EVALUATION OF THE EFFECTS OF ORTHODONTIC TOOTH MOVEMENT ON THE LEVELS OF INTERLEUKIN – 10 IN GINGIVAL CREVICULAR FLUID

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CERTIFICATE

This is to certify that this dissertation titled **" EVALUATION OF THE EFFECTS OF ORTHODONTIC TOOTH MOVEMENT ON THE LEVELS OF INTERLEUKIN – 10 IN GINGIVAL CREVICULAR FLUID "** is a bonafide record of work done by **DR. C.RAVANTH KUMAR** under my guidance during his postgraduate study period between 2010–2013.

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

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CONTENTS

ABSTRACT

When orthodontic forces are applied to a tooth, they are transmitted to the periodontal ligament (PDL) and adjacent alveolar bone.

These forces, in turn, initiate a complex cascade of events that result in the remodeling of the surrounding bone and eventual movement of the tooth[2]**.**

While the sequence of events at the tissue and cellular levels of bone remodeling is well described, there remains a lack of comprehensive understanding in the coordination of biochemical events at the molecular level.

While there are numerous interconnected systems in place that control the levels of mature osteoclasts and osteoblasts active during bone remodeling, one predominate pathway uses the cytokine family interleukin -10 (IL -10) as a signal transducer .

Most importantly, IL - 10 plays a critical role in the differentiation of both osteoclasts and osteoblasts. Osteoclasts are derived from hematopoietic precursors in the bone marrow, whereas osteoblasts are mesenchymal in origin[5].

Regardless of the cell's origin, however, IL-10 type cytokines stimulate the differentiation of the respective precursors into osteoblasts and osteoclasts.

Thus the aim of the study was to evaluate the level of INTERLEUKIN – 10 in GINGIVAL CEVICULAR FLUID (GCF) during orthodontic tooth movement with the methodology of assessment using ELISA technique.

Key-words : Bone remodelling, Interleukin – 10, GCF, ELISA technique

INTRODUCTION

Mechanotransduction induced by orthodontic force occurs when external strain induces mechanosensing (the cell senses structural changes in the extracellular matrix, caused by external mechanical loading), transduction and cellular response in several paradental tissues.This process leads to vasculature and extracellular matrix remodeling in the periodontal ligament (PDL), gingiva, and alveolar bone.This remodeling is facilitated by proliferation,differentiation, and apoptosis of local periodontal cells, bone cell precursors, and leukocyte migration from the microvascular compartment.,An understanding of bone remodeling is of particular interest to the profession of orthodontics.

Unperturbed, the native pattern of alveolar bone remodeling maintains a homeostatic state.

The introduction of orthodontic treatment, however, disrupts the equilibrium between bone apposition and bone resorption [103].

When orthodontic forces are applied to a tooth, they are transmitted to the periodontal ligament (PDL) and adjacent alveolar bone.

These forces, in turn, initiate a complex cascade of events that result in the remodeling of the surrounding bone and eventual movement of the tooth[104] .

While the sequence of events at the tissue and cellular levels of bone remodeling is well described, there remains a lack of comprehensive understanding in the coordination of biochemical events at the molecular level [105]. As such, there have been numerous studies that have sought to further elucidate the molecular biology of bone remodeling during orthodontic tooth movement.

In order to examine the molecular biology involved, a cellular understanding of tooth movement is prerequisite. Osteoclasts are the primary bone resorbing cells in the body, whereas osteoblasts are the primary bone forming cells. A large number of osteoclasts are recruited to the resorptive front during tooth movement [106]. As the tooth begins to move into the resorbed area, osteoblasts proliferate and begin to secrete the precursors of new bone in the vacated area [107,108].

Cycling between osteoclast and osteoblast activity, the body must maintain a type of equilibrium in order to obtain efficient bone remodelling during tooth movement.

While there are numerous interconnected systems in place that control the levels of mature osteoclasts and osteoblasts active during bone remodeling, one predominate pathway uses the cytokine family interleukin -10 (IL -10) as a signal transducer .

Most importantly, IL - 10 plays a critical role in the signalling mechanism of both osteoclasts and osteoblasts. Osteoclasts are derived from hematopoietic precursors in the bone marrow, whereas osteoblasts are mesenchymal in origin.[109]

IL-10 also interacts with other factors that are critically involved with bone homeostasis, including the receptor activator of nuclear factor kappa-β (RANK)/receptor activator of nuclear factor kappa-β ligand (RANKL)/ osteoprotegerin (OPG) system, sex steroids, prostaglandin E2 (PGE2), tumor necrosis factor (TNF)-α, parathyroid hormone (PTH), IL- 11, and IL-1b.

While IL 4,6,8 has been extensively studied in other models of bone metabolism, the investigation of the role of IL -10 during orthodontic tooth movement is relatively limited.

The gingival crevicular fluid (GCF) is an osmotically mediated inflammatory exudates found in the gingival sulcus. As an exudate,it tends to increase in volume with inflammation and capillary permeability.[110]

Serum is the primary source of the aqueous component of the GCF. However, the gingival tissue through which the fluid passes, along with bacteria both in tissues and the gingival crevice, can modify its composition. Therefore, its constituent vary according to the condition of the periodontal tissues. In general, cells, immunoglobulins, microorganisms, toxins, and lysosomal enzymes can all be detected in the GCF.[116]

The released molecules evoke cellular responses in the various cell types in and around teeth, providing a favourable microenvironment for tissue deposition or resorption.

Various cell-signalling pathways are activated, which ultimately stimulate PDL turnover, as well as localised bone resorption and bone deposition.

Additionally, since tooth movement is a reliable model of overall bone metabolism, the results of this study will aid in more clearly understanding somatic bone remodeling and repair.

Furthermore, orthodontic tooth movement and periodontal disease involve similar factors, and knowledge of the cytokines involved in both processes may lead to an increased comprehension of the complications involved with tooth movement such as loss of attachment and external apical root resorption. Lastly, future research may provide the clinician with novel techniques that make use of the complex role of IL-10 in orthodontic tooth movement. A convenient and non-invasive method to study molecules involved with tooth movement is gingival crevicular fluid (GCF) analysis. Using this approach, the presence of many inflammatory factors has been identified, and the role these factors play in orthodontic tooth movement has become more clearly understood.

Previously bone resorptive markers were studied extensively using the gcf samples from rats.

Other forms of bone remodelling studies have been studied using cone beam computed tomography (cbct) ,but the drawbacks in those study were the radiation dosage was a factor of concern to the orthodontic profession[114] . so a non-invasive methodology to study bone remodelling in patients have to be studied in this regard so the gcf study was undertaken.

Although studies have been done in the past using different markers ,very few studies have been done using IL – 10 marker. So the present study was done to quantify bone remodelling based on IL – 10.

Prior to this study many studies based on orthodontic tooth movement evaluation using gcf was carried out in subjects who were in retraction stage or end of retraction stage .In those studies bone remodelling was identified with elevation of the pro-inflammatory and anti –inflammatory markers.

In this study we are assessing bone remodelling at the end of levelling and alignment stage itself by quantifying the elevation of IL – 10 marker between the two time points T0 and T1and between upper and lower arches which is unique to this study.

Thus the aim of this study was to evaluate the effects of orthodontic tooth movement on the levels of interleukin 10 in GCF.

REVIEW OF LITERATURE

Reitan (1951)[1] stated that the first stage occurs when tooth movement is initiated by the increased mechanical loading of an orthodontic force. The initial movement of the tooth within the periodontal ligament space results in a positive strain on the bone apposition side of the periodontal ligament. The side opposite of the appositional side, on the other hand, undergoes a negative strain within the periodontal ligament, and is commonly referred to as the pressure side.

Midgett *et al* (1981)[2] stated that the periodontal ligament is compressed on the side opposite of the force causing an increase in pressure, whereas the periodontal ligament on the same side of the force increases in tension . The changes in pressure and tension within the periodontal ligament space initiate cellular changes produced by chemical messengers.

On the pressure side, there is a decrease in cellular replication as a result of vascular constriction, whereas on the tension side, there is an increase in cellular replication because of the stimulation afforded by the stretching of the fiber bundles of the periodontal ligament.

Rygh *et al* (1986)[3] stated that the side of bone apposition is often referred to as the tension side in the pressure-tension theory but may be more accurately described as the appositional side. This positive strain creates a stretching of the collagen fibers connecting the tooth to the bone, which in turn, can activate residing periodontal cells such as osteoblasts and fibroblasts.

Davidovitch *et al* (1988)[4] stated that pathways stimulate the production of mediators such as interleukins, nitric oxide, and prostaglandins that activate several types of cells.

