoclastogenic potential of activated RANKL-expressing lymphocytes in periodontal disease tissue. These results suggest that activated T and B cells can be the cellular source of RANKL for bone resorption in periodontal diseased gingival tissue.

Rolando Vernal, Nicolás Dutzan, Marcela Hernández, Sabrita Chandía, Javier Puente, Rubén León, Leyre García, Ignacio Del Valle, Augusto Silva and Jorge Gamonal (2006)[59] demonstrated that gingival CD4(+) T cells are the main cells responsible for higher levels of RANKL observed in human chronic periodontitis patients. Gingival biopsies were obtained from 33 chronic periodontitis patients and 20 healthy controls. RANKL mRNA and protein levels were determined by quantitative RT-PCR and enzyme-linked immunosorbent assay (ELISA) in gingival-cell culture supernatants. Gingival leukocytes were quantified by flow cytometry. RANKL and CD4 immunoreactivity were analyzed by flow cytometry and confocal microscopy. They found that RANKL mRNA levels were higher in patients with periodontitis than in healthy subjects, and spontaneous and lipopolysaccharide (LPS)- and phytohemagglutinin (PHA)-stimulated RANKL synthesis were higher also in patients than controls. CD4(+) T lymphocytes were the predominant infiltrate cell subset present in gingival

tissues of periodontitis patients. Furthermore, an association between RANKL and CD4(+) T cells was determined by double-staining flow cytometry and confocal microscopy.

N. Bostanci, T. Ilgenli, G. Emingil, B. Afacan, B. Han, G. Atilla, F.J. Hughes, and G.N. Belibasakis (2007)[46] compared the concentrations of RANKL, OPG and their relative ratio in gingival crevicular fluid (GCF) of patients with periodontal disease. They found that there was no significant difference in RANKL and OPG levels between chronic and generalized aggressive periodontitis groups. The RANKL/OPG ratio was elevated by approximately 200 fold in both chronic periodontitis and generalized aggressive periodontitis, compared to healthy patients. This increase in ratio in both forms of periodontitis, may be a key factor in determining attachment loss in periodontitis.

Wara-aswapati N, Surarit R, Chayasadom A, Boch JA, Pitiphat W (2007)[82] showed for the first time to an association between upregulated RANKL levels and the number of P. gingivalis in clinically obtained periodontal tissues. They studied the expression of RANKL and OPG mRNA and the relationship between these factors and periodontopathic bacteria in periodontal tissue. **G**ingival tissue and subgingival plaque samples were collected from 15 patients with chronic periodontitis and 15 periodontally healthy subjects. RNA, extracted from the tissue was subjected to reverse

transcription-polymerase chain reaction (RT-PCR) using primers specific for RANKL or OPG. Their results showed increased levels of RANKL mRNA in chronic periodontitis tissues. The RANKL/OPG expression ratio was significantly higher in the periodontitis group compared to the healthy control group (P = 0.001). Also, the expression of RANKL (P <0.001), but not OPG (P = 0.20), was significantly correlated with increased numbers of P. gingivalis.

Bostanci N, Ilgenli T, Emingil G, Afacan B, Han B, Töz H, Berdeli A, Atilla G, McKay IJ, Hughes FJ, Belibasakis GN (2007)[7] demonstrated that RANKL and osteoprotegerin expression are differentially regulated in various forms of periodontitis, and the relative RANKL/osteoprotegerin ratio appears to be indicative of disease occurrence. Gingival tissue was obtained from nine healthy subjects and 41 patients, who had gingivitis, chronic periodontitis, generalized aggressive periodontitis, and chronic periodontitis and were receiving immunosuppressant therapy. Quantitative real-time polymerase chain reaction was employed to evaluate the mRNA expression of RANKL and osteoprotegerin in these tissues. Their results showed that compared with healthy individuals, patients in all periodontitis groups, but not those with gingivitis, exhibited stronger RANKL expression and a higher relative RANKL/osteoprotegerin ratio. In addition, osteoprotegerin expression was weaker in patients with chronic periodontitis. When patients with

generalized aggressive periodontitis and chronic periodontitis were compared, the former exhibited stronger RANKL expression, whereas the latter exhibited weaker osteoprotegerin expression, and there was no difference in their relative ratio. When chronic periodontitis patients were compared with chronic periodontitis patients receiving immunosuppressant therapy, osteoprotegerin, but not RANKL, expression was stronger in the latter.

Tang TH, Fitzsimmons TR, Bartold PM (2009)[73] compared the levels of the soluble receptor activator of nuclear factor kappa B ligand (sRANKL), osteoprotegerin (OPG) and their relative ratio in gingival crevicular fluid (GCF) among periodontitis patients with varying smoking histories. They found that RANKL, OPG and their relative ratio were not statistically significant among the never smokers, former smokers and current smokers. However, OPG was significantly reduced and subsequently the sRANKL:OPG ratio was significantly increased in the high pack-years group as compared with never smokers. They, thus concluded that an increased lifetime exposure to cigarette smoking above a minimum threshold suppresses OPG production and leads to increased sRANKL:OPG. This may partially explain increased bone loss in smoking-related periodontitis.

The effect of external force application on the RANKL/OPG ratio

Kanzaki H, Chiba M, Shimizu Y, Mitani H. (2002)[31] examined how mechanical stress affects the osteoclastogenesis-supporting activity of PDL cells. PDL cells were compressed continuously and then cocultured with peripheral blood mononuclear cells (PBMCs) for 4 weeks. PDL cells under mechanical stress up-regulated osteoclastogenesis from PBMCs. Furthermore, the expression of RANKL mRNA and protein in PDL cells increased with compressive force in parallel with the change in the number of osteoclasts. In addition, cyclo-oxygenase 2 (COX-2) mRNA expression was induced by compressive force, and indomethacin inhibited the RANKL up-regulation resulting from compressive force. PDL cells under compressive force exhibited significantly increased prostaglandin E2 (PGE2) production in comparison with control PDL cells. Exogenous PGE2 treatment increased RANKL mRNA expression in PDL cells. OPG expression remained constant throughout compressive force or PGE2 treatment. They concluded that compressive force up-regulated RANKL expression in PDL cells. Furthermore, RANKL up-regulation in mechanically stressed PDL cells was dependent on PGE2.

Tsuji K, Uno K, Zhang GX, Tamura M (2004)[78] studied the mRNA expression of osteoprotegerin (OPG), receptor activator of NF-kappa B ligand (RANKL), tissue inhibitor of matrix metalloprotease (TIMP)-1 and -2, and matrix metalloprotease (MMP)-1 and -2 by human periodontal ligament

(PDL) cells under intermittent tensile stress using a Flexercell Strain Unit. Analysis by reverse transcriptase-polymerase chain reaction showed that mechanical force upregulated OPG mRNA. They also demonstrated that the protein concentration of OPG in conditioned medium increased upon loading with tensile stress, as determined by enzyme-linked immunosorbent assay. TIMP-1 and -2 mRNA levels also increased, whereas levels of RANKL, MMP-1, and MMP-2 mRNA were barely affected. We further examined the effect of loading with tensile stress and addition of Salmonella abortus equi lipopolysaccharide (LPS) on the mRNA expression of PDL cells. The amount of OPG mRNA induced by mechanical strain was found to decrease with the addition of LPS to cultures. The induction of OPG mRNA expression by stretching was inhibited in the presence of indomethacin or genistein, whereas TIMP-1 mRNA expression induced by stretching was inhibited by the addition of cycloheximide, suggesting that tensile stress regulates cyclooxygenase activities, tyrosine phosphorylation, and de novo protein synthesis in PDL cells through the induction of OPG and TIMP-1 mRNA expression. These results provide evidence that the mechanical stimulus of stretching is responsible for the observed regulation of bone resorption and tissue degradation in PDL tissue.

Ogasawara T, Yoshimine Y, Kiyoshima T, Kobayashi I, Matsuo K, Akamine A, Sakai H. (2004)[51] suggested that an autocrine mechanism of RANKL-RANK exists in osteoclast, which is heightened in the pathological

conditions. They examined the in situ expression of receptor activator of nuclear factor-kappaB ligand (RANKL), receptor activator of nuclear factor-kappaB (RANK), osteoprotegerin, interleukin-1beta (IL-1beta) and tumor necrosis factor alpha (TNFalpha) in the osteoclasts of rat periodontal tissue. Four-week-old Wistar rats were used. Tooth movement was performed by the Waldo method, and the pathological bone resorption was induced. The demineralized maxillae and mandiblae were embedded with paraffin. In situ hybridization was performed to detect RANKL, RANK, osteoprotegerin, IL-1beta, and TNFalpha mRNAs in osteoclasts and other cells using the specific RNA probes, respectively. Both RANKL and RANK were concomitantly expressed in some osteoclasts. RANKL was also positive in osteoblasts and PDLs. No IL-1beta- and TNFalpha-positive osteoclast was noted. The positive signals of osteoprotegerin were detected in almost all osteoblasts, PDLs and odontoblasts. No osteoprotegerin-positive osteoclasts were observed. The number and the distribution pattern of RANKL- and RANK-expressing osteoclasts changed when orthodontic excessive force was applied to periodontal tissue. In addition, IL-1beta and TNFalpha were shown to be expressed in osteoclasts under pathological status. They inferred that an autocrine mechanism of RANKL-RANK exists in osteoclast, which is heightened in the pathological conditions. Furthermore, the autocrine mechanism of IL-1beta and TNFalpha is also provided in osteoclast under pathological condition. These autocrine mechanisms therefore seem to regulate the osteoclast function in both physiological and pathological conditions.

