Cementoblastic Response to High vs. Low Level of Mechanical Force in Vitro

Natalie Nicole Mullally
Marquette University

CEMENTOBLASTIC RESPONSE TO HIGH VS. LOW LEVEL OF MECHANICAL FORCE *IN VITRO*

By

Natalie Nicole Mullally, DMD

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ABSTRACT

CEMENTOBLASTIC RESPONSE TO HIGH VS. LOW LEVEL OF MECHANICAL FORCE *IN VITRO*

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One of the possible complications of orthodontic treatment is apical root resorption. During orthodontic treatment, as the teeth are being moved, the alveolar bone is continually being modeled to accommodate the teeth. This process activates specific cells that are responsible for bone resorption and can have the unwanted effect of resorbing the apex of the tooth root adjacent to the bone. It is unclear exactly what aspects of orthodontic treatment may trigger the resorptive process. A positive correlation, however, between root resorption and mechanical loading applied during orthodontic tooth movement has been established implicating orthodontic treatment in this adverse effect. Since cementum is the mineralized tissue covering the tooth root, it is poised to play a role in this process. Cementoblasts, sharing many characteristics of osteoblasts, have been shown to express various bone regulatory proteins such as osteopontin (OPN), receptor activator of NFκB ligand (RANKL), cyclooxygenase-2 (COX-2) and sclerostin (SOST). How the expression of these proteins varies in response to mechanical loading is unclear. As cementum has been shown to have reparative properties, it is uncertain whether a certain level of mechanical loading may have a resorptive or antiresorptive effect. Can a low level of force provide a protective effect on the tooth root, while a higher force level precipitate resorption to occur? To study the role of cementoblasts in external apical root resorption, we examined changes in ATP release and protein production of molecular bone biomarkers in OCCM-30 cells (murine cementoblastic cell line) following application of mechanical loading by fluid shear stress (FSS) for one hour at two different force levels (12 dynes/cm^2, 18.5dynes/cm^2). FSS is an *in vitro* model for applying a mechanical load to cells. We found a significant increase in ATP release following FSS at both levels and a significant decrease of RANKL and OPN protein at 12 dynes/cm^2. RANKL promotes the differentiation, activation and survival of osteoclasts, while OPN serves to attach osteoclast cells to bone or the root surface to begin resorption. Our findings suggest that cementoblasts play an active role in the mechanical adaptation of cementum in the process of orthodontic root resorption.

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I also must say a heartfelt "thank you" to my husband and manager of the "support team." It is only with his endless support and encouragement that I have managed to complete, not only this project, but the last 6 years of school, all while having a family and raising our three spirited daughters.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

External apical root resorption (EARR) is one of the side effects resulting from orthodontic treatment. When this occurs, a portion of the tooth root is resorbed, which can lead to tooth mobility, loss, or liability. Although severe EARR is rare, it is uncertain to which patients it will affect. The application of orthodontic force is most likely the cause of EARR, however the mechanism still remains unknown (Brezniak and Wasserstein 1993; Baumrind 1996). During orthodontic treatment, mechanical forces are applied to move the teeth to healthier, better functioning and more esthetic positions. Orthodontic forces are distributed through the teeth, to the periodontal ligament, and ultimately to the alveolar bone, producing a compression zone where the alveolar bone is resorbed and a tension zone where additional bone is added during the tooth movement (Henneman 2008). As has been demonstrated, a complex network of molecular signals orchestrates numerous cellular events to resorb the alveolar bone to move teeth and occasionally the cementum as a side effect known as root resorption (Krishnan and Davidovitch 2009).

As cementum covers the outer layer of the tooth root, it bears the majority of the dynamic mechanical load during orthodontic force application and may trigger or participate in the root resorption and repair process. Cementoblasts are a group of cells depositing cementoid onto the root surface and are eventually embedded in the mineralized cementum to become cementocytes (Avery 2000). Due to the difficulty in isolating the cementoblasts, the interaction between mechanical force and cementoblastic cells during root resorption is still unknown. At this time, there is little

evidence of the role cementoblasts may play in root resorption and repair, and of the cellular and molecular mechanisms involved in the responses of cementoblasts to mechanical loading.

The purpose of this study was to examine the changes in various mineralized tissue (bone) biomarkers in cementoblasts following application of mechanical loading *in vitro*. This study employed the use of a specific apparatus that was designed and fabricated to apply fluid shear stress (FSS) (a form of mechanical force) to the cells *in vitro*. Cells were subjected to two different levels of FSS and then examined for the changes in ATP release and protein production and to determine whether the cells respond differentially to different levels of mechanical force. The results of this study will shed light on the role of mechanical force in the formation and repair of EARR.

Root Resorption

As with any medical or dental procedure there are certain risks involved and a list of possible complications that may occur. Orthodontic treatment is no different in this regard, therefore treatment risks and complications must be considered prior to starting orthodontic treatment. One of the possible complications of orthodontic treatment with fixed appliances is apical root resorption (also referred to as root shortening) (Figure 1-1). During orthodontic treatment, as the teeth are being moved,

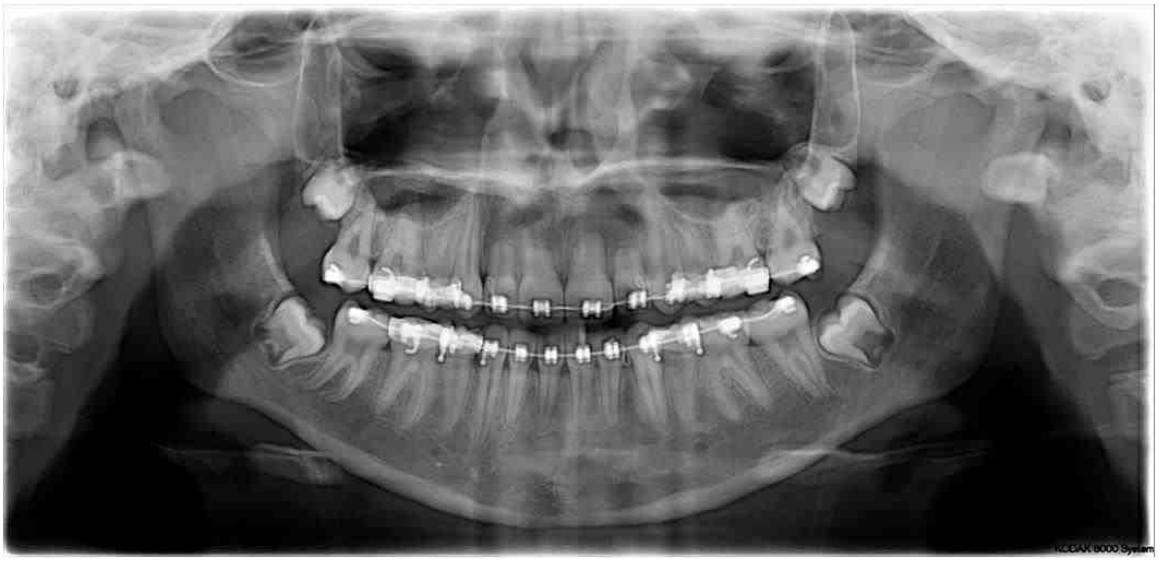

Figure 1-1: Apical Root Resorption during Orthodontic Tooth Movement. Panoramic X-ray of a 15 year old female, showing severe root resorption of the maxillary incisors following orthodontic treatment with full fixed appliances.

the alveolar bone is continually being modeled to accommodate the teeth. This process requires the activation of specific cells (osteoclasts) that are responsible for bone resorption and can have the unwanted effect of resorbing the apex of the tooth root adjacent to the alveolar bone. To date, research shows a great variability in both the cause and severity of root resorption without much consensus on what parameters may be used to predict future occurrences (Sameshima and Sinclair 2001). Most of the research conducted on root resorption has been clinical studies, case reports, and animal studies with few randomized clinical trials. Fortunately, the studies that have been done have shown that few patients actually experience severe root resorption. Although as much as 88% of orthodontic patients may show apical resorption of 1mm or less, only 5% of patients actually had more than 5mm of resorption (Killiany 1999). The most common teeth to be affected are the maxillary incisors, with very little resorption occurring in the buccal segments (Sameshima and Sinclair 2001). Although severe resorption of 5mm or greater is relatively rare, it creates an unfavorable situation for the affected tooth leading to increased mobility, decreased stability and eventually may compromise its longevity.