Davidovitch (1991)[5] stated that cell strain is the next step in the response to orthodontic tooth movement.

Hynes (1992)[6] also acknowledged that cell strain is the next step in the response to orthodontic tooth movement.

Weinbaum *et al* (1994)[7] stated that the fluid flow on both the appositional and resorptive sides may activate entrapped osteocytes within the bone through canaliculi.

Cowin *et al* (1995)[8] stated that the fluid flow on both the appositional and resorptive sides may activate entrapped osteocytes within the bone through canaliculi.

Klein-Nulend *et al* (1995)[9] also stated that pathways stimulate the production of mediators such as interleukins, nitric oxide, and prostaglandins that activate several types of cells.

Klein-Nulend *et al* (1995)[10] also found that this concept is often referred to as fluid shear stress theory and is supported by both *in vitro* data.

Pitsillides *et al* (1995)[11] also stated that this concept is often referred to as fluid shear stress theory and is supported by both *in vivo* data.

Turner *et al* (1995)[12] also stated that this concept is often referred to as fluid shear stress theory and is supported by both *in vivo* data.

Ajubi *et al* (1996)[13] also stated that this concept is often referred to as fluid shear stress theory and is supported by both *in vitro* data.

Beertsen *et al* (1997)[14] stated that collagen fiber tension and fluid flow cause deformation of periodontal ligament cells through a network of cell-matrix attachments . These cells include both fibroblasts and osteoblasts.

Mak *et al* (1997)[15] stated that the fluid flow on both the appositional and resorptive sides may activate entrapped osteocytes within the bone through canaliculi.

Owan *et al* (1997)[16] also stated that this concept is often referred to as fluid shear stress theory and is supported by both *in vitro* data. He also stated that cytokines likely stimulate the production and differentiation of osteoblasts which are the primary cells associated with bone deposition. While not the focus of this review, it is important to note that the degradation and production of extracellular matrix material also plays an important role in orthodontic tooth movement. The periodontal fibroblasts react in a similar way as osteoblasts in that they react to a

number of mediators, including prostaglandins, MMPs, cathepsins, interleukin-1 beta, and transforming growth factor-β.

Teitelbaum *et al* (1997)[17] stated that It is at this site that hydrogen ions are released to dissolve the bone, and enzymes resorb residual organic matrix.

Hill (1998)[18] stated that the clear zone isolates the bone surface; the body of the osteoclast contains an extensive lysosomal system; and the ruffled border is the site of actual bone resorption.

Matsuda *et al* (1998)[19] stated that as the bone undergoes resorption, space is created to allow movement in the direction of force application. The appositional side, meanwhile, is undergoing bone formation that is mediated by the production of alkaline phosphatase, osteocalcin, and other non-collagenous proteins .

Howard *et al* (1998)[20] also stated that cytokines likely stimulate the production and differentiation of osteoblasts which are the primary cells associated with bone deposition. While not the focus of this review, it is important to note that the degradation and production of extracellular matrix material also plays an important role in orthodontic tooth movement. The periodontal fibroblasts react in a similar way as osteoblasts in that they react to a number of mediators, including prostaglandins, MMPs, cathepsins, interleukin-1 beta, and transforming growth factor-β

Melsen (1999)[21] also stated that the side of bone apposition is often referred to as the tension side in the pressure-tension theory but may be more accurately described as the appositional side. This positive strain creates a stretching of the collagen fibers connecting the tooth to the bone which in turn, can activate residing periodontal cells such as osteoblasts and fibroblasts. This term, however, can be somewhat misleading as the collagen fibers on this side actually relax upon force application due to the negative strain.

Westbroek *et al*(2000)[22] also stated that pathways stimulate the production of mediators such as interleukins, nitric oxide, and prostaglandins that activate several types of cells. He also stated that this concept is often referred to as fluid shear stress theory and is supported by both *in vitro* data.

Knothe Tate *et al* (2000)[23] also stated that this concept is often referred to as fluid shear stress theory and is supported by both *in vivo* data.

Nomura and Takano-Yamamoto (2000)[24] found that these osteoclast associated regulating cytokines have been shown *in vitro* and *in vivo* to include colony-stimulating factor, receptor activator of nuclear factor kappa β ligand (RANKL), osteoprotegerin (OPG), and bone morphogenic proteins.

Gay and Weber (2000)[25] stated that after osteoclast differentiation has begun, the osteoclasts are stimulated by osteoblast and osteocyte released osteopontin to begin attaching to the bone surface by specific integrins.

You *et al* (2000)[26] also stated that cytokines likely stimulate the production and differentiation of osteoblasts which are the primary cells associated with bone deposition. While not the focus of this review, it is important to note that the degradation and production of extracellular matrix material also plays an important role in orthodontic tooth movement. The periodontal fibroblasts react in a similar way as osteoblasts in that they react to a number of mediators, including prostaglandins, MMPs, cathepsins, interleukin-1 beta, and transforming growth factor-β.

Alhashimi *et al* (2001)[27] also stated that pathways stimulate the production of mediators such as interleukins, nitric oxide, and prostaglandins that activate several types of cells.

Shiotani *et al* (2001)[28] stated that these osteoclast associated regulating cytokines have been shown *in vitro* and *in vivo* to include colony-stimulating factor, receptor activator of nuclear factor kappa β ligand (RANKL), osteoprotegerin (OPG), and bone morphogenic proteins.

Binderman *et al* (2002)[29] also stated that this term, however, can be somewhat misleading as the collagen fibers on this side actually relax upon force application due to the negative strain.

Zhao *et al* (2002)[30] stated that these osteoclast associated regulating cytokines have been shown *in vitro* and *in vivo* to include colony-stimulating factor, receptor

activator of nuclear factor kappa β ligand (RANKL), osteoprotegerin (OPG), and bone morphogenic proteins.

Bakker *et al (*2003)[31] also stated that pathways stimulate the production of mediators such as interleukins, nitric oxide, and prostaglandins that activate several types of cells.

Apajalahti *et al., (*2003)[32] also stated that cytokines likely stimulate the production and differentiation of osteoblasts which are the primary cells associated with bone deposition. While not the focus of this review, it is important to note that the degradation and production of extracellular matrix material also plays an important role in orthodontic tooth movement. The periodontal fibroblasts react in a similar way as osteoblasts in that they react to a number of mediators, including prostaglandins, MMPs, cathepsins, interleukin-1 beta, and transforming growth factor-β.

Takahashi *et al (*2003)[33] also stated that cytokines likely stimulate the production and differentiation of osteoblasts which are the primary cells associated with bone deposition. While not the focus of this review, it is important to note that the degradation and production of extracellular matrix material also plays an important role in orthodontic tooth movement. The periodontal fibroblasts react in a similar way as osteoblasts in that they react to a number of mediators, including prostaglandins, MMPs, cathepsins, interleukin-1 beta, and transforming growth factor-β.

Mullender *et al* (2004)[34] also stated that pathways stimulate the production of mediators such as interleukins, nitric oxide, and prostaglandins that activate several types of cells.

Yoo *et al* (2004)[35] in his study found that osteocytes release cytokines, which stimulate the differentiation of precursors into osteoclasts on the resorptive side.

Phan *et al* (2004)[36] stated that It is at this site that hydrogen ions are released to dissolve the bone, and enzymes resorb residual organic matrix.

Nahm *et al* (2004)[37] also stated that cytokines likely stimulate the production and differentiation of osteoblasts which are the primary cells associated with bone deposition. While not the focus of this review, it is important to note that the degradation and production of extracellular matrix material also plays an important role in orthodontic tooth movement. The periodontal fibroblasts react in a similar way as osteoblasts in that they react to a number of mediators, including prostaglandins, MMPs, cathepsins, interleukin-1 beta, and transforming growth factor-β.

Yamaguchi *et al* (2004)[38] also stated that cytokines likely stimulate the production and differentiation of osteoblasts which are the primary cells associated with bone deposition. While not the focus of this review, it is important to note that the degradation and production of extracellular matrix material also plays an important role in orthodontic tooth movement. The periodontal fibroblasts react in a similar way as osteoblasts in that they react to a number of

mediators, including prostaglandins, MMPs, cathepsins, interleukin-1 beta, and transforming growth factor-β.

Ozaki *et al* (2005)[39] stated that as the bone undergoes resorption, space is created to allow movement in the direction of force application. The appositional side, meanwhile, is undergoing bone formation that is mediated by the production of alkaline phosphatase, osteocalcin, and other non-collagenous proteins .