Yamamoto T, Kita M, Kimura I, Oseko F, Terauchi R, Takahashi K, Kubo T, Kanamura N (2006)[85] investigated the effect of mechanical stress as hydrostatic pressure (HP) on cytokine expression in human PDL cells. The hPDL cells were obtained from a healthy maxillary third molar. After the third to fourth passage, the cells were exposed to HP ranging from 1 to 6 MPa. Total RNA was extracted and the expression of cytokine mRNA was determined by RT-PCR. The exposure to 6 MPa of HP caused no morphological changes of hPDL cells, and did not affect the cellular viability. No expression of IL-1beta, IL-6, IL-8, TNF-alpha, receptor activator of NF-lambdaB (RANK), receptor activator of NF-lambdaB ligand (RANKL), or osteoprotegerin mRNA was observed in the control cells under atmospheric pressure, whereas, in hPDL cells treated with HP, a pressure-dependent enhancement of IL-6, IL-8, RANKL, and OPG mRNA expression was observed between 10 and 60 min after the exposure to HP. These results suggest that hPDL cells may play a role in the production of cytokines in response to mechanical stress in vivo.

H. Kanzaki, M. Chiba, A. Sato, A. Miyagawa, K. Arai, S. Nukatsuka, and H. Mitani (2006)[24] subjected a conditioned media of PDL cells to cyclical tensile force. They demonstrated that cyclical tensile force up-regulated not only RANKL mRNA expression, but also OPG mRNA expression in PDL cells. Tensile force up-regulated TGF-beta expression in PDL cells as well. Administration of neutralizing antibodies to TGF-beta inhibited OPG

upregulation under cyclical tensile-force stimulation in a dose-dependent manner. Additionally, the osteoclastogenesis-inhibitory effect of the conditioned media of PDL cells under cyclical tensile force was partially rescued by the administration of TGF-beta neutralizing antibodies. They concluded that tensile force inhibited the osteoclastogenesis-supporting activity of PDL cells by inducing the up-regulation of OPG *via* TGF-beta stimulation.

Tatsumi S, Ishii K, Amizuka N, Li M, Kobayashi T, Kohno K, Ito M, Takeshita S, Ikeda K (2007)[74] suggested that at least in response to mechanical forces, osteocytes regulate the recruitment of osteoclasts to sites of bone resorption by inducing the expression of RANKL by osteoblastic cells in the local micro-environment.

Nakajima R, Yamaguchi M, Kojima T, Takano M, Kasai K (2008)[48] applied a compression force of 0.5-4.0 g/cm2 to human periodontal ligament cells for 0-24 h and the amounts of soluble RANKL (sRANKL) and fibroblast growth factor-2 were measured using an enzyme-linked immunosorbent assay. They found that the applied compression force induced higher levels of sRANKL and fibroblast growth factor-2 in both a time- and magnitude-dependent manner.

Kook SH, Son YO, Choe Y, Kim JH, Jeon YM, Heo JS, Kim JG, Lee JC (2009)[37] examined how mechanical force affects the nature of human gingival fibroblasts to produce osteoprotegerin and inhibit osteoclastogenesis. Human gingival fibroblasts were exposed to mechanical force by centrifugation for 90 min at a magnitude of approximately 50 g/cm(2). The levels of osteoprotegerin, receptor activator of nuclear factor-kappaB ligand (RANKL), interleukin-1beta and tumor necrosis factor-alpha were measured at various time-points after applying the force. Centrifugal force stimulated the expression of osteoprotegerin, RANKL, interleukin-1beta and tumor necrosis factor-alpha by the cells, and produced a relatively high osteoprotegerin to RANKL ratio at the protein level. Both interleukin-1beta and tumor necrosis factor-alpha accelerated the force-induced production of osteoprotegerin, which was inhibited significantly by the addition of anti-(interleukin-1beta) immunoglobulin Ig isotype; IgG (rabbit polyclonal). However, the addition of anti-(tumor necrosis factor-alpha) immunoglobulin Ig isotype; IgG1 (mouse monoclonal) had no effect. Centrifugal force also had an inhibitory effect on osteoclast formation. They concluded that application of centrifugal force to human gingival fibroblasts accelerates osteoprotegerin production by these cells, which stimulates the potential of human gingival fibroblasts to suppress osteoclastogenesis.

Sanuki Rina, Shionome Chieko, Kuwabara Akiko, Mitsui Narihiro, Koyama Yuki, Suzuki Naoto, Zhang Fan Shimizu, Noriyoshi, Maeno, Masao (2010)[61] examined the effect of compressive force on the production of PGE_2, cyclooxygenase-2 (COX-2), macrophage colony-stimulating factor (M-CSF), receptor activator of NF-*ê*B ligand (RANKL), and osteoprotegerin (OPG) using osteoblastic MC3T3-E1 cells. They found that as the compressive force increased, PGE_2 production and the expression of COX-2, M-CSF, and RANKL increased, whereas OPG expression decreased. Celecoxib, a specific inhibitor of COX-2, blocked the stimulatory effect of CF on TRAP staining and the production of PGE_2, M-CSF, RANKL, and OPG.

Zhang F, Wang CL, Koyama Y, Mitsui N, Shionome C, Sanuki R, Suzuki N, Mayahara K, Shimizu N, Maeno M. (2010)[89] evaluated the effects of compressive force during orthodontic tooth movement on cytokines released from periodontal ligament fibroblasts. Their results indicate that compressive force induces the expression of IL-17s and their receptors in osteoblast-like cells and that IL-17s and their receptors produced in response to compressive force may affect osteoclastogenesis through the expression of RANKL, M-CSF, and OPG.

The involvement of RANKL and OPG in bone remodeling during orthodontic tooth movement

Eva Low, Hans Zoellner, Om Prakash Kharbanda, and M. Ali Darendeliler (2005)[14] showed by densitometric analysis that an increase in background levels of OPG mRNA was evident in bony tissues subjected to orthodontic forces. RANKL and OPG levels were seen to increase in the environment during root resorption with the application of heavy forces. They might play a significant role during root resorption processes after orthodontic tooth movement.

Y Nishijima, M Yamaguchi, T Kojima, N Aihara, R Nakajima, K Kasai (2006)[50] conducted a study to determine the levels of the receptor activator of NFkB ligand (RANKL) and osteoprotegerin (OPG) in the gingival crevicular fluid (GCF) during orthodontic tooth movement and to investigate the effect of compression force on RANKL and OPG production from human periodontal ligament (hPDL) cells. Enzyme-linked immunosorbent assay (ELISA) kits were used to determine RANKL and OPG levels in the GCF collected at the distal cervical margins of the experimental and control teeth 0, 1, 24, and 168 h after the retracting force was applied. GCF levels of RANKL were significantly higher, and the levels of OPG significantly lower, in the experimental canines than in the control teeth at 24 h, but there were no such significant differences at 0, 1, or 168 h. In vitro study indicated that the compression force significantly increased the secretion of RANKL and

decreased that of OPG in hPDL cells in a time and force magnitude-dependent manner. The compression stimulated secretion of RANKL increased approximately 16.7-fold and that of OPG decreased 2.9-fold, as compared with the control.

Kawasaki K, Takahashi T, Yamaguchi M, Kasai K (2006)[33] compared the levels of the receptor activator of NF*k*B ligand (RANKL) and osteoprotegerin (OPG) in the gingival crevicular fluid (GCF) during orthodontic tooth movement in juvenile and adult patients. GCF was collected from the distal cervical margins of the experimental and control teeth at 0, 1, 24, and 168 h after application of a retracting force. Enzyme-linked immunosorbent assay kits were used to determine RANKL and OPG levels in the GCF samples. The amount of tooth movement for juveniles was larger than for adults after 168 h. Further, after 24 h RANKL levels were increased and those of OPG decreased in GCF samples from the compression side during orthodontic tooth movement in both juveniles and adults. The RANKL/OPG ratio in GCF from adult patients was lower than that in the juvenile patient samples. Their results suggest that the age-related decrease in amount of tooth movement may be related to a decrease in RANKL/OPG ratio in GCF during the early stages of orthodontic tooth movement.

Nakao K, Goto T, Gunjigake KK, Konoo T, Kobayashi S, Yamaguchi K (2007)[49] effectively demonstrated that intermittent force induces high RANKL expression in human periodontal ligament cells. They examined the

molecular mechanism in human periodontal ligament (PDL) cells stimulated by an intermittent force. PDL cells were subjected to compressive force (2.0 or 5.0 g/cm(2)) for 2-4 days. Continuous or intermittent force was applied all day or for 8 hrs per day, respectively. At days 3 and 4, cell damage was less with intermittent force than with continuous force. At day 4, RANKL and IL-1beta expressions were greater with intermittent force than with continuous force. An IL-1 receptor antagonist inhibited the compressive force-induced RANKL expression. These findings indicate that IL-1beta is an autocrine factor regulating compressive force-induced RANKL expression in PDL cells, and that intermittent force can effectively induce RANKL in PDL cells with less cell damage.