Researchers have tried to correlate the severity of root resorption with various factors including treatment mechanics or appliance type, amount of force, duration of treatment, extractions, and previous resorption (Gonzales 2000; McNab 2000; Mohandeson 2007; Roberto de Freitas 2007). There have been some disagreements in the articles published to date on what factors show the most promise in predicting root resorption (Sameshima and Sinclair 2001). In addition, there is great variability in an

individual's response (genetically determined) to orthodontic treatment and root resorption, adding to the difficulty in predicting when root resorption will occur (Owman-Moll 1995). Currently, the most accepted predictive factor of severe resorption is the occurrence of mild resorption early in orthodontic treatment (Artun 2005). This stresses the importance of regular "progress" radiographs to monitor resorption and if necessary alter the treatment plan to avoid the continual destruction of the roots. To date, it is still unclear what exact aspects of orthodontic treatment may trigger the resorptive process of a tooth root. A positive correlation, however, between root resorption and mechanical loading applied during orthodontic tooth movement has been established, implicating orthodontic force application in this adverse sequela (Brezniak and Wasserstein 1993; Baumrind 1996).

Root Resorptive Process and Repair

Orthodontic treatment uses the body's inflammatory response to its advantage to move teeth. The force applied to the teeth causes a local aseptic inflammatory reaction inducing the four defining characteristics of inflammation; pain, heat, redness, and swelling (Krishnan and Davidovitch 2009). This inflammatory response also sets off a cascade of cellular and molecular signals to regulate bone activity and ultimately tooth movement. In the direction of the force application, there forms a compression zone of periodontal ligament (PDL) fibers and ultimately bone resorption via osteoclasts (multinucleated bone resorbing cells). When the applied force per area is proper (light

enough), there is a decrease in strain as the periodontal fibers relax and therefore an unloading of PDL and alveolar bone occurs which leads to disuse-induced osteocyte apoptosis and resorption. In contrast, opposite to the direction of force application, the PDL fibers are stretched and under tension which causes an active loading of bone, activating osteoblastic (bone-forming) cells and yielding new bone (Melsen 2001; Henneman 2008) (Figure 1-2). This is congruent with the orthopedic dogma: loading of bone builds new bone while unloading results in resorption. Mechanical forces, if heavy, applied to the teeth can also cause a local zone of tissue necrosis on the resorption side when blood flow is obstructed (>20-26 g/cm^2), causing hypoxia to the cells. Tooth movement can only proceed when this necrotic (or "hyalinized") tissue is removed by phagocytic cells such as macrophages and osteoclasts (Hennenman 2009).

The inflammatory process involved in moving teeth is also the key component in orthodontically induced root resorption. Root resorption occurs as part of the necrotic tissue (hyalinized zone) elimination process that occurs during tooth movement (Brudvik and Rygh 1993). During the removal of the hyalinized zone by macrophage-like cells and multinucleated tartrate resistance acid phosphatase (TRAP) positive cells, the adjacent outer surface of the tooth root (cementum) can also be resorbed. As these phagocytic cells remove the necrotic tissue, the nearby cementum is attacked (Hellsing and Hammarstrom 1996) (Figure 1-3). When the cementoblastic covering of the root is damaged, the mineralized cementum is exposed. The pressure from the orthodontic force may also directly damage the cementum layer thereby requiring its removal and

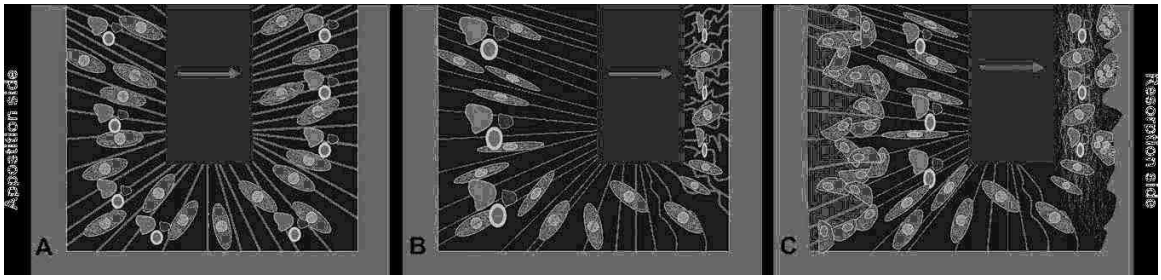

Figure 1-2: Diagram showing the PDL fibers as they connect the tooth to bone. (A) shows the equilibrium of PDL fibers; (B) As the force is applied, fibers on the resorptive side are compressed and no longer under tension leading to bone resorption by osteoclasts. (C) In contrast, on the apposition side, fibers are stretched and creating tension, which triggers bone formation by osteoblasts. (Hennenman, 2009)

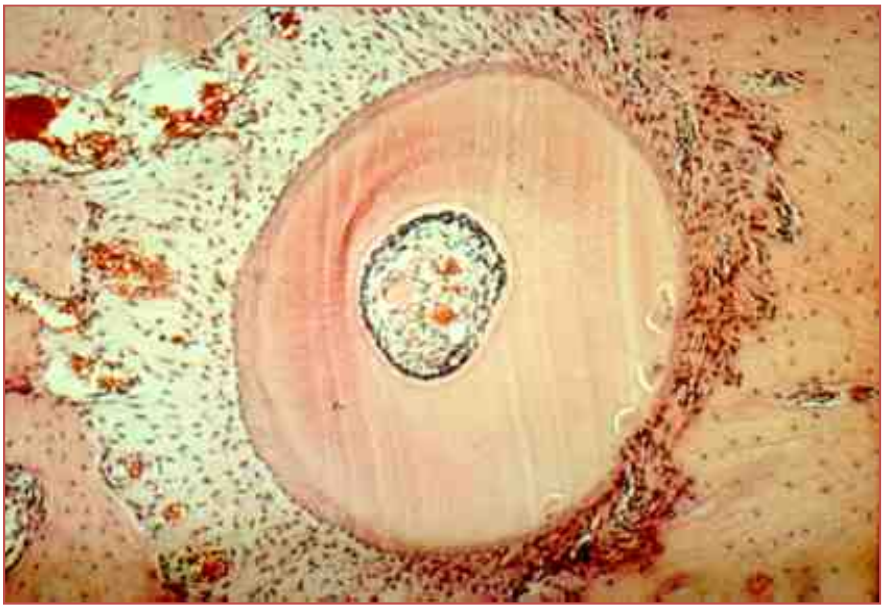

Figure 1-3: Cross sectional view of root slice showing resorption of both alveolar bone and cementum by osteoclastic cells under compression (Proffit 2007).

repair (Brezniak and Wasserstein 2002). These TRAP positive cells involved in necrotic tissue removal are initially present without the characteristic ruffled border (common to osteoclasts), but upon further mechanical stimulus they can be differentiated into functional osteoclasts or odontoclasts (or called cementoclasts) capable of resorbing bone or root (Brudvik and Rygh 1993). Brezniak states that there are three degrees of resorption that can occur: (Quoted from Brezniak and Wasserstein 2002)

1. Cemental or surface resorption with remodeling. In this process only the outer layers are resorbed and are later fully regenerated and remodeled.
2. Dentinal resorption with repair (deep resorption). In this process, the cementum and the outer layers of the dentin are resorbed and usually repaired with cementum material. The final shape of the root … may not be identical to the original form.
3. Circumferential apical root resorption. …full resorption of the hard tissue components of the root apex occurs and root shortening is evident. …No regeneration is possible.

The repair of the damaged cementum following root resorption prevents any communication between the periodontal and pulpal tissue (Hellsing and Hammarstrom 1996). The process of cementum repair begins when the force application is discontinued or drops below a certain level. Repair can begin as early as one week after the removal of orthodontic force and by eight weeks 82% of resorption was repaired (Owman-Moll and Kurol 1995; 1998). The early stages of cementum repair are characterized by deposition of acellular cementum, while the later stages (and the majority of repair) are marked by cellular cementum (Owman-Moll and Kurol 1998). The ability of the body to repair the damaged cementum depends on the extent of damage that occurred. If the resorption only occurs in small amounts and in distinct

lacuna, repair of the damaged cementum usually restores the original contours of the root. If the damage is severe enough that small islands of cementum are separated from the body of the tooth root, the reparative process cannot rejoin the separated cementum and it will be subsequently resorbed leading to apical shortening (Proffit 2007).

Cellular and Molecular Regulation of EARR

Although severe root resorption with apical shortening is not common and not a concern for the majority of orthodontic cases, it is, however, both alarming and concerning to the orthodontist and patient when it happens. It would be beneficial for practitioners to have a better understanding of what causes resorption to occur. Insight into the molecular mechanisms of root resorption and repair could possibly lead to preventive or therapeutic strategies for dealing with this unwanted side effect. Recent investigations into the molecular pathways of bone resorption have shed some light on the specific proteins involved in root resorption. One pathway that has recently been explored to help clarify the molecular regulation of root resorption is the OPG/RANKL/RANK system.

The functions of OPG/RANKL/RANK axis have been well established in bone physiology and more recently outlined for orthodontic tooth movement (Khosla 2001; Roberts 2004; Yamaguchi 2009) and root resorption (Tyrovola 2008; Hartsfield 2009).