Ingman *et al* (2005)[40] also stated that cytokines likely stimulate the production and differentiation of osteoblasts which are the primary cells associated with bone deposition. While not the focus of this review, it is important to note that the degradation and production of extracellular matrix material also plays an important role in orthodontic tooth movement. The periodontal fibroblasts react in a similar way as osteoblasts in that they react to a number of mediators, including prostaglandins, MMPs, cathepsins, interleukin-1 beta, and transforming growth factor-β.

Tang *et al* (2006)[41] also stated that pathways stimulate the production of mediators such as interleukins, nitric oxide, and prostaglandins that activate several types of cells.

Tan *et al* (2006)[42] also stated that this concept is often referred to as fluid shear stress theory and is supported by both *in vitro* data.

Wang and Thampatty (2006)[42] stated that this strain is then transferred within the cell by the focal adhesion complex to the cytoskeleton and protein kinases that initiate intracellular signaling pathways.

Kurata *et al* (2006)[43] stated that these osteoclast associated regulating cytokines have been shown *in vitro* and *in vivo* to include colony-stimulating factor, receptor activator of nuclear factor kappa β ligand (RANKL), osteoprotegerin (OPG), and bone morphogenic proteins.

Yang *et al* (2006)[44] stated that as the bone undergoes resorption, space is created to allow movement in the direction of force application. The appositional side, meanwhile, is undergoing bone formation that is mediated by the production of alkaline phosphatase, osteocalcin, and other non-collagenous proteins .

Takahashi *et al* (2006)[45] also stated that cytokines likely stimulate the production and differentiation of osteoblasts which are the primary cells associated with bone deposition. While not the focus of this review, it is important to note that the degradation and production of extracellular matrix material also plays an important role in orthodontic tooth movement. The periodontal fibroblasts react in a similar way as osteoblasts in that they react to a number of mediators, including prostaglandins, MMPs, cathepsins, interleukin-1 beta, and transforming growth factor-β.

Henneman (2008)[46] stated that therefore, it may be more accurately referred to as the resorptive side. As negative strain occurs on the resorptive side, the fluid

within the periodontal ligament space undergoes an almost immediate fluid flow. The last two stages occur simultaneously and in conjunction with one another. The mediators released by the activated periodontal cells stimulate osteocytes and a variety of periodontal ligament cells including osteoblasts, fibroblasts, and osteoclast-precursors.

Molecular Level of Tooth Movement

Simpson *et al* (1997)[47]in his study stated that Cytokines are often described as pleiotropic due to the variety of effects they can stimulate depending on the nature of the target cell Some cytokines may elicit agonistic or antagonistic effects on the same target cell depending on the surrounding factors. In addition to the pleiotropism that they may exhibit, cytokines often display overlapping biological activities and may even share the same receptors .

Simonet *et al* (1997)[48] stated that a system of cytokines of particular significance in understanding the relationship between osteoclastogenesis and osteoblastogenesis in tooth movement is the OPG/RANKL/RANK system. The discovery of osteoprotegerin was the first step in understanding this complex interaction. The molecule was discovered independently by two groups conducting contrasting experiments. While studying therapeutic utilities of TNF receptor-related molecules, the Amgen, Inc. group stumbled upon a relatively innocuous protein whose overexpression caused marked osteopetrosis in rats.

Yasuda *et al* (1998)[49] stated that It was found that this was due to a decrease in osteoclasts, indicating that OPG played a clear role in regulating osteoclastogenesis. The Snow Brand Milk Group, meanwhile, was systematically searching for a theoretical molecule fulfilling the role that OPG occupies now when they discovered the same protein as the Amgen, Inc. group

Heinrich *et al* (1998)[50] stated that as in most biological functions, cytokines are the soluble factors primarily involved in the coordinate activities of osteogenic cells. While typically only present in nanomolar-to-picomolar concentrations , cytokines regulate the proliferative, differentiative, and maturation events in most cells throughout the body. Unlike hormones, which are stored for later secretion, cytokines are usually rapidly synthesized locally and then immediately secreted when stimulated . When the two communicating cells are in close proximity, the rapidity of synthesis and secretion can make cytokine detection difficult.

Khosla (2001)[51] stated that OPG has since undergone significant research to further characterize the protein.

RANK/RANKL/OPG System

Anderson *et al* (1997)[52] stated that RANK had previously been identified as the receptor for RANKL, so its involvement in osteoclastogenesis regulation became readily apparent.

Lacey *et al* (1998)[53] stated that the discovery of OPG warranted the search for an osteoclast differentiation factor expressed on osteoblastic cells that was essential for osteoclast development. The identification of RANKL as this protein was quickly realized by the two previously mentioned groups. RANKL was shown to play a key role in osteoclast differentiation stimulation, osteoclast activity, and the inhibition of osteoclast apoptosis.

Yasuda *et al (1998)*[54] stated that the discovery of OPG warranted the search for an osteoclast differentiation factor expressed on osteoblastic cells that was essential for osteoclast development. The identification of RANKL as this protein was quickly realized by the two previously mentioned groups.

Fuller *et al (1998)*[55]stated that RANKL was shown to play a key role in osteoclast differentiation stimulation, osteoclast activity, and the inhibition of osteoclast apoptosis. Nakagawa *et al (1998)* If OPG is not present, however, then RANKL is free to bind to its natural receptor, RANK .

Shalhoub V, Faust J, Boyle WJ, Dunstan CR, Kelley M, Kaufman S, Scully S, Van G, Lacey DL(1999)[56] 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)[57] 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)[58] 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)[59] 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)[60] 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)[61] 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.

Malyankar *et al* (2000)[62] stated that RANKL was shown to play a key role in osteoclast differentiation stimulation, osteoclast activity, and the inhibition of osteoclast apoptosis.

Khosla (2001)[63] stated that the interaction between the three proteins is actually quite simple. Osteoblasts produce both osteoclastogenesis-stimulating RANKL and osteoclastogenesis-inhibiting OPG.

H. Kanzaki, M. Chiba, Y. Shimizu, and H. Mitani (2001)[64] 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)[65] 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)[66] 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)[67] 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)[68] 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)[69] 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)[70] 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)[71] 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)[72] 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)[73] evaluated the ligature-induced bone loss (BL) and quality of tooth-supporting lveolar 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.

Kanzaki H, Chiba M, Shimizu Y, Mitani H. (2002)[75] 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)[76] 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 up regulated 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)[77] 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)[78] 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)[78] 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)[79] 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)[80]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)[81] 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)[82] examined the effect of compressive force on the production of PGE2, 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, PGE2 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 PGE2, 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)[83] 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)[84] 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)[85] 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)[86] 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)[87] 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)[88] 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)[89] 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)[90]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)[91] 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)[92] 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)[93] 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)[94] 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)[95]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.

INTERLEUKIN – 10 AND ORTHODONTIC TOOTH MOVEMENT

Alhashimi n et al (2000)[96] in their study was to analyze effects of mechanical force during orthodontic tooth movement, in the pressure zone, on the induction of interferon-gamma (IFN-gamma) as a proinflammatory cytokine of Th1 type and interleukin-4 (IL-4)/IL-10 as anti-inflammatory cytokines of Th2 type. In 12 Wistar rats 40-45 days old, the maxillary first molar was moved mesially by means of a closed coil spring for 3, 7, and 10 days. The contralateral side served as a control. IFN-gamma, IL-4, and IL-10 mRNA were determined by in situ, hybridization, and protein levels of IFN-gamma was measured by immunohistochemistry. Induction of IFN-gamma at both mRNA and protein levels was significantly higher on the experimental side than on the contralateral control side on day 3. The signal gradually became stronger on day 7 and remained high on day 10. Cytokines of the Th2 type (IL-4 and IL-10) were not detected at all examined time points in both pressure and contralateral control sides. Considering the potential immunoregulatory roles played by IFN-gamma, our data suggest that IFN-gamma may be involved in periodontium remodeling during orthodontic tooth movement.

Andrade I jr et al (2009)[97] in their study aimed to investigate whether the CCR5-receptor influences these events and, consequently, orthodontic tooth movement. An orthodontic appliance was placed in wild-type mice (WT) and CCR5-deficient mice (CCR5(-/-)). The expression of mediators involved in bone remodeling was evaluated in periodontal tissues by Real-time PCR. The number of TRAP-positive osteoclasts and the expression of cathepsin K, RANKL, and MMP13 were significantly higher in CCR5(-/-). Meanwhile, the expression of two osteoblastic differentiation markers, RUNX2 and osteocalcin, and that of bone resorption regulators, IL-10 and OPG, were lower in CCR5(-/-). Analysis of the data also showed that CCR5(-/-) exhibited a greater amount of tooth movement after 7 days of mechanical loading. The results suggested that CCR5 might be a down-regulator of alveolar bone resorption during orthodontic movement.