Matthew D. Dunn, Chan Ho Park, Paul J. Kostenuik, Sunil Kapila, and William V. Giannobile (2007)[44] examined the role of OPG in regulating mechanically induced bone modeling in a rat model of orthodontic tooth movement. The maxillary first molars of male Sprague-Dawley rats were moved mesially using a calibrated nickel–titanium spring attached to the maxillary incisor teeth. Two different doses (0.5 mg/kg, 5.0 mg/kg) of a recombinant fusion protein (OPG-Fc), were injected twice weekly mesial to the first molars. Tooth movement was measured using stone casts that were scanned and magnified. Changes in bone quantity were measured using micro-computed tomography and histomorphometric analysis was used to quantify osteoclasts and volumetric parameters. The 5.0 mg/kg OPG-Fc dose showed a

potent reduction in mesial molar movement and osteoclast numbers compared to controls (p<0.01). The molar movement was inhibited by 45.7%, 70.6%, and 78.7% compared to controls at days 7, 14, and 21 respectively, with the high dose of OPG. The 0.5 mg dose also significantly (p<0.05) inhibited molar movement at days 7 (43.8%) and 14 (31.8%). The 5.0 mg/kg OPG-Fc dose showed a potent reduction in mesial molar movement and osteoclast numbers compared to controls (p<0.01). The molar movement was inhibited by 45.7%, 70.6%, and 78.7% compared to controls at days 7, 14, and 21 respectively, with the high dose of OPG. The 0.5 mg dose also significantly (p<0.05) inhibited molar movement at days 7 (43.8%) and 14 (31.8%). They concluded that local delivery of OPG-Fc inhibits osteoclastogenesis and tooth movement at targeted dental sites.

Hilal Uslu Toygar; Beyza Hancioglu Kircelli; Sule Bulut; Nurzen Sezgin; Bahar Tasdelen (2008)[26] investigated the level of osteoprotegerin (OPG) in gingival crevicular fluid (GCF) during tooth movement. Twelve patients (13–17 years of age) requiring canine distalization participated in the study. GCF sampling was done at baseline, 1 hour, 24 hours, 168 hours, 1 month, and 3 months from the distal sites of the test and with control teeth after the application of mechanical stress. OPG concentration was detected by enzyme-linked immunosorbent assay. OPG concentrations in distal sites of the test teeth were decreased in a time-dependent manner. The decrease is significant when compared with the baseline measurements (P < 0.038).

Variability was detected in the levels of OPG concentration in the distal sites of the control tooth throughout the experimental period. They concluded that OPG is one of the key mediators responsible for alveolar bone remodeling during tooth movement.

Fujita S, Yamaguchi M, Utsunomiya T, Yamamoto H, Kasai K. (2008)[18] examined the effects of low-energy laser irradiation on expressions of RANK, RANKL, and OPG during experimental tooth movement. To induce experimental tooth movement in rats, 10 g of orthodontic force was applied to the molars. Next, a Ga-Al-As diode laser was used to irradiate the area around the moved tooth and the amount of tooth movement was measured for 7 days. Immunohistochemical staining with RANK, RANKL, and OPG was performed. Real time PCR was also performed to elucidate the expression of RANK in irradiated rat osteoclast precursor cells in vitro. They found that in the irradiation group, the amount of tooth movement was significantly greater than in the non-irradiation group by the end of the experimental period. Cells that showed positive immunoreactions to the primary antibodies of RANKL and RANK were significantly increased in the irradiation group on day 2 and 3, compared with the non-irradiation group. In contrast, the expression of OPG was not changed. Further, RANK expression in osteoclast precursor cells was detected at an early stage (day 2 and 3) in the irradiation group.

Garlet TP, Coelho U, Repeke CE, Silva JS, Cunha Fde Q, Garlet GP (2008)[84] investigated the pattern of mRNAs expression encoding for osteoblast and osteoclast related chemokines, and further correlated them with the profile of bone remodeling markers in palatal and buccal sides of tooth under orthodontic force, where tensile (T) and compressive (C) forces, respectively, predominate. Real-time PCR was performed with periodontal ligament mRNA from samples of T and C sides of human teeth submitted to rapid maxillary expansion, while periodontal ligament of normal teeth were used as controls. Results showed that both T and C sides exhibited significant higher expression of all targets when compared to controls. Comparing C and T sides, C side exhibited higher expression of MCP-1/CCL2, MIP-1alpha/CCL3 and RANKL, while T side presented higher expression of OCN. The expression of RANTES/CCL5 and SDF-1/CXCL12 was similar in C and T sides. Their data demonstrate a differential expression of chemokines in compressed and stretched PDL during orthodontic tooth movement, suggesting that chemokines pattern may contribute to the differential bone remodeling in response to orthodontic force through the establishment of distinct microenvironments in compression and tension sides.

Yamaguchi (2009)[84] found that concentrations of RANKL in GCF increased during orthodontic tooth movement, and the ratio of concentration of RANKL to that of OPG in the GCF was significantly higher than in control sites. The study has shown the presence of RANKL and RANK in periodontal

tissues during experimental tooth movement of rat molars, and that PDL cells under mechanical stress may induce osteoclastogenesis through upregulation of RANKL expression during orthodontic tooth movement.

Lijun Tan; Yijin Ren; Jun Wang; Lingyong Jiang; Hui Cheng; Andrew Sandham; Zhihe Zhao (2009)[39] tested the null hypothesis that increased tooth displacement in ovariectomized rats is not related to differential expressions of OPG and RANKL in the periodontium. Eighty-four 12-week female rats were used; half were ovariectomized and half were not. Three months later, the maxillary first molar was moved mesially. Groups of rats were sacrificed at days 0, 1, 3, 5, 7, 10, and 14 after activation. Tooth movement was measured at each time point. OPG and RANKL expressions were examined through immunohistochemistry. Ovariectomized and nonovariectomized rats showed three-phase tooth movement. In both groups, OPG expression increased at the tension area and RANKL increased at the pressure area. The OPG/RANKL ratio coincided with tooth movement, especially in the linear phase from 7 to 14 days. They concluded that the increased rate of tooth movement in ovariectomized rats was related to differential expressions of OPG and RANKL.

Patricia Joyce Brooks; Dorrin Nilforoushan; Morris Frank Manolson; Craig A. Simmons; Siew-Ging Gong (2009)[56] employed a rat model of early orthodontic tooth movement using a split-mouth design (where contralateral side serves as a control) and performed immunohistochemical

staining to map the spatial expression patterns of RANKL at 3 and 24 hours after appliance insertion. They observed increased expression of RANKL, a molecule associated with osteoclastic differentiation, in the compression sites of the periodontal ligament subjected to 3 hours of force. The early RANKL expression indicates that at this early stage cells are involved in osteoclast precursor signaling.

Joanna Tyrovola, Despoina Perea, Dimitrios Halazonetis, Ismene Dontas, Ionnis Vlachos, Margarita Makou (2010)[30] carried out a study to determine the levels of OPG and soluble RANKL in blood serum and GCF relative to the degree of orthodontic root resorption in a rat model. They demonstrated a positive linear correlation between the initial concentration of RANKL in the blood serum and the degree of root resorption. The ratio of the initial concentrations of OPG to RANKL in the blood serum proved to be an independent prognostic factor of the degree of root resorption. The concentration of OPG in blood serum decreased significantly in cases of severe root resorption.

Materials and Methods

MATERIALS AND METHODS

Informed consent was obtained from all the subjects and the study protocol was reviewed by the Institutional Review Board (IRB) of Ragas Dental College and Hospital, Chennai.

The study group consisted of 10 subjects with the following inclusion criteria:

1. All of them were treated by post-graduate students in the Department of Orthodontics, Ragas Dental College and Hospital, Chennai.
2. These subjects, 4 females and 6 males ranged in age from 15 yrs to 25 yrs.
3. All the patients were treated using .022 Roth Ovation fully programmed brackets.
4. All the subjects were indicated for upper and lower first bicuspid extraction.
5. Space closure in these patients was carried out using sliding mechanics.
6. All subjects were in good general health with healthy periodontal tissues.
7. Probing depths were < 3mm with no radiographic evidence of periodontal bone loss.

Subjects were excluded if:

1. They had antibiotic therapy during the previous six months; or
2. They had taken anti-inflammatory medication during the month preceding the start of the study.

METHODS

Gingival crevicular fluid was collected at the following stages:

T1 - End of Stage I-Leveling and aligning.

T2 - Three weeks after initiation of Stage II- Space closure.

The samples were collected at T1 and T2, from the anterior sextants of the maxillary and mandibular arches, amounting to a total of 4 samples per subject.

At the beginning of Stage I, all the subjects were bonded with fully programmed .022 Roth Ovation brackets following upper and lower first bicuspid extraction.

Leveling and aligning was carried out through a sequence of arch wires beginning with .014 Niti and culminating in .019 x .025 SS.

Stage I was completed on an average within 6-8 months.

Samples were collected at this point from the anterior sextants of both the arches and were labeled as T1 (Figure 3).

Stage II-Space closure, was initiated using sliding mechanics. Hooks were soldered distal to the lateral incisors on the .019x.025 SS archwires. The six anterior teeth were consolidated using .008 inch SS ligature wire. Retraction force was applied with elastic ties. With the help of a Dontrix gauge (ETM Corporation, Glendora CA, USA) (Figure 5), the force was standardized at 300 gms per side.

Retraction was carried out for 21 days, following which another sample was collected from both the arches and labeled as T2 (Figure 6).