Osteoprotegerin (OPG), receptor activator of nuclear kappa beta ligand (RANKL) and their receptor activator of nuclear kappa beta (RANK) are members of the tumor necrosis factor (TNF) superfamily and are important in the control of osteoclastogenesis and bone remodeling (Khosla 2001). Preosteoblastic cells help regulate osteoclastogenesis by expressing two proteins, OPG and RANKL, that bind to RANK on the surface of preosteoclasts. These two proteins work in opposition of each other – one promotes osteoclastogenesis while the other inhibits osteoclastogenesis and promotes bone apposition. When expressed on the surface of preosteoblastic cells, RANKL binds to its cognate receptor RANK on the surface of preosteoclastic cells and promotes the differentiation, activation and survival of osteoclasts. In contrast, when OPG, a soluble protein, is secreted by preosteoblastic cells, it acts as a decoy receptor, binding to RANKL, preventing the action of RANKL to promote osteoclastogenesis, and stimulating osteoclast apoptosis (Khosla 2001) (Figure 1-4). These proteins have also shown to be expressed by periodontal ligament (PDL) cells and participate in bone modeling during orthodontic tooth movement and root resorption (Ogasawara 2004; Low 2005; Yamaguchi 2006). Changes in OPG and RANKL have been shown in PDL cells when subjected to orthodontic forces (Low 2004). Studies have also shown that under tensile strain, as in the side behind orthodontic tooth movement, there is an increase in OPG mRNA in PDL cells and a decrease in RANKL which is congruent with bone apposition. On the resorptive side, the compressed PDL cells produce more RANKL. Since there are many similarities between the cellular mechanisms of root resorption

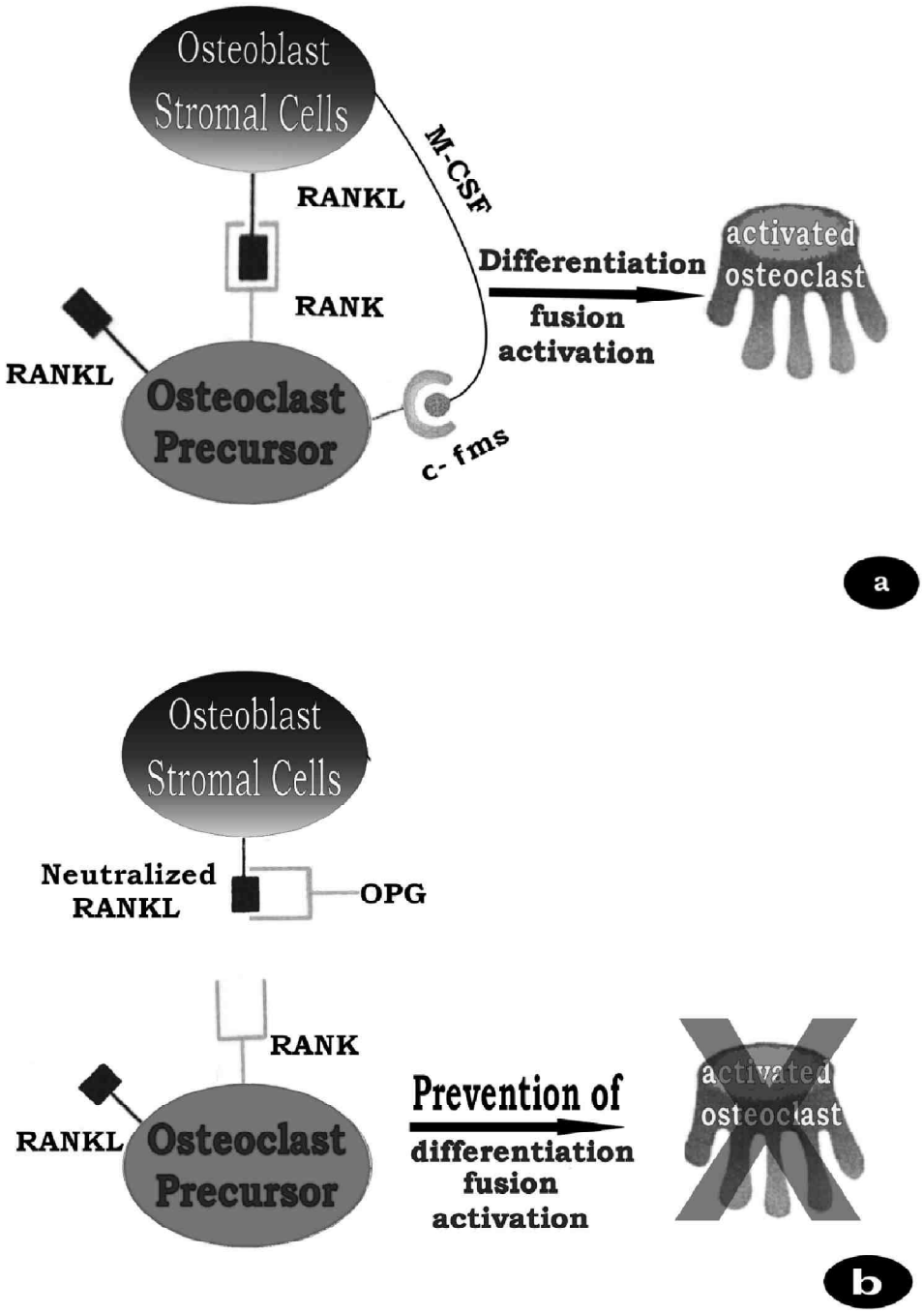

Figure 1-4: Schematic illustration showing regulation of osteoclastic precursor cells by RANKL and OPG. A) RANKL secreted or expressed on the surface of osteoblasts binds to RANK on osteoclastic precursor cells to differentiate and activate osteoclasts. B) OPG, a soluble protein is also secreted from osteoblasts and binds to RANKL, blocking the action of RANKL, thereby preventing the activation of osteoclasts (Tyrovolo 2008).

and osteoclastic bone resorption, it is expected that OPG/RANKL/RANK axis will be affected in cases of orthodontic treatment that exhibit apical root resorption as well (Yamaguchi 2006). In fact, cases of severe apical root resorption show an even greater increase in RANKL, which stimulates osteoclastogenesis and subsequently resorption of the root in patients (Yamaguchi 2006) (Figure 1-5). This increase in RANKL can also be seen in samples of gingival crevicular fluid of orthodontic patients that exhibit apical root resorption (George and Evans 2009). The ratio between RANKL and OPG, therefore, can be indicative of the direction of bone modeling that is occurring.

Another bone regulatory molecule involved in osteoclastogenesis and linked to root resorption is osteopontin (OPN). OPN is a non-collagenous glycoprotein that is produced by osteocytes, osteoblasts, osteoclasts and odontoclasts (Terai 1999; Liu 2004; Chung 2007). Its primary role in bone regulation is to aid in the attachment of osteoclasts to the bone mineral matrix and promote osteoclastogenesis (Terai 1999). It also acts as a chemoattractant for nearby osteoclastic precursors and helps osteoclasts develop their distinctive ruffled border (Terai 1999). It has been shown that OPN is responsive to mechanical loading (Terai 1999; Liu 2004; Kuroda 2005). By using *in situ* hybridization, Terai found that the main cell expressing OPN was the osteocyte, but that it was also expressed by osteoblasts and bone-lining cells. All of the cells that expressed OPN, however, were located on the pressure side of the applied force. Two studies with OPN genetic knockout mice have shown both a decrease in the number of osteoclasts in the alveolar bone and a decrease in odontoclasts and resultant root resorption (Fujihara 2006; Chung 2007). Analysis of genetic polymorphisms of OPN in patients with root