Melissa grant et al (2012)[98] in their study investigated changes in cytokines and biomarkers of bone and tissue metabolism within gingival crevicular fluid (GCF) from patients undergoing orthodontic treatment. GCF was collected on Periopaper™ strips (Oraflow Inc., USA) from 20 volunteers at baseline, before tooth extraction and appliance placement and then at intervals during orthodontic treatment. Samples were taken 10 weeks following first appliance placement: at four hours; 7 days; and 42 days after application of distalising forces to maxillary canine teeth. Cytokines (GM-CSF, interferon-gamma, IL-1beta, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10 and TNFalpha), tissue biomarkers (MMP-9, TIMP-1 & 2) and bone metabolism indicators (RANKL and OPG) were measured in GCF using multiplex assays. Tension sites adjacent to canines showed significant increases in IL-1beta, IL-8, TNFalpha, MMP-9 and TIMPs 1 and 2 across all time points

following force application, while compression sites exhibited increases in IL-1beta and IL-8 after 4 hours, MMP-9 after 7 and 42 days and RANKL after 42 days. These data demonstrate that high levels of pro-inflammatory cytokines and biomarkers of tissue and bone metabolism in GCF are associated with orthodontic force application. Elevated levels were evident at 4 hours but continued for periods of up to 6 weeks. The data suggest that GCF biomarker analysis may help optimise orthodontic forces for individual patients.

INTERLEUKIN – 10 GENERAL FUNCTIONS

Mei hua hong et al (2000)[99] stated that Interleukin-10 (IL-10) inhibits osteoclast (OC) formation in rat and mouse systems. However, little is known concerning the mechanism of this inhibitory effect. Using a coculture system of mouse bone marrow cells and primary osteoblastic cells (POB), we evaluated the potential target cells for IL-10 and components of the IL-10 activating pathway. In the coculture system, IL-10 treatment abolished OC differentiation in a dose-dependent manner. This inhibitory effect occurred regardless of the stage of cellular proliferation and differentiation, suggesting that IL-10 may act on a variety of genes participating in OC formation. IL-10 specifically abrogated the production of IL-6 by enriched bone marrow-derived mononuclear cells (BMM) but not by osteoblastic cells. IL-10 treatment also stimulated the binding of a protein in the BMM to an IL-10 response element, whereas no such activation was induced in osteoblastic cells. In contrast, interferon g (IFN-g), another inhibitory factor, stimulated tyrosine-phosphorylated proteins to bind to an IL-10 response element in both monocytes and osteoblastic cells. These data suggest that the

BMM are the direct target of IL-10 action. Importantly, oligonucleotide-specific precipitation confirmed that IL-10 treatment strongly augmented 88, 85, and 70 kDa tyrosine-phosphorylated proteins in BMM. Taken together, these data show that IL-10 inhibits mouse OC formation by acting directly on hemopoietic OC precursor, through a novel signal transduction and activation pathway.

Giorgio Trinchieri (2007)[100] in his study stated that Interleukin (IL)-10 is a cytokine that Modulates both innate and adaptive immunity, primarily by exerting antiinfl ammatory effects. IL-10 was originally thought to be produced only by T helper (Th)2 cells, but is now known to be made by a variety of cell types. During many infections, CD4+ T cells produce both interferon (IFN)-γ, the signature Th1 cytokine, and IL-10. New data now show that the IL-10 produced by effector Th1 cells helps limit the collateral damage caused by exaggerated infl ammation. But this control may also limit the effectiveness of the immune response, resulting in a failure to fully eliminate pathogens.

David L. Cochran (2008)[101] stated that Inflammation and bone loss are hallmarks of periodontal disease (PD). The question is how the former leads to the latter. Accumulated evidence demonstrates that PD involves bacterially derived factors and antigens that stimulate a local inflammatory reaction and activation of the innate immune system. Proinflammatory molecules and cytokine networks play essential roles in this process. Interleukin-1 and tumor necrosis factor-alpha seem to be primary molecules that, in turn, influence cells in the lesion. Antigen-stimulated lymphocytes (B and T cells) also seem to be important. Eventually, a cascade of events leads to osteoclastogenesis and subsequent bone loss via the

receptor activator of nuclear factor-kappa B (RANK)–RANK ligand (RANKL)–osteoprotegerin (OPG) axis. This axis and its regulation are not unique to PD but rather are critical for pathologic lesions involving chronic inflammation. Multiple lines of evidence in models of PD clearly indicate that increases in RANKL mRNA expression and protein production increase the RANKL/OPG ratio and stimulate the differentiation of macrophage precursor cells into osteoclasts. They also stimulate the maturation and survival of the osteoclast, leading to bone loss. OPG mRNA expression and protein production do not generally seem to be increased in the periodontitis lesion. Studies of RANKL and OPG transgenic and knockout animals provide further support for the involvement of these molecules in the tissue loss observed in PD. Ironically, periodontal practitioners have focused on the bacterial etiology of PD and believed that plaque removalwas aimed at eliminating specific bacteria or bacterial complexes. However, it seems that the reduction of inflammation and attenuation of the host's immune reaction to the microbial plaque, eventually leading to a decrease in the ratio of RANKL/OPG and a decrease in associated bone loss, are the actual and desired outcomes of periodontal therapy.

S. R. Goldring (2003)[102] stated that Inflammatory disorders such as rheumatoid arthritis (RA), may have profound effects on skeletal homeostasis. In contrast to physiologic remodeling in which mechanical influences and/or systemic endocrine hormones initiate the remodeling process, in disorders such as RA the recruitment of macrophage lineage cells to sites of inflammation and the action of local osteoclastogenic cytokines associated with the inflammatory process initiate the remodeling process. In both physiologic and pathologic remodeling, osteoclasts

appear to be the principal cell type responsible for the bone resorption. In addition, many of the same cytokines and mediators are involved in physiologic and pathologic bone remodeling. These observations have important implications with respect to the development of therapeutic strategies to prevent bone loss in inflammatory conditions.

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. Patients with class 1 malocclusion with bidental protrusion, who has not undergone previous orthodontic treatment.
2. These subjects, 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. All subjects were in good general health with healthy periodontal tissues.
6. Probing depths were < 3mm with no radiographic evidence of periodontal bone loss.
7. All of them were treated by post-graduate students in the Department of Orthodontics, Ragas Dental College and Hospital, Chennai.

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.

3. Patients with active periodontal problem , Syndromic or reports of allergic reaction
4. Patients who underwent orthodontic treatment before

METHODS

Gingival crevicular fluid was collected at the following stages:

T0 – Before initiation of treatment

T1 - 92 days after levelling and alignment stage

The samples were collected at T0,T1 from the anterior sextants of the maxillary and mandibular arches, amounting to a total of 4 samples per subject. At the beginning of treatment, T0 – GCF samples were collected from upper and lower arches. 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. Once the stage 1 was completed on an average within 92 days from start of leveling & alignment, Samples were collected at this point from the anterior sextants of both the arches and were labeled as T1.

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) 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 .
- The collected GCF was stored in sterile Eppendorf tubes at -65°C.

The collected samples were analysed at Hubert Enviro Care Pvt. Ltd, Chennai

ELISA Testing for Interleukin - 10

The collected GCF samples were tested for interleukin – 10 using the Gen-Probe Diaclone Human IL – 10 ELISA kit (Gen-Probe Diaclone sas, FRANCE). The microtiter plate provided in this kit has been pre-coated with an antibody specific to IL – 10.

Assay Procedure

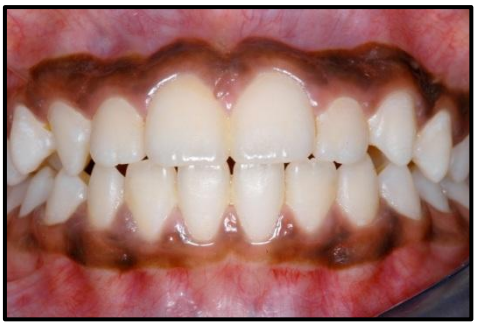
1. Each of the collected samples was diluted using the sample diluents provided in the kit.
2. The diluents used were the Phosphate Buffered Saline (PBS)100µl of sample was added per well. The wells were covered with adhesive strips and incubated for 15 - 20 hours at 37°C.
3. The liquid from each well was removed, not washed.
5. The plate is incubated at 4^{0}C for 10 min.
6. 100ml of diluted detection antibody is added to every well and incubated at room temperature for 1 hour 30 minutes.