GCF collection

- To avoid contamination of the GCF samples, small deposits of plaque were removed with a periodontal probe, and heavy deposits with a sickle scaler.
- Following the isolation and drying of a site, disposable micropipettes (Ringcaps® -Hirschmann Labergerate, Hauptstr) (Figure 1) were inserted into the entrance of the gingival crevice.
- 4-5µl of GCF was collected from the six anterior teeth in both the maxillary and mandibular arches (Figure 4a, 4b).
- The collected GCF was stored in sterile Eppendorf tubes (Figure 2) at -65°C.
- The collected samples were analysed at Hubert Enviro Care Pvt. Ltd. Chennai

ELISA Testing for RANKL

The collected GCF samples were tested for RANKL using the RayBio® Human RANKL ELISA kit (Ray Biotech,Inc. Norcross, GA) (Figure 7). The microtiter plate provided in this kit has been pre-coated with an antibody specific to RANKL.

Assay Procedure

1. Each of the collected samples was diluted using the sample diluents provided in the kit.
2. 100µl of sample was added per well. The wells were covered with adhesive strips and incubated for 2 hours at 37°C.
3. The liquid from each well was removed, not washed.
4. 100µl of Biotin-antibody working solution was added to each well and incubated for 1 hour at 37°C.
5. Each well was aspirated and washed, repeating the process three times for a total of three washes.
6. 100µl of HRP (Horseradish Peroxidase) -avidin working solution was added to each well and the microtiter plate was covered with a new adhesive strip. It was then incubated for 1 hour at 37°C.
7. Each well was again aspirated and washed, repeating the process three times for a total of three washes.

8. 90µl of TMB (3,3',5,5' tetramethyl-benzidine) substrate was then added to each well and incubated for 10-30 minutes at 37°C.

9. 50µl of Stop Solution was then added to each well. The enzyme-substrate reaction is terminated by the addition of the sulphuric acid stop solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm using an ELISA plate reader (Figure 9).

ELISA Testing for OPG

The collected GCF samples were tested for RANKL using the RayBio® Human OPG ELISA kit (Ray Biotech,Inc. Norcross,GA) (Figure 8). The microtiter plate provided in this kit has been pre-coated with an antibody specific to OPG.

Assay Procedure

1. Each of the collected samples was diluted using the sample diluents provided in the kit.

2. 100µl of sample was added per well. The wells were covered with adhesive strips and incubated for 2.5 hours at 37°C.

3. The liquid from each well was removed, not washed.

4. 100 µl of prepared biotinylated antibody was added to each well. The microtiter plate was then incubated for 1 hour at room temperature with gentle shaking.

5. Each well was aspirated and washed, repeating the process three times for a total of three washes.

6. 100 µl of prepared Streptavidin solution was added to each well and again incubated for 45 minutes at room temperature with gentle shaking.

7. Each well was again aspirated and washed, repeating the process three times for a total of three washes.

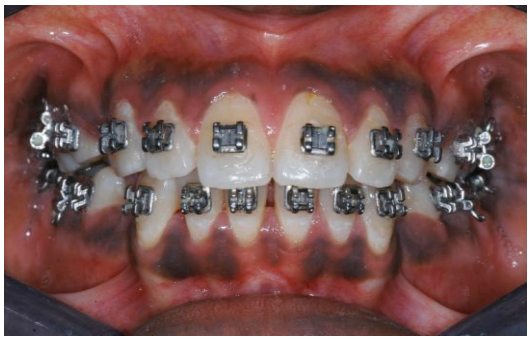
8. 100 µl of TMB One-Step Substrate Reagent was then added to each well. The plate was then incubated for 30 minutes at room temperature in the dark with gentle shaking. A blue color develops in proportion to the amount of Osteoprotegerin bound.

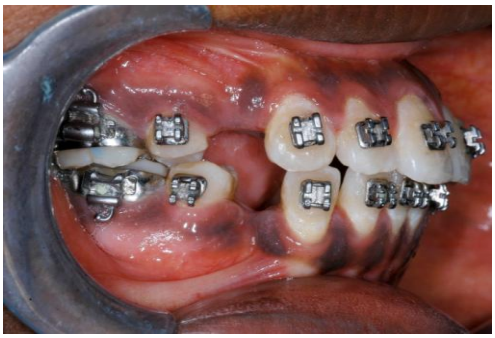
9. 50 µl of Stop Solution was added to each well. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm using an ELISA plate reader (Figure 9).

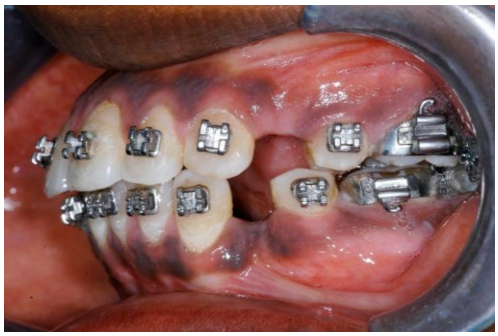
10.FIGURE 1: MICROPIPETTE FOR GCF COLLECTION

11.FIGURE 2: EPPENDORF TUBES

12. FIGURE 3: END OF STAGE I-LEVELING & ALIGNING (T1)







13.FIGURE 4a: GCF COLLECTION (UPPER ARCH)

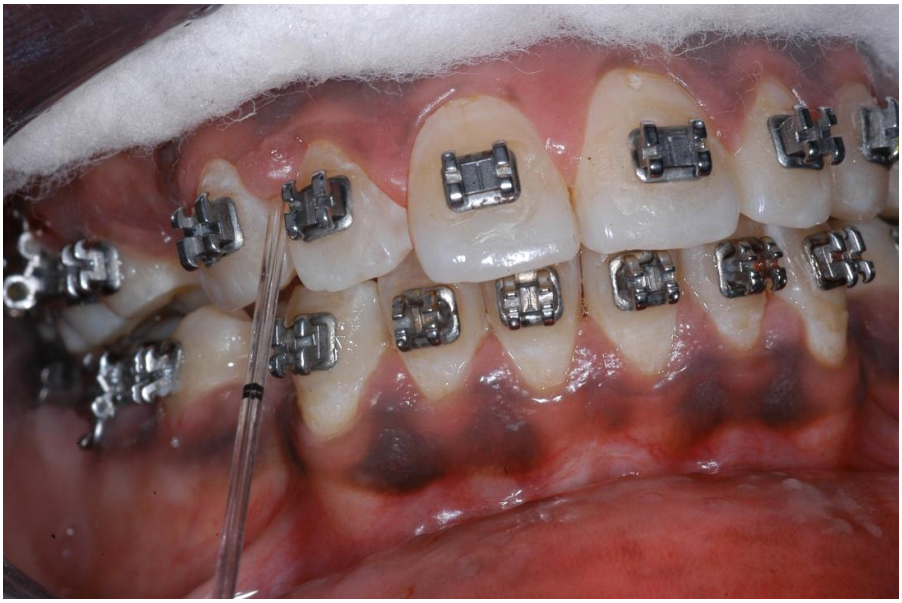


FIGURE 4b: GCF COLLECTION (LOWER ARCH)