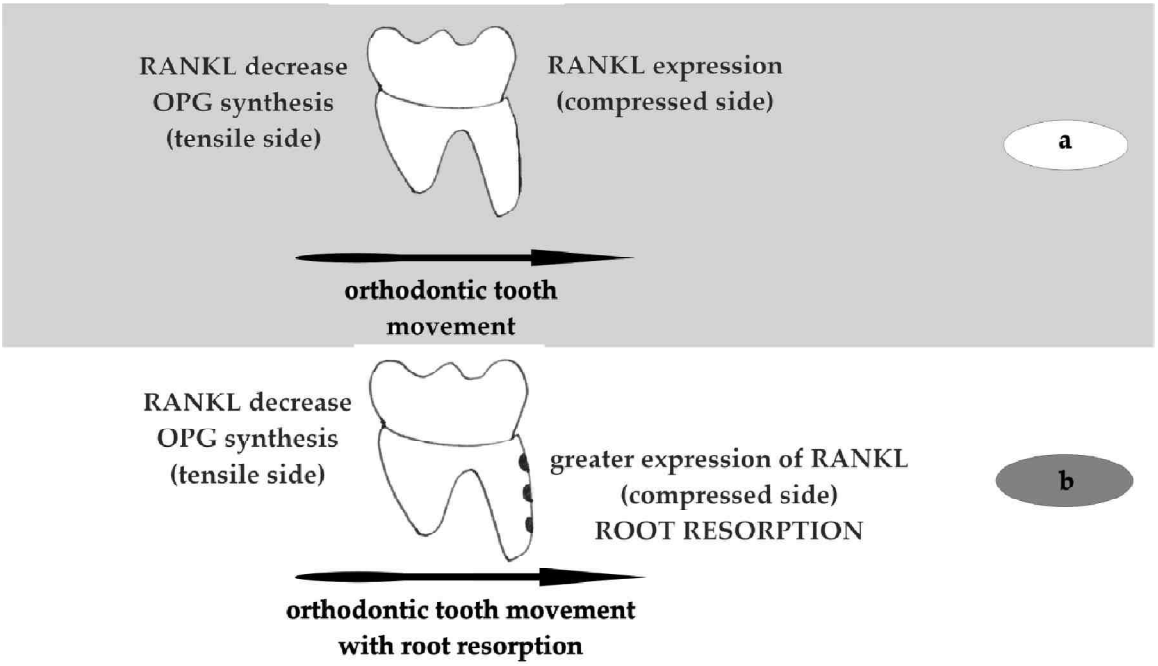

Figure 1-5: Changes in OPG and RANKL during orthodontic tooth movement and root resorption (Tyrovolo 2008).

resorption has also shown a significant association between OPN production and root resorption (Hartsfield Submitted).

The exact mechanism for OPN upregulation is still unclear but adenosine triphospate (ATP) has recently been linked to the induction of OPN in response to mechanical stress (Wongkhantee 2008). ATP is known to be an important intra and extracellular signaling molecule. Its actions inside the cell are mediated by cAMP while extracellular activity is mediated through the family of P2 purinoceptors present on target cells (Hoebertz 2002). Although the actions of ATP in bone regulation are not as defined as those of RANKL or OPG, osteoblasts have been shown to increase levels of ATP in response to mechanical stress (Genetos 2005; Wongkhantee 2008). Following mechanical stress, ATP released from osteoblasts inhibits OPG induction while upregulating RANKL and OPN from periodontal cells thus inhibiting bone formation and stimulating osteoclastogenesis (Hoebertz 2002; Buckley 2002; Wongkhantee 2008). Liu et al also showed that extracellular ATP is released from cementoblasts in response to fluid shear stress, a form of mechanical loading *in vitro* (In Press). This data suggests that ATP plays an important role in pressure-induced bone modeling and is likely involved in both orthodontic tooth movement and root resorption.

Similar to ATP, prostaglandin E_2 (PGE_2) is also known to have an effect on osteoclastogenesis by mediating the effects of RANKL (Han 2005). PGE_2 is produced from arachidonic acid, which is located in the cellular plasma membrane, and synthesized via the enzyme cyclooxegenase-2 (COX-2). The availability of COX-2

perpetuates the action of RANKL and facilitates the differentiation of monocytes, osteoclastic precursors, into functional osteoclasts capable of bone resorption (Han 2005). Cementoblasts, similar to osteoblasts, have been shown to express the genes for COX-2 and PGE_2. When cementoblasts in culture were incubated with exogenous PGE_2 *in vitro*, expression of COX-2 and RANKL increased, while OPG expression was decreased thus promoting cementoclastogenesis (Oka 2007).

Another inhibitor of bone formation is a protein called sclerostin. The activity of sclerostin was identified in patients who lacked this protein and exhibited sclerosteosis, a disorder characterized by bone overgrowth and increased bone mass (van Bezooijen 2004). SOST, the gene that produces sclerostin, is expressed mainly in osteocytes, and inhibits bone formation by inhibiting osteoblast differentiation. It is known to be expressed by osteocytes within the lacunae and transmitted via cell to cell contact to surface-lining osteoblasts where it inhibits further bone apposition. Both SOST transcripts and sclerostin protein were significantly reduced in response to mechanical loading *in vivo* (Robling 2008). Sclerostin has not been localized in osteoclasts, however, and appears to have no affect on bone resorption (van Bezooijen 2004). Just recently, sclerostin has also been identified in cementocytes, and cells of the PDL as well, suggesting a role for this protein in orthodontically induced bone modeling and root resorption (Jager 2010).

Fluid Shear Stress

To study bone adaptation to mechanical load at tissue and cell levels, many different methods have been tested and employed including mechanical strain and fluid shear stress (FSS). Two principle theories have been used to explain how mechanical loading generates an osteogenic cellular response. The first concept suggests that osteocytes and osteoblasts, in response to mechanical load, deform under the physical strain which sets off an array of intra and extracellular signaling pathways to control bone metabolism (Owan 1997). In contrast, fluid flow within the bone tissue can vary due to hydrostatic pressure changes and can affect cellular metabolism (Owan 1997). To understand the process by which fluid flow affects bone cells, it is necessary to briefly review the microstructure of bone.

Osteocytes, the dominate cell type in bone, become trapped in the mineralized matrix as the bone tissue develops. The bodies of osteocytes are located in a structure called lacuna which are connected to each other or to bone lining cells by long cellular processes located in a structure called canaliculi (Figure 1-6) (Akst 2009). These canaliculi form a network that penetrates the entire bone matrix. The space between the plasma membrane and the bone matrix is the periosteocytic space. Extracellular fluid flows through this space and through the canalicular network and can be affected by bone matrix compression or tension. This fluid flow allows exchange of nutrients and signaling molecules (such as RANKL, OPG, OPN) with nearby cells and surrounding tissues and can create shear forces that are directly involved in mechanosensing and

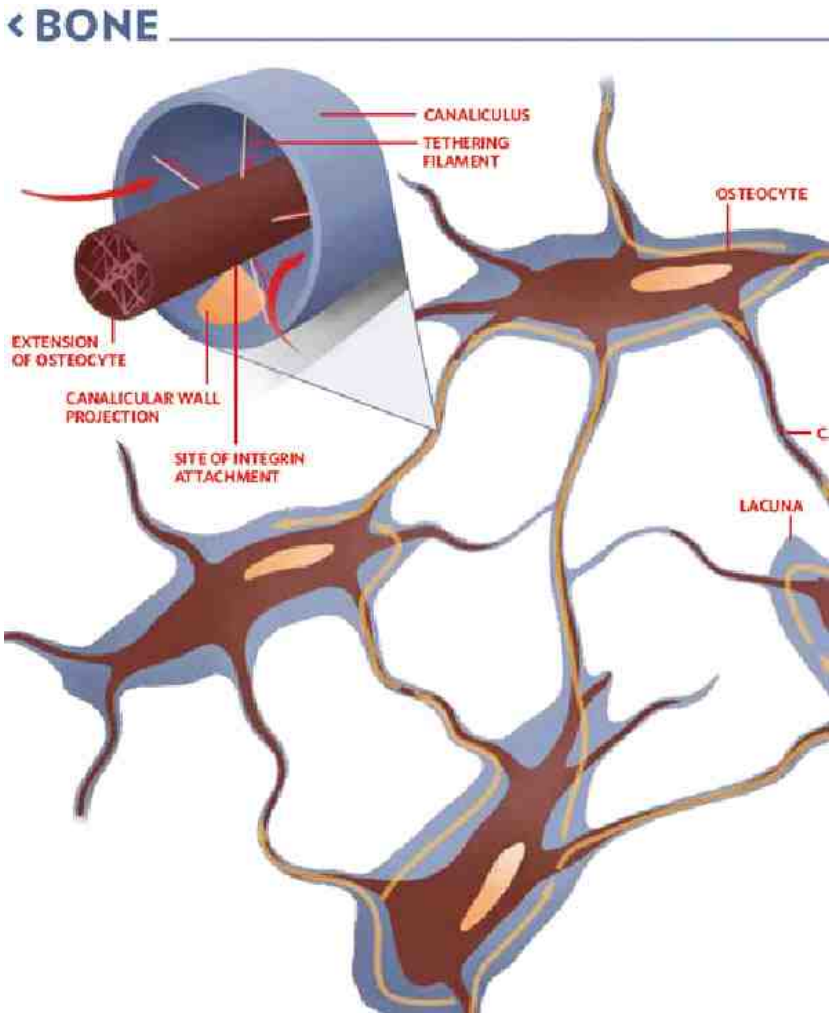

Figure 1-6: Microanatomy of bone. Osteocytes are situated within lacuna of the calcified bone matrix. Cells are connected via a network of canals called canaliculi. As interstitial fluid passes through these canals it can transmit and amplify mechanical signals to the cells (Akst 2009).

regulation of bone remodeling (Tan 2007; Henneman 2008; You 2008). The framework of the canalicular network and the location of osteocytes, within the lacunae where extracellular fluid flow is detected, allow these cells to respond to mechanical load and alter bone remodeling activity by recruiting osteoclasts to sites where bone resorption is required.