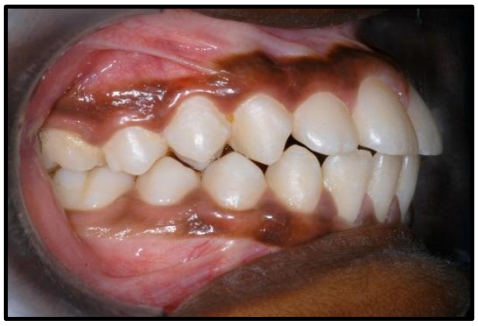
4. After washing the plate for 3 times 100ml of diluted Streptavidin-AP conjugate is added to every well and incubated at room temperature.
7. 100ml of ready-to-use 5-Bromo-4-Chloro-Indolyl-Phosphatase/ nitroblue tetrazolium buffer is added to every well.
8. Then the plate is incubated for 5-15 min monitoring spot formation visually throughout the incubation period to assess sufficient colour development.
9. The frequency of the resulting coloured spots corresponding to the cytokine producing cells can be determined using an appropriate ELISpot reader.

FIGURE 1: MICROPIPETTE FOR GCF COLLECTION

FIGURE 2: EPPENDORF TUBES

FIGURE 3: BEFORE START OF THE TREATMENT (T0)





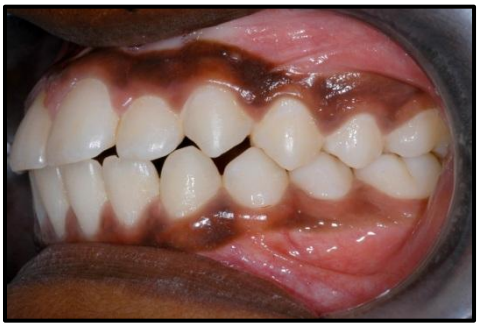


FIGURE 4: END OF STAGE I-LEVELING & ALIGNING (T1)

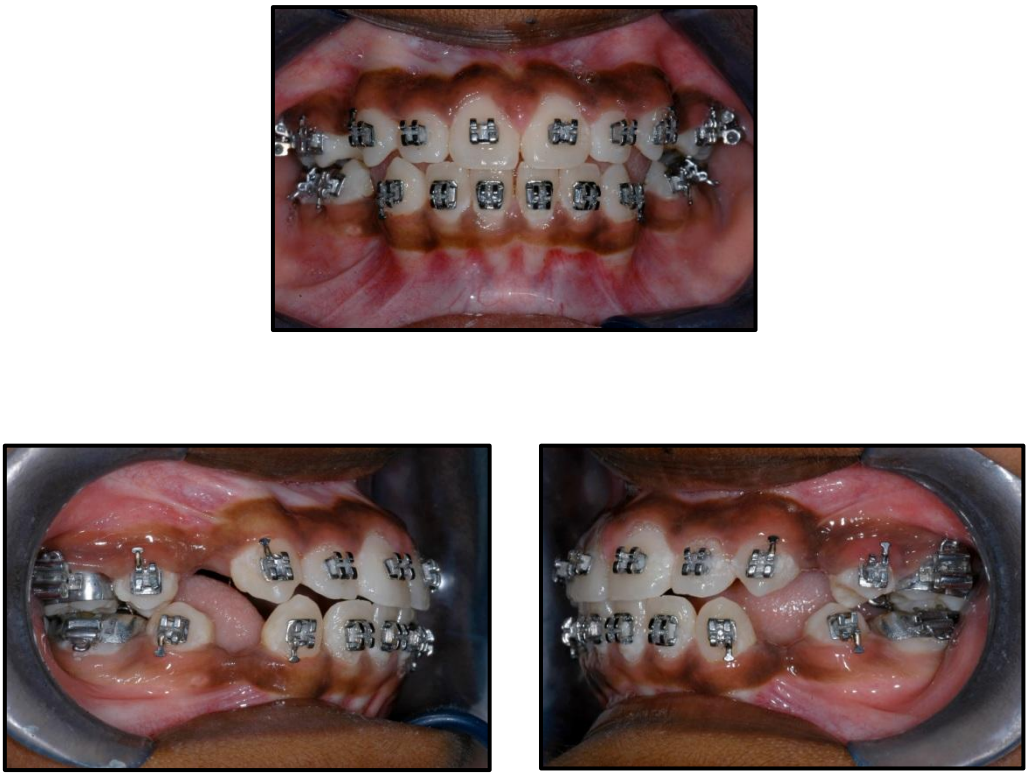


FIGURE 5a: GCF COLLECTION (UPPER ARCH)

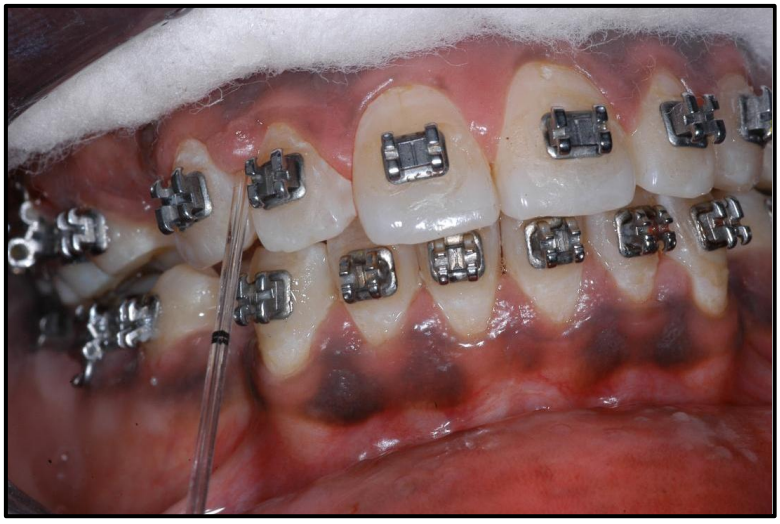


FIGURE 5b: GCF COLLECTION (LOWER ARCH)

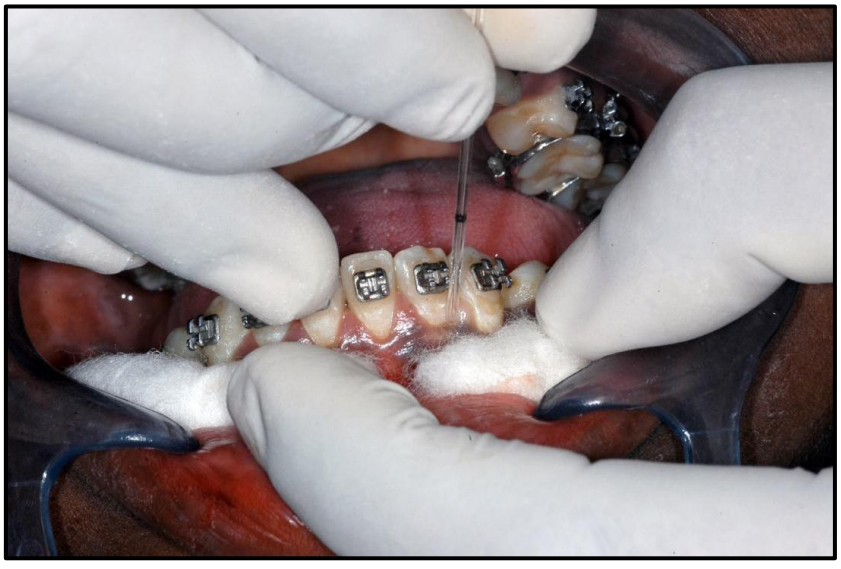


FIGURE 6: ELISA PLATE READER

FIGURE 7 : IL -10 ELISA KIT & REAGENTS

RESULTS

The optical density of the samples was determined using an ELISA plate reader (Figure 9).

The concentration of IL – 10 (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 IL – 10 were determined.

- A Mann – Whitney test was performed.The hypothesis being tested was that the concentration of IL – 10 increases in bone remodelling from baseline to end of levelling and alignment stage between upper and lower arches.
- Wilcoxon – signed ranks test was performed to compare the mean values of concentration of IL – 10 from baseline (T0) to end of levelling and alignment stage (T1).
- The statistical analysis was carried out in the following parts;
    - Mean IL – 10 values in upper arch
    - Mean IL – 10 values in lower arch
    - Mean IL – 10 values in baseline (T0)
    - Mean IL – 10 values in end of levelling and alignment stage (T1)

- 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 IL – 10 value at T0 was 199.7 ± 142.3 pg/ml and T1 was 229.5 ±163.2 pg/ml.