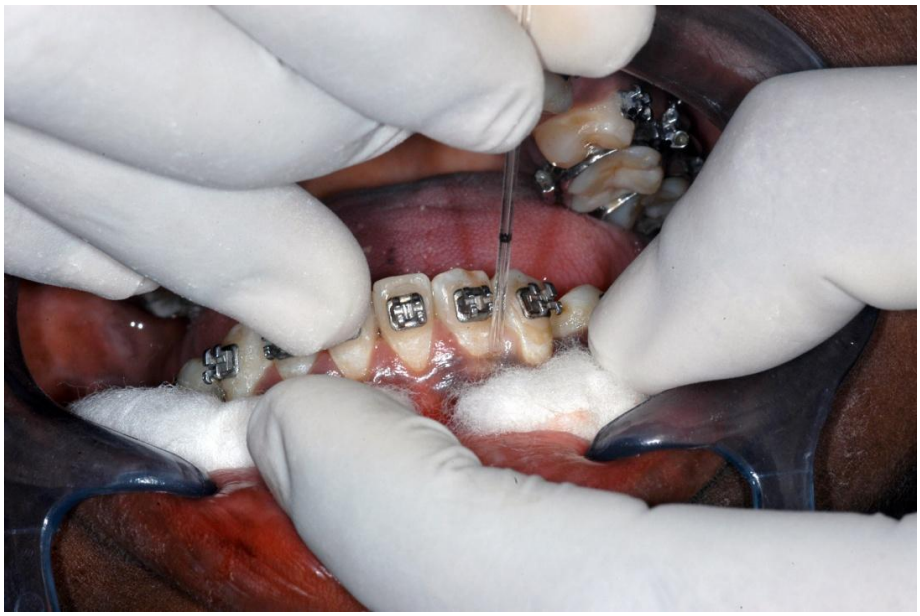


FIGURE 5: DONTRIX GAUGE

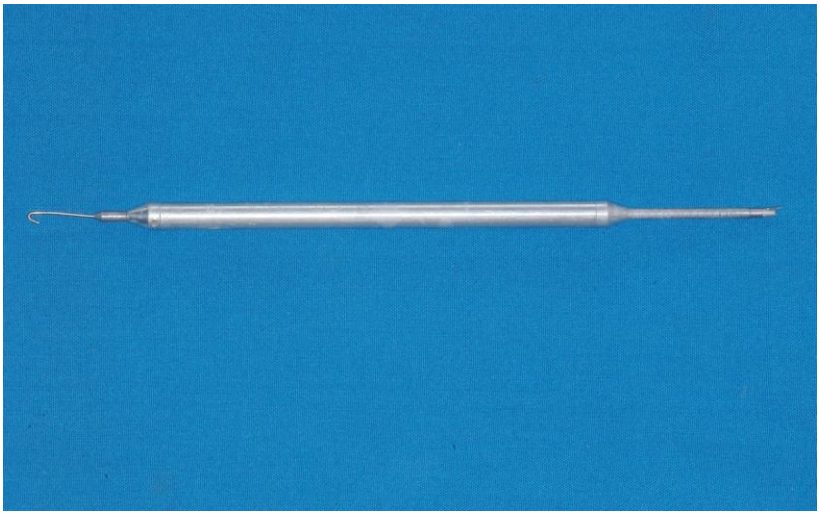
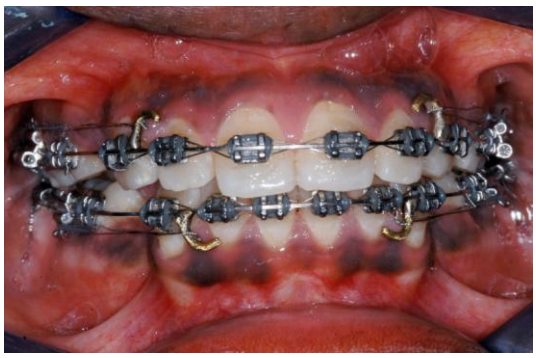
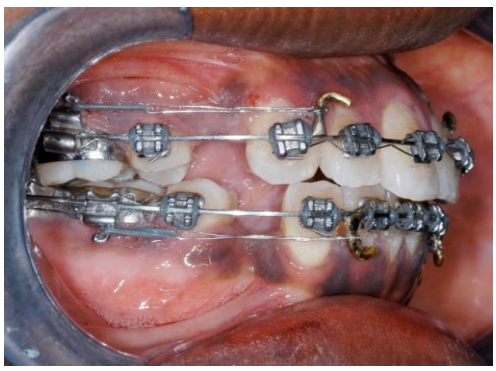


FIGURE 6: THREE WEEKS AFTER INITIATION OF SPACE CLOSURE (T2)





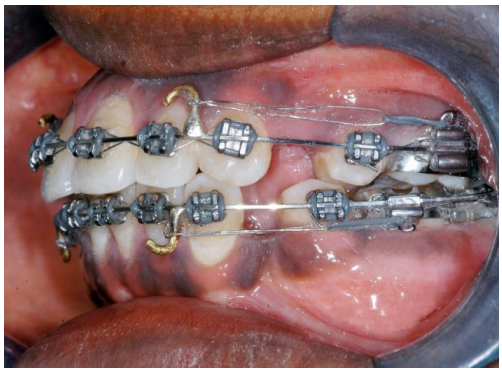
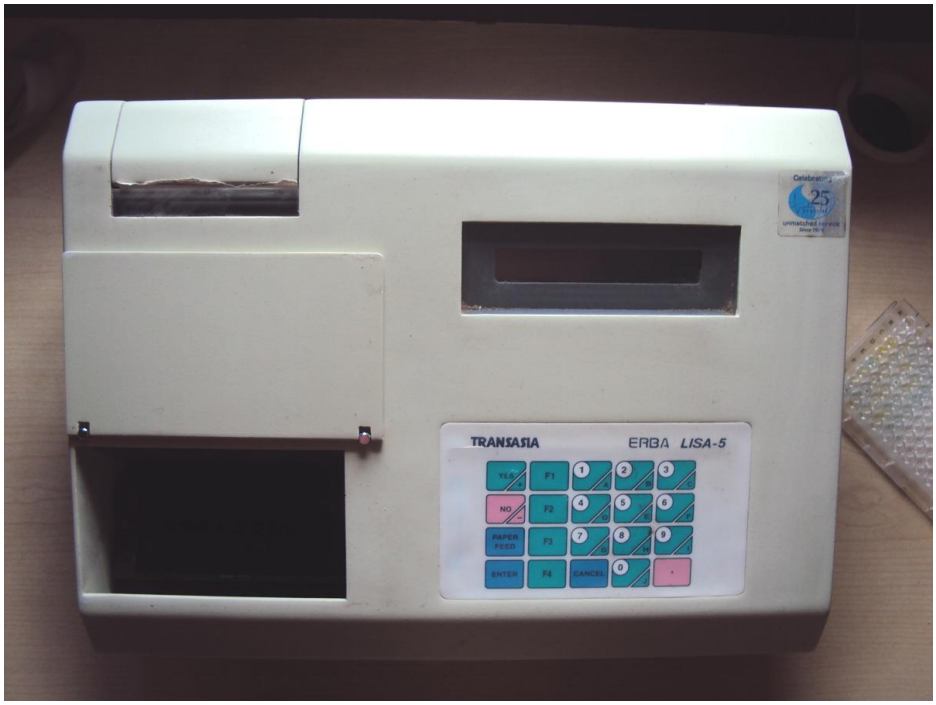


FIGURE 7: HUMAN RANKL ELISA KIT

FIGURE 8: HUMAN OPG ELISA KIT

FIGURE 9: ELISA PLATE READER



Results

RESULTS

The optical density of the samples was determined using an ELISA plate reader (Figure 9). The concentration of RANKL and OPG (pg/ml) in the samples was then determined by comparing the optical density of the samples to standard curves. Linear regression analyses were employed to obtain the standard curves (Figure 10) from which the corresponding concentrations of RANKL and OPG were determined.

- A student's T- Test was performed. The hypothesis being tested was that the concentration of RANKL in the GCF increases on application of retraction force and the concentration of OPG decreases with a concomitant increase in the RANKL/OPG ratio.
- The statistical analysis was carried out in the following parts;
    - Mean RANKL and OPG levels in the upper arch
    - Mean RANKL and OPG levels in the lower arch
    - RANKL/OPG ratio in the Upper and Lower arches
- The level of statistical significance was set at p=0.05.
- If the value of 'p' > 0.05, then the inference is that there is no statistical difference between the variables being compared.
- However, if the value of 'p' < 0.05, then the inference is that there is a statistical difference between the variables being compared.

UPPER ARCH

- The mean RANKL value at T1 was 1520 + 247.3 pg/ml and at T2 it was 1960 + 285 pg/ml.

  A statistically significant increase ($p < 0.001$) in the RANKL concentration was observed at T2 compared to T1. (Table 1, Figure 11)

- The mean OPG value at T1 was 42.8 + 8.3 pg/ml and at T2 it was 29.6 + 8.43 pg/ml.

  A statistically significant decrease ($p < 0.0001$) in the OPG concentration was observed at T2 compared to T1.(Table 1, Figure 13)

- A statistically significant increase in the RANKL/OPG ratio ($p < 0.01$) was observed from T1 (35.5 **:** 1) to T2 (66.2 **:** 1).(Table 3, Figure 15)

LOWER ARCH

- The mean RANKL value at T1 was 1584 + 279.9 pg/ml and at T2 it was 2008 + 276.7 pg/ml.

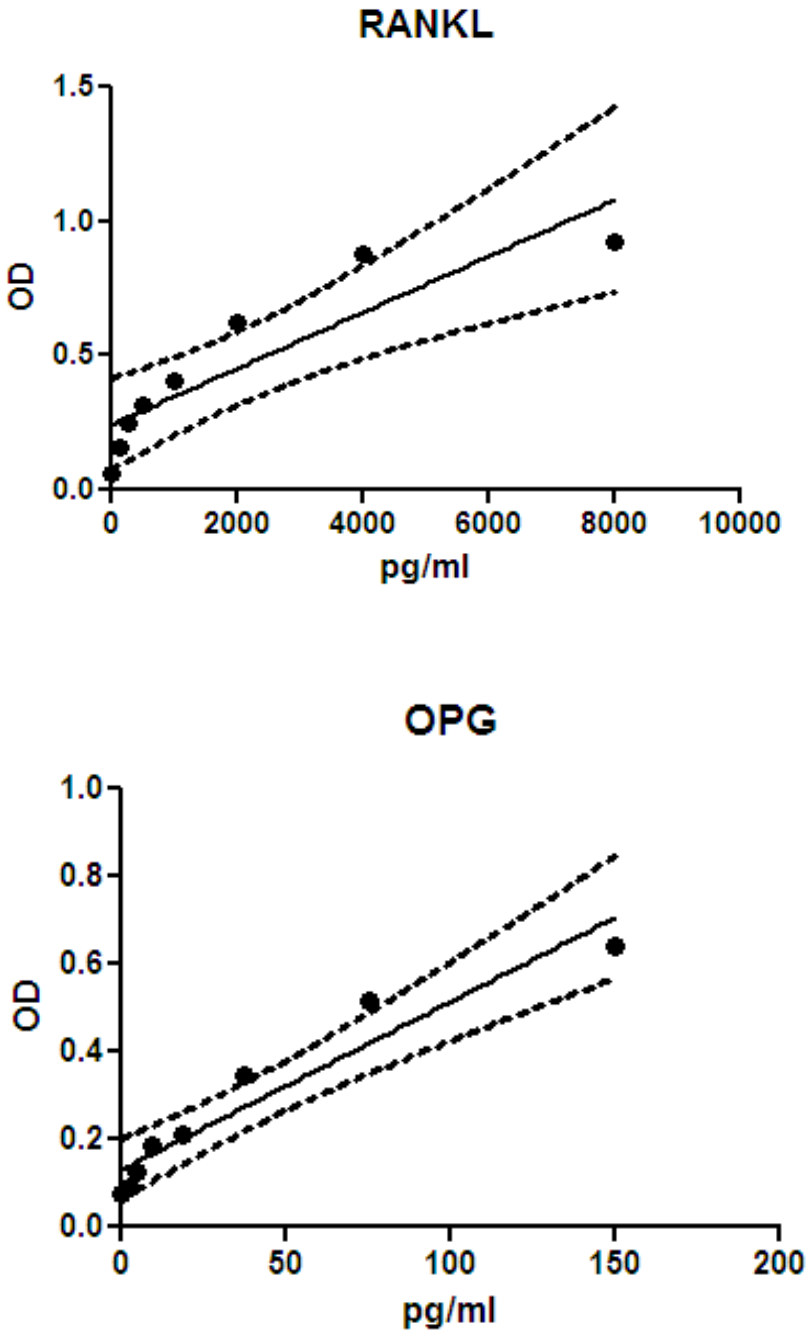
- A statistically significant increase ($p < 0.0001$) in the RANKL concentration was observed at T2 compared to T1. (Table 2, Figure12)

- The mean OPG value at T1 was 40.35 + 7.472 pg/ml and at T2 it was 20.10 + 7.331 pg/ml.