Experimental evidence has shown that fluid flow is a more accurate model for applying a mechanical load to bone cells *in vitro* than compression, or mechanical deformation (Owan 1997). Recent studies have shown that bone cells are indeed acutely responsive to mechanical loading by fluid flow. When osteocytes were subjected to pulsating fluid flow, *in vitro*, an inhibition of osteoclast formation and bone resorption was seen (Tan 2007). You et al. (2008) also found that osteocytes in cell culture were responsive to fluid flow and exhibited an upregulation of RANKL mRNA, but a decrease in RANKL protein levels and prevention of osteoclast formation. These results favor the idea that FSS has an anabolic effect on bone modeling. In contrast, when fluid flow is reduced (as seen in the resorptive side of a tooth moved under compression) osteocytes may undergo apoptosis which encourages the recruitment of osteoclasts and subsequent bone and root resorption (Bakker 2004). Aguirre et al (2006) showed in mice that when bone is unloaded, osteocytes do undergo apoptosis which leads to bone resorption. It is not clear, however, how higher than normal levels of FSS may affect bone remodeling at the molecular level and if this could also lead to bone and root resorption. Frost's (1987) theory on microstrain suggests that bone is resorbed when unloaded, it is formed when physiologically loaded and then fatigues

and fractures when physiological levels of strain are exceeded. Fatigue and fracture in bone can also lead to resorption through a different mechanism (Frost 1987) (Figure 1-7).

Physiological levels of FSS have been established for long bones from 8-30 dynes/cm^2. Although the amount of FSS that occurs in the PDL has not been established (currently under investigation by Dr. Dawei Liu in collaboration with the University of Delaware), 12 dynes/cm^2 is the amount frequently used for studies examining molecular bone regulation (Chen 2000; Chen 2003; Pavalko 2003; Lee 2008; Liu 2008; Rangaswami 2009). The dyne is a unit of measurement often used to describe the surface tension in fluids. One dyne is the force required to cause a mass of one gram to accelerate at a rate of one centimeter per second squared in the absence of other force-producing effects. The application of the fluid flow model in research allows for the examination of cellular responses of bone cells to various environmental stimuli and will help further clarify the molecular regulation pathways involved in bone remodeling and possibly root resorption and repair.

Cementoblasts

As has been reported, osteocytes and osteoblasts play a very prominent role in bone remodeling and regulation. This investigation, however, seeks to find how

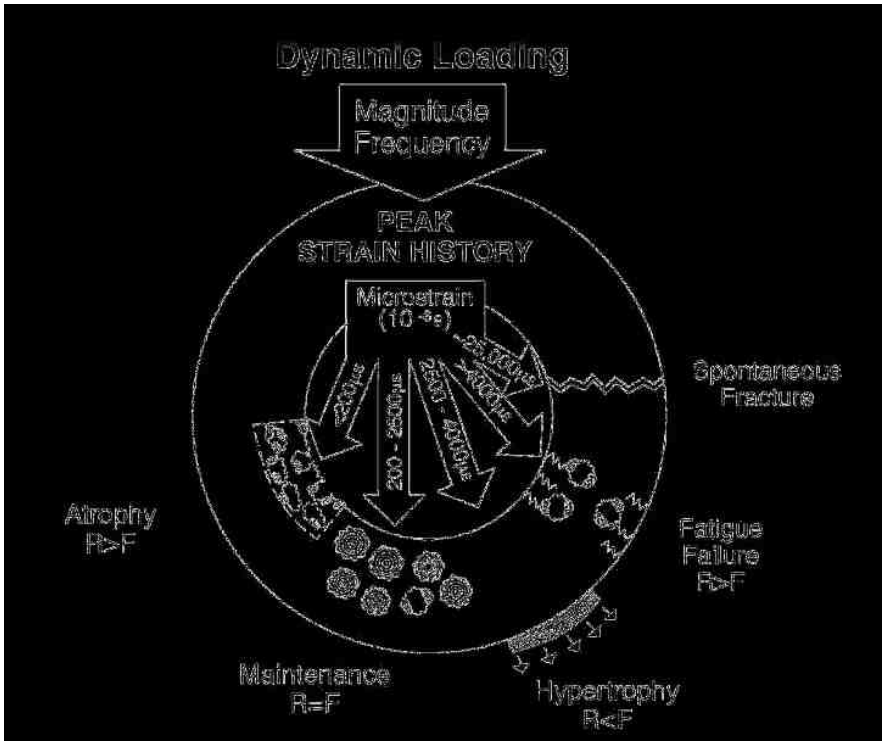

Figure 1-8: Dynamic loading of bone. Mechanostat theory proposed by Frost. R=resorption, F=formation. When bone is loaded in the physiological range, bone is formed. When bone is unloaded or overloaded, resorption occurs (Frost 1987).

cementoblasts, which share many similarities with osteoblasts, are involved in cementum modeling during orthodontic tooth movement and how this involvement may affect root resorption during orthodontic treatment. As the cells that lie on the surface of the tooth root, cementoblasts bear the mechanical load during orthodontic tooth movement and thereby association are poised to participate in the resorptive process. To what degree and in what mechanism, however is still under examination.

Cementoblasts are the matrix producing cells of cementum, the lining that covers the tooth root. Cementum covers the surface of the tooth root and attaches the periodontal ligament (sharpey's) fibers that secure the tooth in alveolar bone. It also serves to repair root defects following resorption or fracture, seal the dentinal tubules and protect the pulp (Bosshardt 2005). Similar to bone, cementoblasts become embedded in the matrix they secrete to become cementocytes. These cells reside in lacunae and are connected to each other via a canalicular network as seen in bone cells (Avery 2000). Unlike bone, however, cementum is avascular and aneural (Avery 2000).

Until recently, the lack of availability of a cementoblast cell line has made the study of these cells in culture and under *in vitro* testing difficult. With the recent development of an immortalized murine cell line, OCCM-30, we are now able to subject cementoblasts to various environmental stimuli to see how they respond. Cementoblasts have recently been shown to express OPN, RANKL, SOST, COX-2, and the P2 receptor for ATP as well as other molecules known for their involvement in bone modeling and root resorption (Dalla-Bona 2008; Huang 2009; Jager 2010; Liu In Press).

Huang et al found that cementoblasts are responsive to mechanical stress and that OPN mRNA is regulated differentially with varying strain levels (2009). Liu et al also found that cementoblasts were responsive to mechanical stress (In Press). Using ultrasound to induce an anabolic response, Dalla-Bona et al showed that only OPG protein production was increased significantly while RANKL protein levels were unchanged (2008). More investigations like these will help to clarify the role of cementoblasts in bone modeling and root resorption following mechanical loading.

Hypothesis

Having shown that a physiological level of FSS can elicit an anabolic response to osteocytes, our working hypothesis is that an equivalent physiological level of FSS applied to OCCM-30 cementoblast cells will produce anabolic responses i.e. a decrease in markers for bone resorption such as RANKL, OPN and COX-2 and the bone formation inhibitor SOST. In contrast, higher levels of FSS may produce catabolic responses i.e. an increase in these markers, promoting osteoclastogenesis.

CHAPTER 2

MATERIALS AND METHODS

Cell Culture

The immortalized murine cementoblastic cell line, OCCM-30 cells were provided by Dr. MJ Somerman (University of Washington). These cells respond to the same factors that are involved in the formation and regeneration of the periodontium and those associated with bone metabolism (Ouyang 2000; Zhao 2003). These cells have also been shown to express RANKL, OPG, OPN, COX-2 and SOST genes and to produce their respective proteins in laboratory experiments (Oka 2007; Jager 2010; Liu In Press). The OCCM-30 cells were cultured in α-minimal essential medium (α-MEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were cultured in T75 cell culture flasks maintained at 37°C with 5% CO_2 in a humidified incubator. Cells were routinely divided and passaged at confluence. Passages 10-20 were used for experimentation. To prepare for FSS experimentation, cells were plated at a density of 5 X 10^4 cells/cm^2 and grown to 90% confluence on 75 X 38 mm^2 glass slides coated with Type I collagen. All cell culture supplies were purchased from Sigma (St. Louis, MO) unless otherwise noted. Prior to FSS experimentation, cells were serum starved with 0.2% FBS containing medium for 24 hours in order to synchronize cell cycles and attain a basal level of metabolic activities.