A statistically significant increase ($p < 0.028$) in the IL -10 concentration was observed at T1 compared to T0.

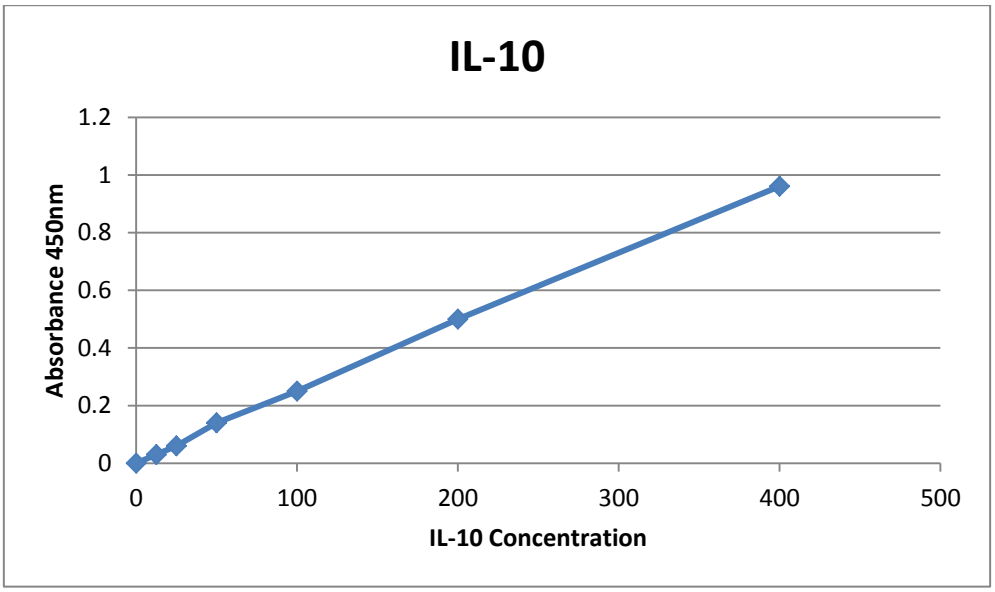
LOWER ARCH

The mean IL – 10 value at T0 was 126 ± 172 pg/ml and T1 was 146 ± 196.8 pg/ml.

A ststistically significant increase ($p< 0.041$) in the IL – 10 concentration was observed at T1 Compared to T0.

According to mann – whitney test no significant statistical differences were observed when compared IL – 10 values between upper and lower arches.

Although in Wilcoxon – signed ranks test IL – 10 values increased from T0 to T1.



OD: OPTICAL DENSITY

Pg/ml: PICOGRAMS/ MILLILITRE

IL-10 STANDARD CURVE

STANDARD	IL-10 CONC pg/ml	Absorbance(450nm)
1	400	0.961
2	200	0.5
3	100	0.25
4	50	0.14
5	25	0.06
6	12.5	0.03
ZERO	0	0

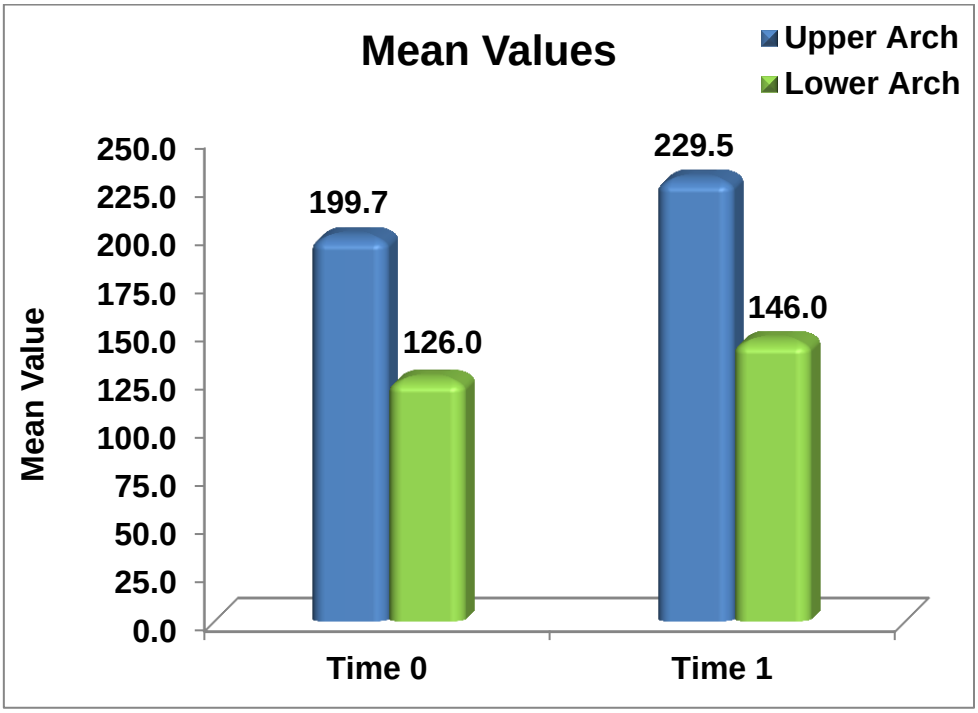
Table 1: Mann-Whitney Test to compare the mean values between Upper and Lower arches

Variables	Group	N	Mean	Std. Deviation	P-Value
Time 0	Upper Arch	6	199.7	142.3	0.229
	Lower Arch	6	126.0	172.0	
Time 1	Upper Arch	6	229.5	163.2	0.374
	Lower Arch	6	146.0	196.8	
Absolute Difference	Upper Arch	6	29.83	30.12	0.194
	Lower Arch	6	20.00	46.06	
Relative Difference	Upper Arch	6	13.09	10.43	0.575
	Lower Arch	6	12.26	13.96	

Table 2: Wilcoxon Signed Ranks Test to compare the mean values between Time 0 and Time 1

Variables	Group	N	Mean	Std. Deviation	P-Value
Upper Arch	Time 0	6	199.7	142.3	0.028
	Time 1	6	229.5	163.2	
Lower Arch	Time 0	6	126.0	172.0	0.041
	Time 1	6	146.0	196.8	

CHART 1: MEAN IL-10 values in upper and lower arch & time variables



DISCUSSION

The study of bone remodeling was introduced in 1892 when German surgeon, Julius Wolff, proposed that a transformation of both the internal and external bone architecture occurs in response to prolonged stresses acting upon it (Frost, 2004). Since that time, Wolff's Law has been updated, modified, and expounded upon as researchers continue to explore the complex field of bone biology. At first glance, it may appear that orthodontic tooth movement can be explained by simple physics: a force is applied to a tooth until friction is overcome and the tooth begins to move. Whereas this may explain the interface between the tooth and the orthodontic appliance, it does not address the biological mechanisms involved.

Orthodontic tooth movement is a unique phenomenon where a solid object moves through a solid medium. Therefore the biological intricacies involved with this type of movement are novel and not entirely understood. As orthodontic forces are applied, the mechanical loading within the system increases, and a complex cascade of biological events lead to the remodeling of the surrounding tissues and eventual movement of the tooth (Masella and Meister, 2006)[111].understanding of bone remodeling is of particular interest to the profession of orthodontics.

Previously bone resorptive markers were studied extensively using the GCF samples from rats.[123] Other forms of bone remodelling studies have been studied using cone beam computed tomography (cbct) ,but the drawbacks in those study were the radiation dosage was a factor of concern to the orthodontic

profession[114] . So a non-invasive methodology to study bone remodelling in patients have to be studied in this regard so the GCF study was undertaken.

Although studies have been done in the past using different markers , very few studies have been done using IL – 10 marker. So the present study was done to quantify bone remodelling based on IL – 10. Prior to this study many studies based on orthodontic tooth movement evaluation using gcf was carried out in subjects who were in retraction stage or end of retraction stage. In those studies bone remodelling was identified with elevation of the pro-inflammatory and anti – inflammatory markers.

In this study we are assessing bone remodelling at the end of levelling and alignment stage itself by quantifying the elevation of IL – 10 marker between the two time points T0 and T1 and between upper and lower arches which is unique to this study. Leveling and alignment is the first stage of an orthodontic treatment sequence. During this stage, a flexible archwire usually a nickel titanium (NiTi) wire was tied into the bracket slot.