  A statistically significant decrease ($p < 0.0001$) in the OPG concentration was observed at T2 compared to T1. (Table 2, Figure 14)

- A statistically significant increase in the RANKL/OPG ratio ($p < 0.001$) was observed from T1 (39.3 : 1) to T2 (99.1 : 1).(Table 4, Figure 16)

FIGURE 10: STANDARD CURVES FOR CONVERSION OF OPTICAL DENSITY TO PG/ML



OD: OPTICAL DENSITY

Pg/ml: PICOGRAMS/ MILLILITRE

Table 1: Paired Samples T-Test for the Upper Arch

		Mean (pg/ml)	N	Std. Error	T - value	P - value
RANKL	T1	1520	10	247.3	5.485	<0.001
	T2	1960	10	285		
OPG	T1	42.8	10	8.3	10.54	<0.0001
	T2	29.6	10	8.43		

Table 2: Paired Samples T-Test for the Lower Arch

		Mean (pg/ml)	N	Std. Error	T - value	P - value
RANKL	T1	1584	10	279.9	7.940	<0.0001
	T2	2008	10	276.7		
OPG	T1	40.35	10	7.472	16.2	<0.0001
	T2	20.10	10	7.331		

Table 3: RANKL/OPG ratio- Upper Arch

		Mean	N	Std. Error	T - value	P - value
RANKL/ OPG	T1	40.52 (35.5 : 1)	10	6.256	3.168	<0.01
	T2	104.9 (66.2 : 1)	10	26.11		

Table 4: RANKL/OPG ratio- Lower Arch

		Mean	N	Std. Error	T - value	P - value
RANKL/ OPG	T1	43.78 (39.3 : 1)	10	8.144	4.848	<0.001
	T2	111.5 (99.1 : 1)	10	20.64		