FSS Experimentation

Individual glass slides were loaded into the specially designed parallel plate flow chamber and connected via tubing to the closed flow loop apparatus (Cytodyne, San

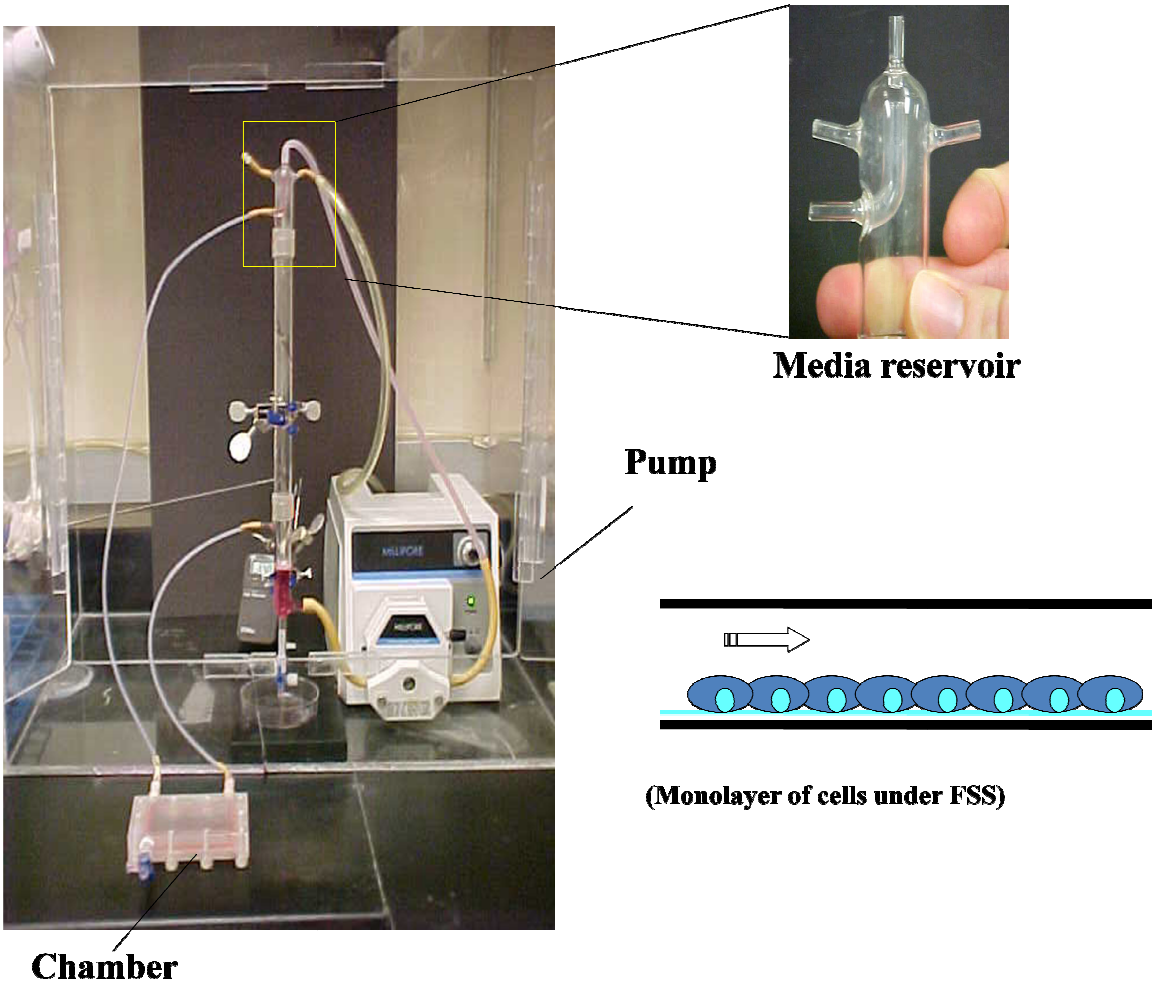

Figure 2-1: Fluid Shear Stress (FSS) system showing the experimental apparatus used for applying a mechanical load to the cells, including the parallel plate chamber for holding the glass slide with attached cells, the medium reservoir for loading flow buffer, and the pump that provides the fluid flow.

Diego CA) (Figure 2-1). The apparatus was maintained at 37°C and 5% CO_2, identical to growth conditions in the cell culture incubator, by the use of an enclosed, heated box and a 5% CO_2 supply line direct to the flow system. The monolayer of cells was subjected to a steady laminar flow shear stress of 12 dynes/cm^2 and 18.5 dynes/cm^2, respectively. The level of FSS applied to the cells varied by regulating the height of the column in the flow set-up (Discussed below). FSS was applied to each glass slide for one hour at a time. Flow medium for the system was 25ml of 0.2% FBS containing medium. Control groups were kept at identical conditions but not subjected to the fluid flow. Six glass slides of cells were run for each FSS level and control group. Experimental and control groups were set up according to the Table below (Table 2-1).

Sample Collection

At one minute after the onset of FSS, 0.5ml of flow medium was collected into a 1.5 ml centrifuge tube and stored at -80°C to be analyzed for the amount of ATP released. By the end of 1 hour of FSS, the glass slide was removed from the parallel plate chamber and returned to a new cell culture dish filled with 2ml of 0.2% FBS containing medium added on top of the glass slide and incubated for an additional hour at 37°C and 5% CO_2. The glass slide was then carefully rinsed with phosphate buffered saline (PBS) twice. To collect RNA, 1 ml of Trizol reagent (Invitrogen, Carlsbad CA) was applied by pipette to only half surface area of the glass slide. Cells were scraped and the solution was transferred to a 1.5 ml centrifuge tube. The samples were mixed

Experimental Samples

		Force Level	
	Control	**Low FSS** 12 dynes/cm^2	**High FSS** 18.5 dynes/cm^2
Protein	12	6	6
ATP	6	6	6

Table 2-1: Experimental samples for the different force levels used in the Low FSS and High FSS groups (12 dynes/cm^2 and 18.5 dynes/cm^2) and the number of samples in each group.

thoroughly by vortex and were stored at -80°C for future use. Protein was collected by adding 100µL of 2X lysis buffer to the other half surface area of the glass slide. The 2X lysis buffer contained 5mM HEPES (pH 7.9), 150mM NaCl, 26% glycerol (v/v), 1.5mM $MgCl_2$, 0.2mM ethylenediaminetetraacetic acid (EDTA), 0.5mM dithiothreitol and 0.5mM phenylmethylsulfonyl fluoride. Slides were scrapped and the solution was moved to a 1.5ml centrifuge tube. The protein samples were mixed by vortex and boiled at 100°C for 5 minutes to deactivate proteinases then stored at -80°C until analysis. Control samples were processed in the same manner as experimental samples. Flow medium was also collected and run through a 0.2 µm filter (VWR International, Batavia, IL) to separate any cellular debris and potential bacterial contamination from the flow medium that may have occurred during the FSS experiment. Medium samples were then placed in a separating column (Millipore, Billerica, MA) and centrifuged at 5000 rpm for 15 minutes to concentrate the samples to 1.5 ml for use in a future resorption activity assay.

Calculating FSS Levels

The amount of FSS applied to the monolayer of cells during the FSS experiment varies depending on the height of the column set-up in the FSS apparatus. The height of the small chamber was 21.75cm tall and was 64cm from the stand base. The height of the large chamber was 43.5cm tall and was 64cm above the stand base. To calculate the actual levels of FSS that were used for the experiment, the apparatus was set up identical to experimental conditions described above. Fluid from the system was

collected in a glass beaker for a measured period of time. This was done three times for each level of FSS used. Volume of medium was measured by pipette. The amount of volume collected per minute was calculated and averaged (Table 2-2). Using the FSS calibration chart, the amount of dynes/cm^2 was determined to be 12 dynes/cm^2 for the small column height and 18.5 dynes/cm^2 for the large column height (Figure 2-2 Provided by Dr. Robling – Indiana University).

ATP Release

To measure ATP release, we used the ATP Bioluminescence Assay Kit HS II from Roche (Indianapolis, IN). This kit uses the enzyme luciferase to catalyze the reaction from D-luciferin into oxyluciferin and light. This reaction requires ATP as a co-factor. The light produced by the reaction is directly related to the ATP concentration in each sample. The resulting luminescence was measured using a Berthold Sirius Luminiometer detection system (Zylux Corp, Huntsville AL). Experimental samples were compared to 0.2% FBS as a control. Samples were run in triplicate and results were normalized to total cell protein determined using the amino black method**.** Final values represent concentrations at pmol level.

Protein Production

Following storage, protein samples were centrifuged at 14,000g for 10 min to remove any cellular debris. Protein concentration of the whole cell lysate was

Calculating FSS force level

LOW

Time (sec)	15.7	15.5	15.7	**Avg (sec)**	15.63
Volume (ml)	14.42	14.21	14.96	**Avg (ml)**	14.53
				ml/min	55.78
				Dynes/cm^2	12.0

HIGH

Time (sec)	10.3	9.4	10.1	**Avg (sec)**	9.93
Volume (ml)	14.61	13.83	13.86	**Avg (ml)**	14.10
				ml/min	85.20
				Dynes/cm^2	18.5

Table 2-2: Time and volume output data from FSS apparatus during fluid flow experiment used to calculate force levels.