The NiTi archwire exerts a light continuous force between 30 and 60 g onto the teeth in order to level and align the dentition. A tipping movement is produced through combination of bracket-archwire system in a fixed orthodontic appliance. Consequently, tooth will tend to rotate around its centre of resistance compressing the periodontal ligaments near the apex of the root on the same side of the force applied. On the other side, PDL around the crest of alveolar bone will be compressed.

This insight into the bone remodelling at the levelling and alignment stage itself will provide in depth knowledge of the bone remodelling mechanism itself in initial stages of tooth movement.

IL-10 PRODUCTION

Interleukin (IL)-10 is a cytokine that modulates both innate and adaptive immunity, primarily by exerting anti inflammatory effects[117]. IL-10 was originally thought to be produced only by T helper (Th)2 cells, but is now known to be made by a variety of cell types. During many infections, CD4+ T cells produce both interferon (IFN)-γ, the signature Th1 cytokine, and IL-10. New data now show that the IL-10 produced by effector Th1 cells helps limit the collateral damage caused by exaggerated inflammation. But this control may also limit the effectiveness of the immune response, resulting in a failure to fully eliminate pathogens.

IL-10 is also made by CD4+ Foxp3+ CD25+ "natural" regulatory T (T reg) cells and IL-10–induced CD4+ T reg cells (Tr1 cells).

IL – 10 AND OSTEOCLASTS

Osteoclasts are terminally differentiated multinuclear cells derived from monocyte macrophage lineage cells and are responsible for bone resorption[125]. OC-like cells, produced in vitro by coculturing mouse bone marrow cells and primary osteoblastic cells (POBs) in the presence of osteotropic hormones(1) exhibit many of the characteristics of authentic OCs including tartrate-resistant acid phosphatase (TRAP) activity, abundant calcitonin (CT) receptors, and the ability to form resorption lacunae on dentine slices.(2) The differentiation and

function of OCs are regulated tightly by hormones, in particular, calcium regulating hormones such as parathyroid hormone (PTH) and 1,25-dihyroxyvitamin D3[1,25(OH)2D3], as well as cytokines such as interleukin-1 (IL-1), tumor necrosis factor a (TNFa), and the IL-6 family.

In a study by hong et al reveals that IL-10 inhibits osteoclastogenesis by directly targeting bone marrow– derived mononuclear cells (BMM), which have been recognized as the origin of OC precursor cells, through a receptor and signaling pathway specific for IL-10. First, IL-10 inhibits IL-6 production by BMM but not by osteoblasts. Second,STAT 1–like molecules were activated by IL-10 only in the treatment of BMM with IL-10 did not alter the protein profile. Interestingly, a 70-kDa phosphoprotein also was reported previously in peripheral monocytes and treated with IL-10 and this protein was recognized by an antibody to STAT 3.

O'Farrell et al. also reported that IL-10 inhibited macrophage proliferation through the STAT 3–dependent pathway. However, we found that the 70-kDa phosphoprotein activated by IL-10 in BMM was recognized by an antibody to STAT 1 but not by an antibody to STAT 3. Therefore, different STAT(s) may be activated by IL-10 in BMM and peripheral monocytes. This finding suggests that IL-10 may activate STAT like proteins other than STAT 1 and STAT 3 such as 88, 85, and 70 kDa proteins in the BMM cells. These STAT-like proteins remain to be identified. IL-6 also has shown a strong activity in OC formation in vitro in the presence of soluble IL-6 receptor(29) and in response to dexamethasone that induces IL-6 receptor production in osteoblasts.(30) However, the inhibitory

effect of IL-10 on OC formation is unlikely to becaused by inhibition of IL-6 production. First, osteoblast derived IL-6 production was 10 times greater than that derived from BMM cells, and IL-10 did not inhibit IL-6 production by the POB while it decreased by 50% the IL-6 production by BMM cells. Second, IL-6 alone was unable to promote osteoclastogenesis in this mouse coculture system without the supplement of soluble IL-6 receptor.

Recently, molecular cloning technique appears to give an insight into the understanding of the mechanism of osteoclastogenesis. TRANCE/ RANKL/ OPGL/ ODF stimulated by bone resorbing agents such as 1,25(OH)2D3, PTH, and IL-11 family in osteoblastic cells interacts with its receptor molecule, namely, RANK, which is a TNF-a receptor family on the surface of OC precursor cells. Importantly, TRANCE/RANKL/OPGL/ODF knockout mice showed severe osteopetrosis and a combination of OPGL/ODF and M-CSF are sufficient for OC Formation in vitro in the absence of osteoblasts, suggesting that the TRANCE/RANKL/OPGL/ODF is the crucial factor for osteoclastogenesis. Furthermore, NF-kB1 and NF-kB2 double-knockout mice developed osteopetrosis because of a defect of OC differentiation. Because NF-kB is the key component of the RANK signal pathway, NF-kB plays a central role in response to bone resorbing agents in the differentiation and functions of OC. Taken together, these pieces of evidence support the notion that TRANCE/RANKL/OPGL/ODF and M-CFS signal pathways are essential for osteoclastogenesis.

They concluded that we found that the inhibitory effect of IL-10 on OC formation is mediated through OC precursor cells and that novel STAT 1–like proteins may be involved in this inhibitory effect.

IL – 10 AND BONE RESORPTION

Whether bone loss will occur in response to an inflammatory reaction is now known to depend on two critical factors[118]. First, the concentration of inflammatory mediators present in gingival tissue must be sufficient to activate pathways leading to bone resorption. Second, the inflammatory mediators must penetrate gingival tissue to reach within a critical distance to alveolar bone. Achieving critical concentrations of inflammatory mediators that lead to bone resorption depends on the expression of proinflammatory cytokines, such as interleukin (IL)-1, -6, -11, and -17, tumor necrosis factor-alpha (TNF-a), leukemia inhibitory factor, and oncostatin M. The kinins, such as bradykinin and kallidin, and thrombin and various chemokines also have a stimulatory effect on bone resorption.8 This is the opposite of the expression of anti-inflammatory cytokines and other mediators, such as IL-4, -10, -12, -13, and -18, as well as interferon-beta (IFN-b) and -gamma (IFN-g), which serve to inhibit bone resorption.

During an inflammatory response, cytokines, chemokines, and other mediators stimulate periosteal osteoblasts, altering expression levels of a protein called receptor activator of nuclear factor-kappa B ligand (RANKL) on the osteoblast surface. RANKL is expressed by osteoblasts in a membrane-bound protein or cleaved into a soluble form. In addition to osteoblasts, RANKL is

expressed by a number of other cell types, including fibroblasts and T and B lymphocytes.

THE RANKL–RANK–OPG AXIS

Under normal physiologic conditions, there is a balance between bone resorption and bone formation.10This balance promotes bone homeostasis, including the maintenance of structural integrity and calcium metabolism.6 In certain inflammatory bone conditions, the balance is altered such that bone formation is enhanced, as in osteopetrosis, or excessive bone resorption occurs, such as that observed in osteoporosis and periodontitis. Accordingly, excessive 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 may result in a net increase in RANKL and pathologic bone resorption, also known as an increase in the RANKL/OPG ratio. During an inflammatory response, proinflammatory cytokines, such as IL-1b, -6, -11, and -17 and TNF-a, can induce osteoclastogenesis by increasing the expression of RANKL while decreasing OPG production in osteoblasts/stromal cells. In contrast, anti-inflammatory mediators, such as IL-13 and IFN-g, may lower RANKL expression and/or increase OPG expression to inhibit osteoclastogenesis.

IL-10 AND BONE REMODELLING

The structural elements and matrix components of the skeleton are remodelled throughout life. This process is accomplished by the actions of a

complex multi-lineage cellular system involving hematopoietic (osteoclasts) and mesenchymal (osteoblasts) cells[102].

There remains controversy, however, regarding the evolutionary basis and principal functional role of bone remodeling. For example, it has been suggested that remodeling has evolved as a system that permits adaptation of skeletal architecture to changing mechanical environments. Remodeling also provides a mechanism for repair of bone micro-damage sustained during repetitive mechanical loading a process that would impart a distinct selective evolutionary advantage. Finally, it has been suggested that remodeling has evolved as a system for regulating mineral ion homeostasis.

These distinct functional roles of bone remodeling are not necessarily exclusive, and under physiologic or certain pathologic conditions each of these mechanisms may provide the major adaptive drive for recruitment of the cellular elements required for initiation and completion of the remodeling cycle. Differential regulatory systems are involved, depending upon whether bone remodeling is generalized or localized to specific skeletal sites. Generalized remodelling characteristically is initiated by systemic hormones such as parathyroid hormone (PTH). This form of remodelling has been classified as non-targeted since it affects the entire skeleton, although even in this situation there may be preferential involvement of anatomic sites. In contrast, so-called ‘‘targeted remodeling’’ is restricted to distinct skeletal sites. Micro-cracks in the bone matrix and/or high local deformation around osteocytic lacunae have been suggested as potential signals for initiation of this form of remodelling.