FIGURE 11: RANKL LEVELS IN UPPER ARCH

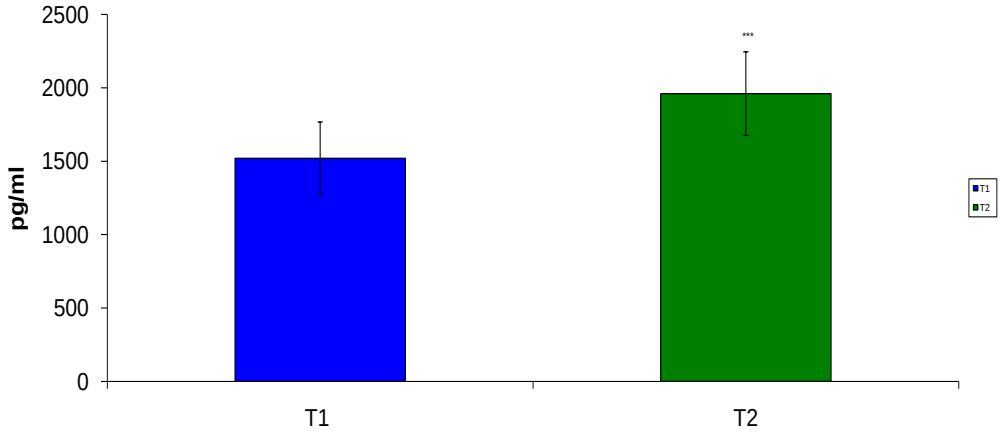


FIGURE 12: RANKL LEVELS IN LOWER ARCH

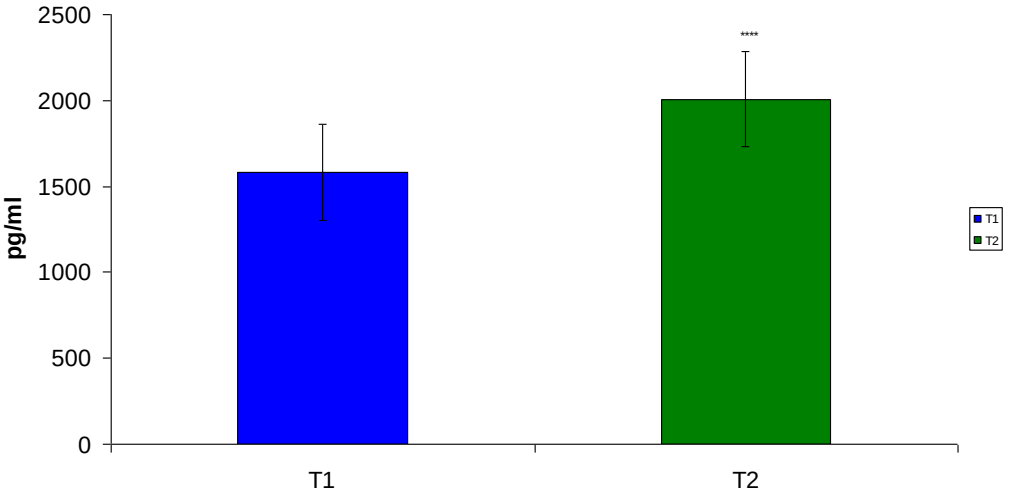


FIGURE 13: OPG LEVELS IN UPPER ARCH

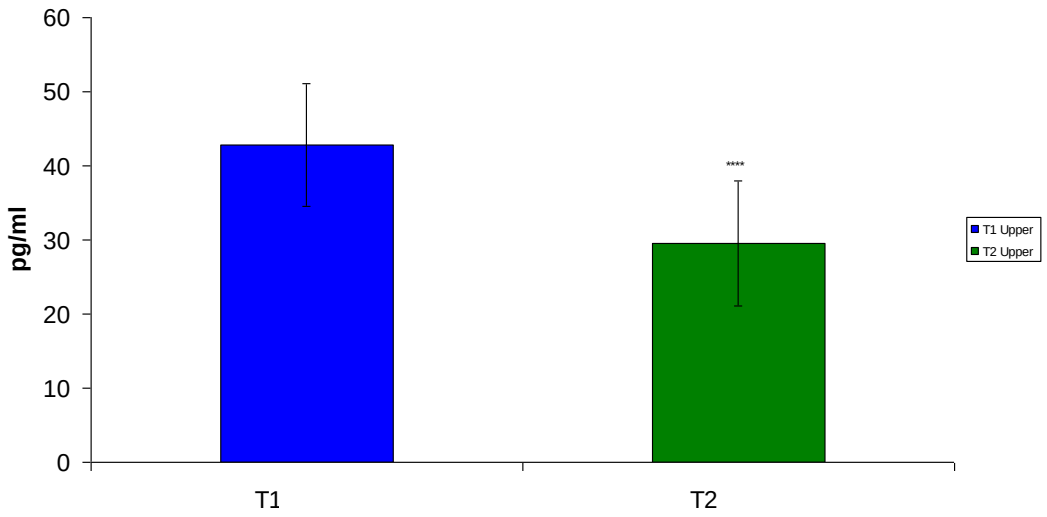


FIGURE 14: OPG LEVELS IN LOWER ARCH

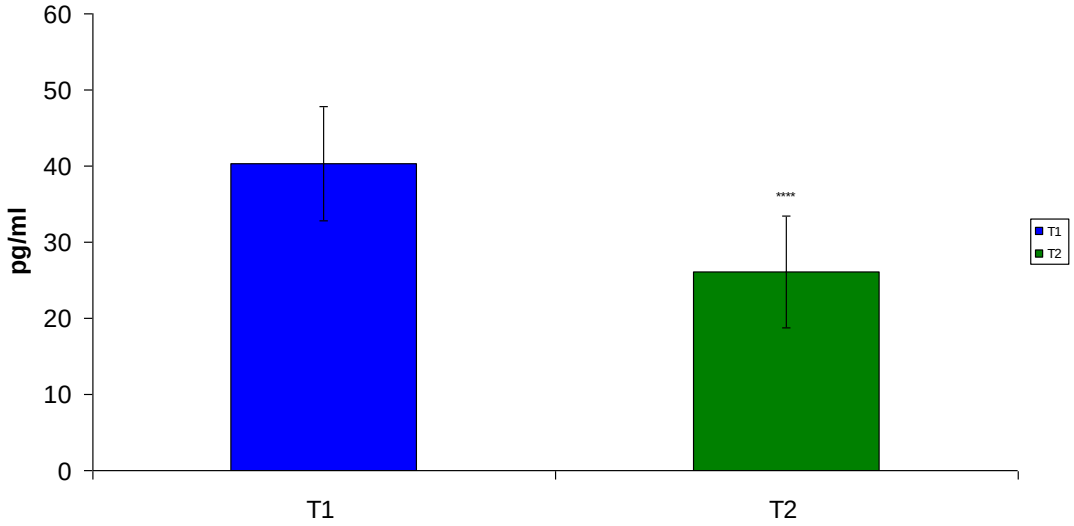


FIGURE 15: RANKL/OPG RATIO IN LOWER ARCH

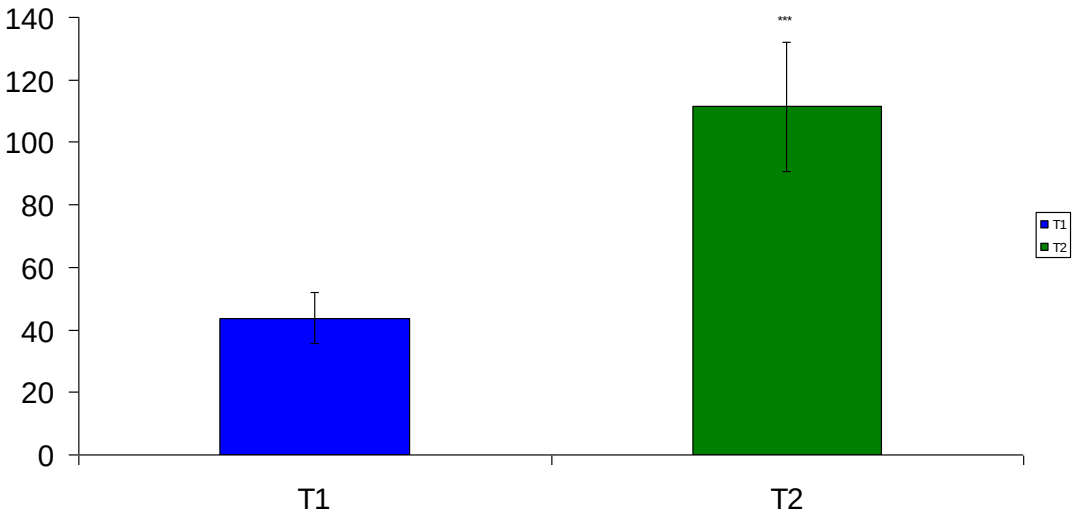
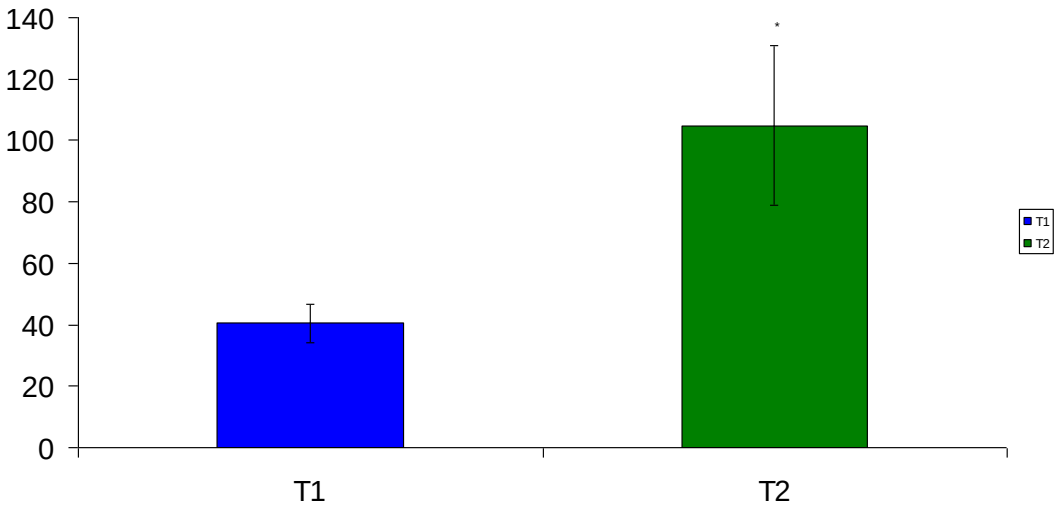


FIGURE 15: RANKL/OPG RATIO IN UPPER ARCH



Discussion

DISCUSSION

Often thought of as a rigid, unchanging entity, skeletal bone is actually the result of a dynamic process involving the secretion and resorption of the bone matrix. These opposing actions are carried out by two cell types — osteoblasts and osteoclasts, respectively — and must be kept in balance to maintain skeletal integrity and calcium metabolism.

Continuously changing functional demands require permanent adaption of the bone structure and microarchitecture. Wolff[83] has observed this principle of functional adaptation already over 100 years ago. The process of where 'form follows function' [83] consists of two activities, namely, bone formation and bone resorption. While these processes are locally separated in modeling [16,17], bone remodeling is characterized by the spatial and temporal coupling of bone formation by osteoblasts and bone resorption by osteoclasts [58].

Osteoclasts are formed from bone-marrow-derived precursor cells in response to the coordinated actions of several protein factors. The study of a group of these proteins –RANK/RANKL/OPG has led to a better understanding of the bone remodeling process.

In the present study, taking into account the various changes in proportion of RANKL and OPG that occur during tooth movement, a ratio

was sought which could act as an indicator to assess the progress of tooth movement. A brief review of the mechanism of action of these cytokines at the cellular level which is necessary to understand how the change in the proportions occur, is discussed here.

RANKL exists as a homotrimeric protein and is typically membrane-bound on osteoblastic and activated T cells or is secreted by some other cells, such as fibroblasts or synovial mesenchymal cells. [34,76] Most of the factors known to stimulate osteoclast formation and activity induce RANKL expression by osteoblastic stromal cells. It is expressed by synovial cells and activated T cells in joints of patients with inflammatory arthritis to contribute at least in part to the joint destruction seen in patients with rheumatoid arthritis. RANKL also stimulates the release of osteoclast precursor cells (OCPs) into the circulation; and recent studies suggest that osteoclasts themselves regulate the emergence of hematopoietic stem cells (HSCs) from niches within the marrow under the control of RANKL.[76]

RANK is a homotrimeric transmembrane protein member of the TNF receptor superfamily. It appears to be expressed in fewer tissues than RANKL at the protein level, but in addition to OCPs, mature osteoclasts and dendritic cells, it is expressed in mammary glands and some cancer cells, including breast and prostate cancers[38], two tumors with high bone metastatic potential.

Simonet et al.[66] discovered a protein which exposed an osteopetrotic phenotype when overexpressed in transgenic mice. Investigating further, they

found that this protein was secreted by preosteoblasts/stromal cells and was capable of inhibiting osteoclast development and activation. Due to its bone-protective effects they named it osteoprotegerin. OPG belongs to the TNF receptor superfamily. It is expressed in a variety of tissues, including lung, heart, kidney, liver, stomach, intestine, brain, spinal cord, thyroid gland, smooth muscle tissue and bone, indicating multiple possible functions including endothelial cell survival[11,43] and vascular calcification[2]. The most prominent role of OPG however, has been assigned to bone protection as OPG acts as scavenger of RANKL, thus inhibiting OC-precursors to become mature OC.[27]

When RANKL expression is enhanced relative to OPG, RANKL is available to bind with RANK on osteoclast precursors, tipping the balance to favor activation of osteoclast formation and bone resorption.[8] The binding of RANKL to osteoclast precursors occurs at a stage when hematopoetic stem cells have differentiated from the colony forming unit for granulocytes and macrophages (CFU-GM) to the colony forming unit for macrophages (CFU-M). Binding of RANKL to RANK on CFU-M in the presence of macrophage colony-stimulating factor induces differentiation of the preosteoclast into a multinucleated cell that becomes a mature osteoclast. [3]

When OPG concentrations are high relative to RANKL expression, OPG binds RANKL, inhibiting it from binding to RANK.[8] Preventing the

binding of RANKL to RANK leads to reduced formation of osteoclasts and apoptosis of existing osteoclasts.[8]

A dynamic interaction also exists between the immune system and bone. Aaron and Choi[1] coined the term "osteoimmunology" and highlighted the interdigitate communication between the immune and skeletal systems especially observed in autoimmune and other inflammatory diseases. During inflammation, activated T cells express more of the RANKL protein and secrete the interferon-γ molecule. RANKL is important for communication between T cells and antigen presenting dendritic cells. It is also essential for the differentiation, activation and survival of osteoclasts. It binds to RANK on osteoclasts to trigger intracellular signaling pathways that promote osteoclast differentiation and bone resorption. The TRAF6 protein is a key intermediate in these pathways.[1] However, if osteoclasts were activated every time T cells were activated, bone loss would be more common than it is. Takayanagi et al.[71] proposed a solution: interferon-γ promotes the degradation of TRAF6, thereby preventing activated T cells from triggering massive bone destruction during inflammation. The effects of RANKL can also be blocked by OPG[27], which is the known soluble decoy receptor. Together, then, RANKL, interferon-γ and OPG maintain the balance between bone deposition and bone resorption.

Microbial biofilms elicit a host-inflammatory process whereby monocytes, macrophages and neutrophils release enzymes and cytokines such

as RANKL and OPG that activate osteoclasts and osteoblasts to stimulate bone remodeling. External mechanical stresses have also been shown to induce the expression of these cytokines.[31,51,85] These proteins then traverse the tissue and exit through the periodontal pocket and can be measured in the gingival crevice fluid.