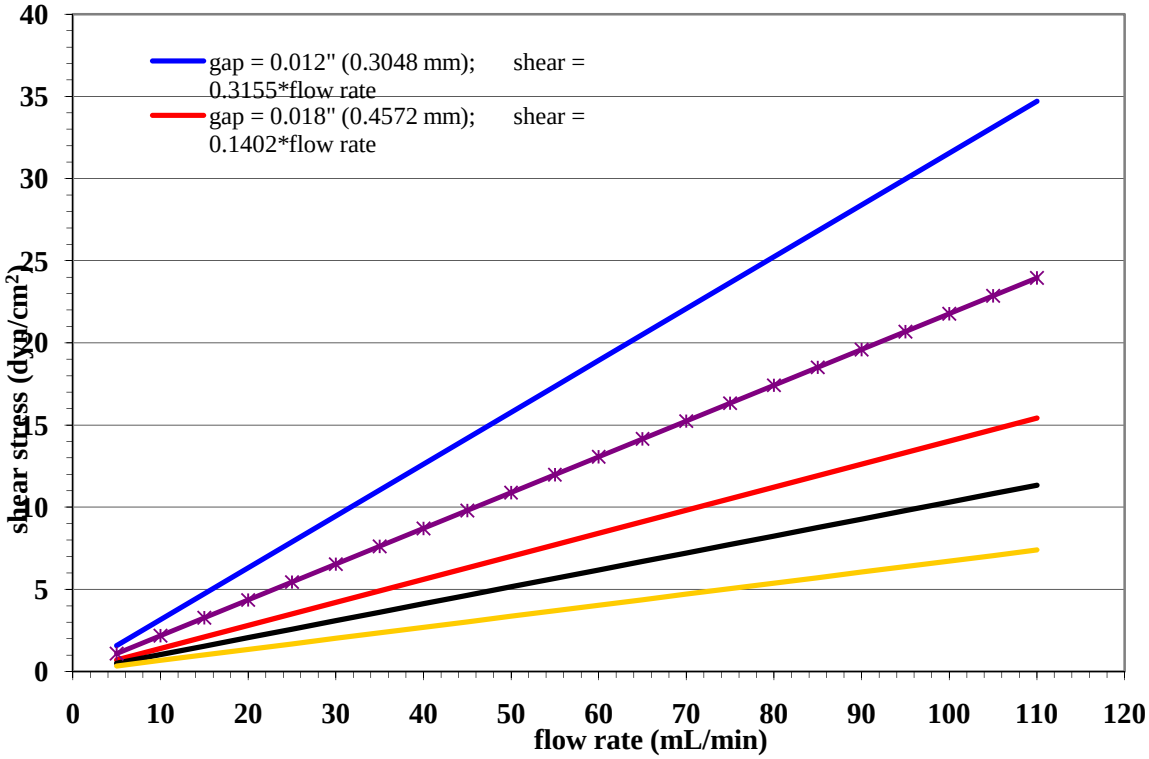

Figure 2-2: Chart used to calculate actual amount of force (dynes/cm^2) applied to cells by fluid shear stress during experiment using ml/min of fluid output generated by FSS apparatus. Based on the size of the flow chamber used in our experiment, the purple line was used for calculating FSS levels (Provided by Dr. Robling – Indiana University).

quantified using the amino black method (Genetos 2005). Proteins were separated by gel electrophoresis by loading 50 µg of whole cell lysate and 5 µl pre-stained molecular weight marker (Bio-Rad Laboratories, Hercules, CA) and running through a 10% SDS gel. For western blotting, separated proteins were transferred overnight to nitrocellulose membranes and then blocked with 1X Tris-buffered saline (TBS) containing 5% nonfat dry milk (Bio-Rad Laboratories, Hercules, CA) and 0.1% Tween-20 (TBST) for 2 hours at room temperature. Membranes were blotted with primary antibodies overnight at 4°C on a shaker. Primary antibodies used were anti-OPN (Assay Designs, Ann Arbor, MI), anti-RANKL (EMD Chemicals Inc, San Diego, CA), anti-COX-2 (Cayman Chemical, Ann Arbor, MI), anti-SOST (R&D Systems Inc, Minneapolis, MN). Membranes were washed three times in 1X TBST and then incubated with secondary antibodies: goat anti-rabbit or goat anti-mouse IgG hydroperoxidase (1:5000) for one hour at room temperature. Protein band images were developed using enhanced chemiluminescence (ECL) method (Pierce, Rockford, IL) and recorded using a FUJIFILM LAS-1000 gel documentation system (Stamford, CT). Protein quantities were normalized by comparing the optical densities of each interested band to that of vinculin as a house keeping protein (internal control).

Statistical Analysis

SPSS version 17.0 software was used to complete the statistical analysis. All samples were averaged and the means for each group were compared using one way analysis of variance (ANOVA) with Tukey's post-hoc comparison to determine where the

significance lies between the different groups. Values were graphed as mean ± standard deviation of the individual groups. Statistical significance was determined at $p < 0.05$.

CHAPTER 3

RESULTS

FSS induces ATP Release

There was an increase in ATP released from OCCM-30 cells following one minute of FSS, compared to controls. ATP released was significantly ($P < 0.05$) elevated for both the low (12 dynes/cm^2) and high (18.5 dynes/cm^2) levels of FSS. ATP release was negligible in control samples while a low level of FSS resulted in an average 785.27 pmol of ATP released into the flow medium and a high level of FSS resulted in 2250.39 pmol which is significantly ($p<0.01$) higher than the lower level of FSS (Figure 3-1, Table 3-1).

Protein Production in OCCM-30 Cementoblasts

Western blot analysis showed a significant decrease in OPN production following the application of a low level of FSS (12 dynes/cm^2) for 1 hour followed by one hour post-FSS incubation, compared to controls. OD values for OPN were decreased from 4.12 to 2.31 ($P<0.01$). There was no significant change in OPN levels between the control group and a high level of FSS (18.5 dynes/cm^2) (Figure 3-2; Table 3-2, 3-3). RANKL protein was also significantly decreased ($P<0.01$) following the application of a low level of FSS (12 dynes/cm^2) for 1 hour followed by one hour post-FSS incubation, compared to controls. OD values for RANKL were decreased from 1.88 to 0.99. No significant difference in RANKL protein levels was seen between a high level of FSS (18.5 dynes/cm^2) and the control. (Figure 3-3; Table 3-2, 3-4). The application of 1 hour of FSS followed by 1 hour of post incubation did not alter protein levels of either COX-2 or SOST significantly (Figure 3-4, 3-5; Table 3-2, 3-5, 3-6).

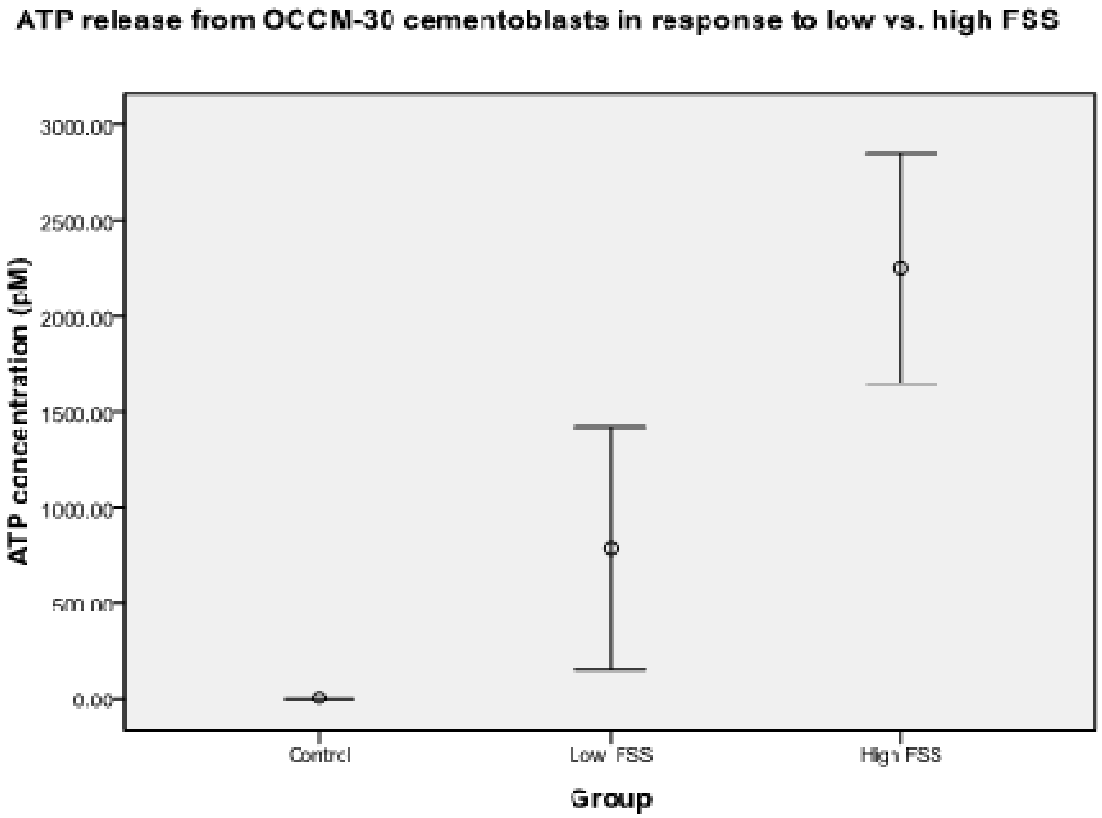

Figure 3-1: Graph showing comparison of ATP release following application of FSS for 1hour. Controls showed no ATP release. ATP release was increased significantly with low and high FSS ($P<0.05$).