IL – 10 IN PERIODONTICS

Anti-inflammatory cytokines, such as IL-10, are widely expressed in diseased periodontal tissues and are associated with lower disease severity. Indeed, IL-10 presents a protective role toward tissue destruction, inhibiting both MMPs and RANK systems. IL-10 characteristically induces the up-regulation of a group of endogenous proteins named tissue inhibitors of metalloproteinases (TIMPs), which are capable of inhibiting almost every member of the MMP family in a nonspecific way. In addition, IL-10 stimulates the production of OPG, a decoy receptor of RANKL, which strongly inhibits bone resorption by preventing RANK-RANKL engagement. In agreement, a positive correlation between the levels of IL-10, TIMPs, and OPG was demonstrated in both human and experimental periodontal diseases.

Gingival Crevicular Fluid

GCF represents a powerful fluid vehicle for the diagnostic clinician since it contains, depending upon the clinical situation an array of biochemical and cellular factors, which feature as biomarkers of the state of the periodontium.The early work of Embery *et al*. (1982), Last *et al*. (1985), and Waddington *et al*. (1994) have described the appearance and identification of a range of glycosaminoglycan, proteoglycan and tissue glycoproteins, which provide biochemical evidence on the underlying state of the biochemical tissues, which elude normal clinical parameters. GCF can variously be described as a transudate or an exudate. It is a fluid that arises at the gingival margin and can be collected by a variety of procedures providing noninvasive, site-specific process. Its collection requires patience on the part of the clinician and ranges from the use of

platinum loops, filter-paper strips, gingival washings, and the use of micropipettes, favoured by ourselves. The advantage of the latter procedure is ready storage, quantitation and application for either electrophoresis of direct chemical assessment.

The outcomes of a number of studies indicate that GCF is a feature of the fine nature of the gingival vasculature where the effect of trauma to the permeability of the arterial and venular capillaries leads to fluid production (Figure 3). In periodontal disease there is an increase in GCF volume, which clearly arises by breaching of the normal integrity conferred by the basement membrane and junctional epithelia. It has been established that this process is accompanied by enlargement of the intercellular spaces of the junctional epithelium and partial destruction of the basement membrane (Freedman *et al*., 1968). Such events will lead to the production of a semi-permeable membrane and an osmotic gradient (Alfono, 1974).

This will draw interstitial fluid from the surrounding capillaries and lymphatic system. The initial exudate is usually discarded, since it is not defined as an inflammatory exudate. However, with time a secondary inflammatory exudate, defined as GCF is evident and provides the basis for identification of constituent biomarkers, which represent the metabolic state of the underlying and deeper-seated tissues of the periodontium.

The value of GCF in the assessment of orthodontic movement will be governed by different parameters as essentially it will be non-inflammatory and non-plaque influenced. The resorptive/synthetic trauma on the deeperseated

tissues of the periodontium will induce a fluid pressure flow, which may be used to assess factors influencing orthodontic assessment. Evidence will be presented later to show that movement of alveolar bone and periodontal ligament induces the production of extracellular matrix factors for use as biomarkers of orthodontic treatment.

ELISA : The enzyme-linked immunosorbent assay (ELISA) is an important tool in for detecting the presence of an antibody or an antigen in a sample. The ELISA's key features are its high level of sensitivity and robustness. It allows easy visualization of results and can be completed without using any radioactive materials because of which it remains a desired assay of choice for routine scientific, medical and veterinary diagnostic assays.

Types of ELISA : There are two basic types known of ELISA:

1) Indirect ELISA : This method is used to determine the presence of a specific antibody in a given specimen. The steps of the general, indirect, ELISA for determining serum antibody concentrations are:

1) The sample containing a known antigen of defined concentration is added to the well of a microtiter plate where it adsorbs to the wells of the plate through charge interactions.
2) A concentrated solution of non-interacting proteins, such as Bovine Serum Albumin (BSA) or casein, is added to the plate wells to block the non-specific adsorption.
3) Test-antiserum is added to these walls and allowed to incubate; if the antibodies in test antiserum are homologous, they bind with adsorbed

antigens forming antigen-antibody complex. The wells are again washed to remove any free antibodies.

4) Enzyme-conjugated (labelled) antibodies are now added which link to the antigen-antibody-complex formed in the previous step.
5) The plate is washed again to remove excess unbound enzyme-antibody conjugates.
6) A chromogenic substrate is applied which converts the enzyme to elicit a signal.
7) The result is viewed/quantified using a spectrophotometer.

2) Direct ELISA : This method employs monoclonal antibodies for detection of a particular antigen in a sample.In this approach, the steps involved are as follows :

1) An unlabeled antibody specific to the antigen of interest is immobilized in microtiter well.
2) The antigen is added and allowed to react with the immobilized antibody to form Ag-Ab complex.
3) An Enzyme conjugate is added (that is specific to the antigen) which forms a double antibody sandwhich
4) The unreacted antibodies are washed away. Thereby, enzyme subsrate is added to elicit a chromogenic or fluorogenic signal.
5) Results are quantified using a spectrophotometer, spectrofluorometer, or other optical device. This procedure is also referred to as, Double Antibody Sandwitch (DAS) ELISA.

Advantages and disadvantages of both Direct and Indirect ELISA :

A. **Advantages of using direct ELISA** : It is relatively quick as only one antibody is used. Hence, fewer steps are involved. Also, cross-reactivity of secondary antibody is eliminated.

B. **Disadvantages of using direct ELISA** : By labeling the primary antibody with enzymes, its Immunoreactivity might be affected. Also the labeling for every specific ELISA is time-consuming and a costly affair. Moreover, this method gives minimum signal amplification compared to indirect method.

C. **Advantages of using indirect ELISA** : Plenty of commercially available labelled secondary antibodies are available. This method generates a stronger signal amplification. Also, many different visualization markers can be used with the same primary antibody.

D. **Disadvantages of using indirect ELISA**: There are chances of cross-reactivity with the secondary antibody which may give non-specific signals. Also, steps are increased in this method. In the present study, we found increased levels of INTERLLEUKIN - 10 in GCF samples collected from areas adjacent to teeth undergoing orthodontic tooth movement. In both the upper and lower arches the increase in IL - 10 levels was found to be statistically non-significant when compared between the arches but statistically significant when comparing between the baseline (T0) and at the end of levelling and alignment stage (T1).(Table 1 & 2).

The IL – 10 levels increased from T0 at 199.7 pg/ml to T1 at 229.5 pg/ml ($p<0.028$) for the

Upper arch and T0 at 126 pg/ml and T1 was 146 pg/ml ($p<0.041$) .(figure 4).

These values enable us to interpret and calculate the relative INTERLEUKIN - 10 which is a major determinant in bone remodeling during orthodontic tooth movement.

Formation of bone may be attributed to an abundance of INTERLEUKIN–10.

In the present study an increase in interleukin – 10 was recorded in both the arches which indicates a definite shift towards osteoblastic activity . (Table 1 & 2)

Available literature amply implicates an increase in the INTERLEUKIN – 10 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.

The detection of local concentration of INTERLEUKIN – 10 in GCF and with levelling and alignment stage force application can help the orthodontist to assess the status of bone remodeling during tooth movement. The concentration of INTERLEUKIN – 10 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 CONCLUSION

This study was undertaken to assess the change in INTERLEUKIN – 10 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; T0- before treatment and T1- at the end of levelling and alignment stage. Disposable micropipettes (5ul) were used for GCF collection and the collected samples were subjected to an ELISA test to determine the concentrations of INTERLEUKIN - 10.

Both the upper and lower arches showed an increase in concentration of IL -10

- The mean IL – 10 value at T0 was 199.7 $\pm$ 142.3 pg/ml and T1 was 229.5 $\pm$ 163.2 pg/ml. A statistically significant increase ($p < 0.028$) in the IL -10 concentration was observed at T1 compared to T0 in the upper arch.
- The mean IL – 10 value at T0 was 126 $\pm$ 172 pg/ml and T1 was 146 $\pm$ 196.8 pg/ml. A statistically significant increase ($p< 0.041$) in the IL – 10 concentration was observed at T1 Compared to T0 in the lower arch.
- The study shows a variation in IL – 10 from T0 to T1 quantifying bone formation at the end of levelling and alignment stage itself. 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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