In the present study, we found increased levels of RANKL and decreased levels of OPG in GCF samples collected from areas adjacent to teeth undergoing orthodontic tooth movement. In both the upper and lower arches the increase in RANKL levels and the decrease in OPG concentration was found to be statistically significant. (Table 1, 2)

The RANKL levels in the upper arch increased from 1520 pg/ml at T1 to 1960 pg/ml at T2, ($p<0.001$) and in the lower arch an increase from 1584 pg/ml at T1 to 2008 pg/ml at T2 ($p<0.0001$) was observed. (Figure 11, 12)

The OPG levels showed a corresponding decrease in concentration with the upper arch levels going from 42.8 pg/ml at T1 to 29.6 pg/ml at T2 ($p<0.0001$). Similarly in the lower arch as well OPG levels declined from 40.35 pg/ml at T1 to 20.10 pg/ml at T2 ($p< 0.0001$). (Figure 13, 14)

These values enable us to interpret and calculate the relative RANKL/OPG which is a major determinant in bone remodeling during orthodontic tooth movement. Generally, bone resorption and formation follow

a specific pattern and an analysis and quantification of the involved cytokines can help us to calculate the relative ratio. Formation of bone may be attributed to an abundance of OPG or reduced expression of RANKL, resulting in a net increase in OPG, also known as a decrease in the RANKL/OPG ratio. Conversely, a relative decrease in concentrations of OPG or increase in RANKL expression results in a net increase in RANKL and bone resorption, also known as an increase in RANKL/OPG ratio.[10]

Bone Resorption:	RANKL → OPG	↑ RANKL =OPG	or	=RANKL ↓OPG
Bone Formation:	RANKL → OPG	= RANKL ↑ OPG	or	↓RANKL = OPG

Thus the relative proportion of RANKL and OPG determines whether RANK is activated, with increasing RANKL/OPG ratios associated with RANK-dependent cellular activation.[15] Whether bone resorption or formation occurs depends critically on the RANKL/OPG ratio, which is a function of the relative expression levels of RANKL and OPG.[10]

In the present study, an increase in the RANKL/OPG ratio was recorded in both the arches which indicates a definite shift towards increased osteoclastic activity. (Table 3,4)

RANKL/OPG ratio in Upper arch at T1: $\frac{1520}{42.8}$ = 35.5 : 1

RANKL/OPG ratio in Upper arch at T2: $\frac{1960}{29.6}$ = 66.2 : 1

RANKL/OPG ratio in Lower arch at T1: $\frac{1584}{40.35}$ = 39.3 : 1

RANKL/OPG ratio in Lower arch at T2: $\frac{2008}{20.10}$ = 37.3 : 1

In the upper arch the ratio of RANKL to OPG increased from 35.5 : 1 at T1 to 66.2 : 1 at T2. ($p<0.01$)(Figure 15), while in the lower arch an increase in the ratio was observed from 39.3 : 1 at T1 to 99.1 : 1 at T2 ($p<0.001$)(Figure 16).

Available literature amply implicates an increase in the RANKL/OPG ratio in the progression of periodontal disease and in bone remodeling during orthodontic tooth literature. However, the factors contributing to this increase in ratio may vary.

Mogi[42] and Bostanci[46] demonstrated that levels of RANKL and OPG in GCF were reciprocally regulated in periodontal disease; i.e., an elevation in RANKL protein and a decrease in OPG were observed in the GCF of individuals with periodontitis compared with healthy controls. In contrast, a study by Lu,[39] demonstrated that RANKL concentrations in GCF of

individuals with periodontitis were increased compared to the controls, whereas the OPG concentration was unchanged. However, the findings of all three studies still showed a net increase in the RANKL/OPG ratio.

The results of the present study are in agreement with the studies conducted by Mogi[42] and Bostanci[46] and with the majority of studies, wherein an increase RANKL levels and a decrease in OPG concentration both contributed to the resultant increase in the RANKL/OPG ratio.

A few variations in the methodology of existing in vivo studies were incorporated into the present study.

The experimental design of existing similar studies by Nishijima et al[50] and Kawasaki et al[33] carried out force application with a power chain which does not provide a continuous and consistent force over the entire experimental period. It is known that elastomeric chains generally lose 50% to 70% of their initial force during the first day of load application.[5] Continuous force application was attributed to present a constant responsive state in the cell biology system in opposition to intermittent force application, which probably creates a fluctuating environment of cellular activity/quiescence.[29,80] Therefore, the experimental design in the present study comprised continuous force application by using e-ties that exert a relatively constant force during the entire experimental period of 3 weeks..

Another point of variation in the study methodology was the duration of force application. Complete orthodontic tooth movement because of the alveolar bone–remodeling process involves several phases[55] over a certain period of time. In most of the existing in vivo studies, different mediators produced from the PDL cells that are responsible for the bone-remodeling mechanism during orthodontic tooth movement were evaluated over a relatively short period of time.[13,33,50,79] A 168-hour investigation such as that undertaken by Nishijima et al,[50] might present data belonging to only the first two phases of the process (displacement and delay phases, respectively) in most circumstances. However, data belonging to the acceleration or linear phase, in which true orthodontic tooth movement is considered to take place, require an observation period of at least 15-21 days. Therefore, we investigated the levels of OPG and RANKL in GCF over a 3 week period.

The enzyme-substrate reaction in the ELISA test was terminated by the addition of a stop solution and the color change was measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of RANKL (pg/ml) in the samples was then determined by comparing the optical density of the samples to the standard curve. Linear regression analyses were employed to obtain the standard curves from which the corresponding concentrations of RANKL and OPG were determined.

For the RANKL assay the detection range was 125 pg/ml - 8000 pg/ml. The standard curve concentrations used were 8000 pg/ml, 4000 pg/ml,

2000 pg/ml, 1000 pg/ml, 500 pg/ml, 250 pg/ml, 125 pg/ml. For the OPG assay the detection range was 2.343 pg/ml – 150 pg/ml. The standard curve concentrations used were 2.343 pg/ml, 4.687 pg/ml, 9.375 pg/ml, 18.75 pg/ml, 37.5 pg/ml, 75 pg/ml, 150 pg/ml.

The detection of local concentration of OPG and RANKL in GCF and calculating the variation in the relative RANKL/OPG ratio with force application can help the orthodontist to assess the status of bone remodeling during tooth movement. The OPG/RANKL ratio may change with the intensity of orthodontic forces. The orthodontist may use this information as a guide for detecting the optimum orthodontic treatment duration to avoid pathologic alveolar bone and root resorption and hyalinization in patients with bone metabolism pathologies such as hypoparathyrodism or hyperparathyroidism and also in patients with inflammatory conditions like periodontitis. At present, there may be many questions about the local response of the bone to the orthodontic forces. In the future, further research and refinement in this field may help to clear this ambiguity.

Summary and Conclusions

SUMMARY AND CONCLUSIONS

This study was undertaken to assess the change in RANKL and OPG levels in the GCF following the application of orthodontic force. GCF samples were collected from the upper and lower arches of orthodontic patients who had undergone upper and lower first bicuspid extractions. The samples were collected at two time points; T1-End of stage I (Leveling & aligning) and T2- Three weeks after application of retraction force. Disposable micropipettes were used for GCF collection and the collected samples were subjected to an ELISA test to determine the concentrations of RANKL and OPG.

- Both upper and lower arches showed an increase in RANKL levels from T1 to T2.

  In the upper arch a statistically significant increase ($p < 0.001$) in the RANKL concentration was observed at T2 (1960 + 285pg/ml) compared to T1 (1520 + 247.3pg/ml).

  In the lower arch as well a statistically significant increase ($p < 0.0001$) in the RANKL concentration was observed at T2 (2008 + 276.7pg/ml) compared to T1 (1584 + 279.9 pg/ml)

- A reciprocal decrease in the OPG concentration was observed in the upper and lower arches.

The upper arch showed a decrease in OPG levels from 42.8 + 8.3 pg/ml at T1 to 29.6 + 8.43 pg/ml at T2. ($p<0.0001$)

The lower arch showed a decrease in OPG levels from 40.35 + 7.472 pg/ml at T1 to 20.10 + 7.331 pg/ml at T2. ($p<0.0001$)

- An increase in the relative RANKL/OPG ratio was seen in both arches.

  In the upper arch, RANKL/OPG ratio increased from 35.5 : 1 at T1 to 66.2 : 1 at T2. ($p<0.01$)

  The lower arch showed an increase in RANKL/OPG ratio from 39.3:1 at T1 to 99.1:1 at T2. ($p<0.001$)

- Both factors, i.e, an increase in the RANKL levels and a decrease in the OPG levels contributed to the observed increase in RANKL/OPG ratio. This is in accordance with existing invivo studies by Mogi et al.[42] and Bostanci et al.[46]

  The present findings differed from a study done by Lu et al.[39] where the OPG levels remained unchanged and only an increase in RANKL concentration contributed to the increase in RANKL/OPG ratio.

- The study design varied from existing studies in the manner and duration of force application.

Instead of elastic chains which were used in previous studies, this study used e-ties to apply the retraction force. This was done to provide a relatively constant force over the entire experimental period.

Existing invivo studies have compared the cytokine levels 168 hours after application of orthodontic force. This study increased the duration of the force application to 3 weeks to obtain data belonging to the linear or acceleration phase of tooth movement which require an observation period of at least 15-21 days

- Further research in this field would help to refine the analysis of these biologic markers of tooth movement in a clinical setting. This sort of advancement would be especially useful in patients with bone metabolic problems as well as in patients suffering from inflammatory periodontal disease.

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