ATP - ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4.902E13	2	2.451E13	22.480	.000
Within Groups	5.561E13	51	1.090E12		
Total	1.046E14	53			

ATP - Post Hoc Comparisons

					95% Confidence Interval	
(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Control	Low FSS	-8.01926E5	3.48073E5	.024	-1.6422E6	38316.1138
	High FSS	-2.29913E6	3.48073E5	.000	-3.1394E6	-1.4589E6
Low FSS	Control	8.01926E5	3.48073E5	.024	-38316.1138	1.6422E6
	High FSS	-1.49720E6	3.48073E5	.000	-2.3374E6	-656958.2751
High FSS	Control	2.29913E6	3.48073E5	.000	1.4589E6	3.1394E6
	Low FSS	1.49720E6	3.48073E5	.000	656958.2751	2.3374E6

*. The mean difference is significant at the 0.05 level.

Table 3-1: Statistical analysis for ATP release. ANOVA and Post-hoc analysis performed by SPSS 17.0 software. Analysis shows a significant ($P<0.05$) difference between Control and Low FSS groups, control and High FSS groups and between Low and High FSS groups.

Protein optical density (OD) values

GROUP	OPN	COX-2	RANKL	SOST
control	4.84	2.3	1.67	0.28
control	4.56	2.16	1.74	0.15
control	4.71	2.94	1.67	0.34
control	4.78	1.89	1.63	0.4
control	4.35	1.7	1.82	0.33
control	4.62	2.04	1.76	0.36
control	3.49	3.87	2.29	0.66
control	3.47	3.59	2.6	0.49
control	3.95	4.39	2.5	0.54
control	3.86	3.72	2.59	0.54
control	3.32	4.25	0.98	0.48
control	3.44	3.98	1.32	0.33
Mean	4.12	3.07	1.88	0.41
Std Dev.	0.59	1.00	0.51	0.14

GROUP	OPN	COX-2	RANKL	SOST
low FSS	2.29	1.76	0.9	0.29
low FSS	2.48	1.82	0.41	0.33
low FSS	2.88	1.67	1.11	0.37
low FSS	1.83	4.56	1.27	0.57
low FSS	2.32	3.98	1.1	0.43
low FSS	2.06	5.93	1.12	0.53
Mean	2.31	3.29	0.99	0.42
Std Dev.	0.36	1.80	0.31	0.11

GROUP	OPN	COX-2	RANKL	SOST
high FSS	5	2	1.75	0.49
high FSS	5.06	1.95	1.73	0.41
high FSS	4.38	1.94	1.46	0.45
high FSS	2.84	5.91	1.2	0.7
high FSS	2.78	4.99	1.84	0.66
high FSS	3.14	4.61	2.06	0.58
Mean	3.87	3.57	1.67	0.55
Std Dev.	1.07	1.81	0.30	0.12

Table 3-2: Optical densitometries (OD values) of protein gel bands following western blot analysis, normalized to vinuclin.

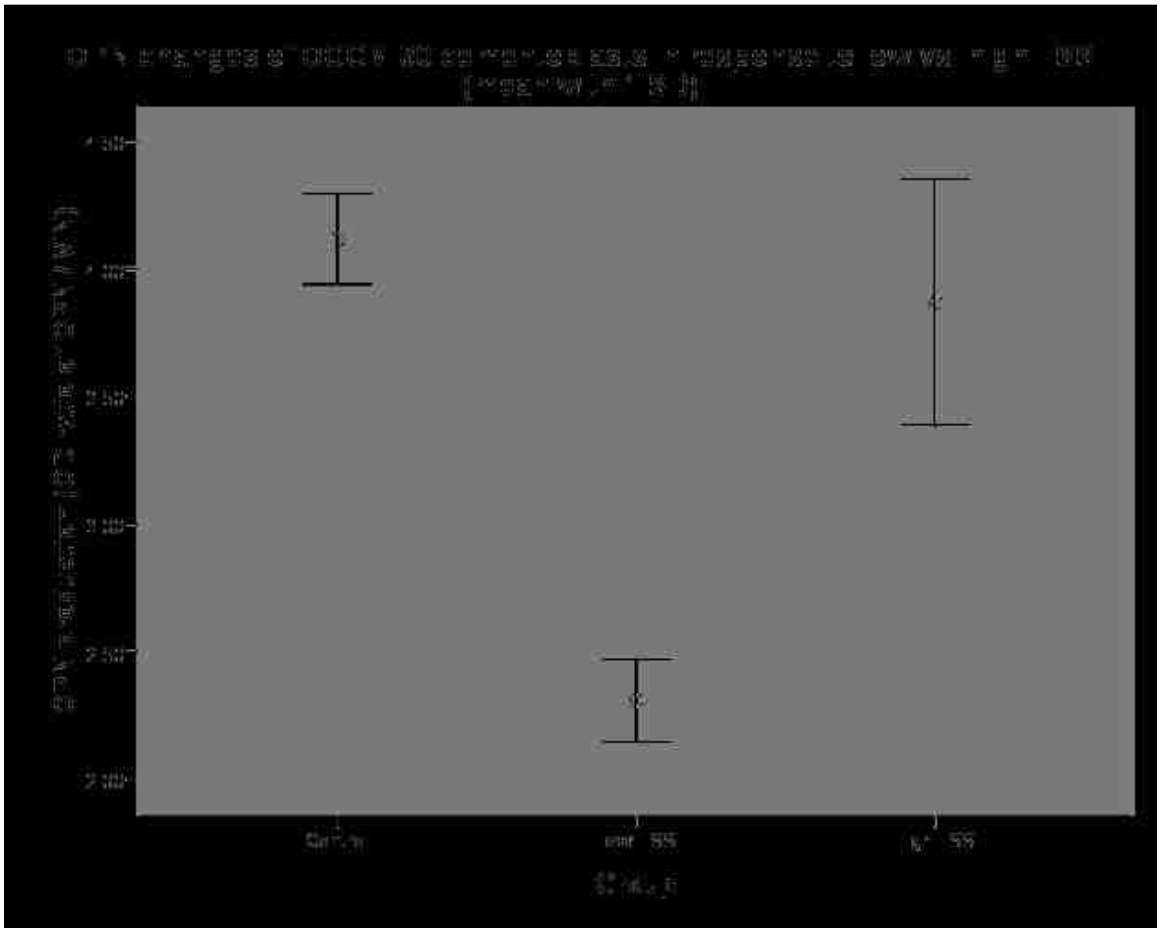

Figure 3-2: Graph showing comparison of OPN protein production following application of FSS for 1hour. OPN decreased significantly with FSS application compared to controls ($P<0.05$).

OPN – ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	13.624	2	6.812	14.025	.000
Within Groups	10.200	21	.486		
Total	23.824	23			

OPN – Tukey's Post hoc Comparison

					95% Confidence Interval	
(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Control	Low FSS	1.80672*	.34847	.000	.9284	2.6851
	High FSS	.24723	.34847	.761	-.6311	1.1256
Low FSS	Control	-1.80672*	.34847	.000	-2.6851	-.9284
	High FSS	-1.55949*	.40237	.002	-2.5737	-.5453
High FSS	Control	-.24723	.34847	.761	-1.1256	.6311
	Low FSS	1.55949*	.40237	.002	.5453	2.5737

Table 3-3: Statistical analysis for OPN protein production. ANOVA and Tukey's Post-hoc analysis performed by SPSS 17.0 software. Analysis shows a significant ($P<0.05$) difference between Control and Low FSS groups and between Low and High FSS groups.

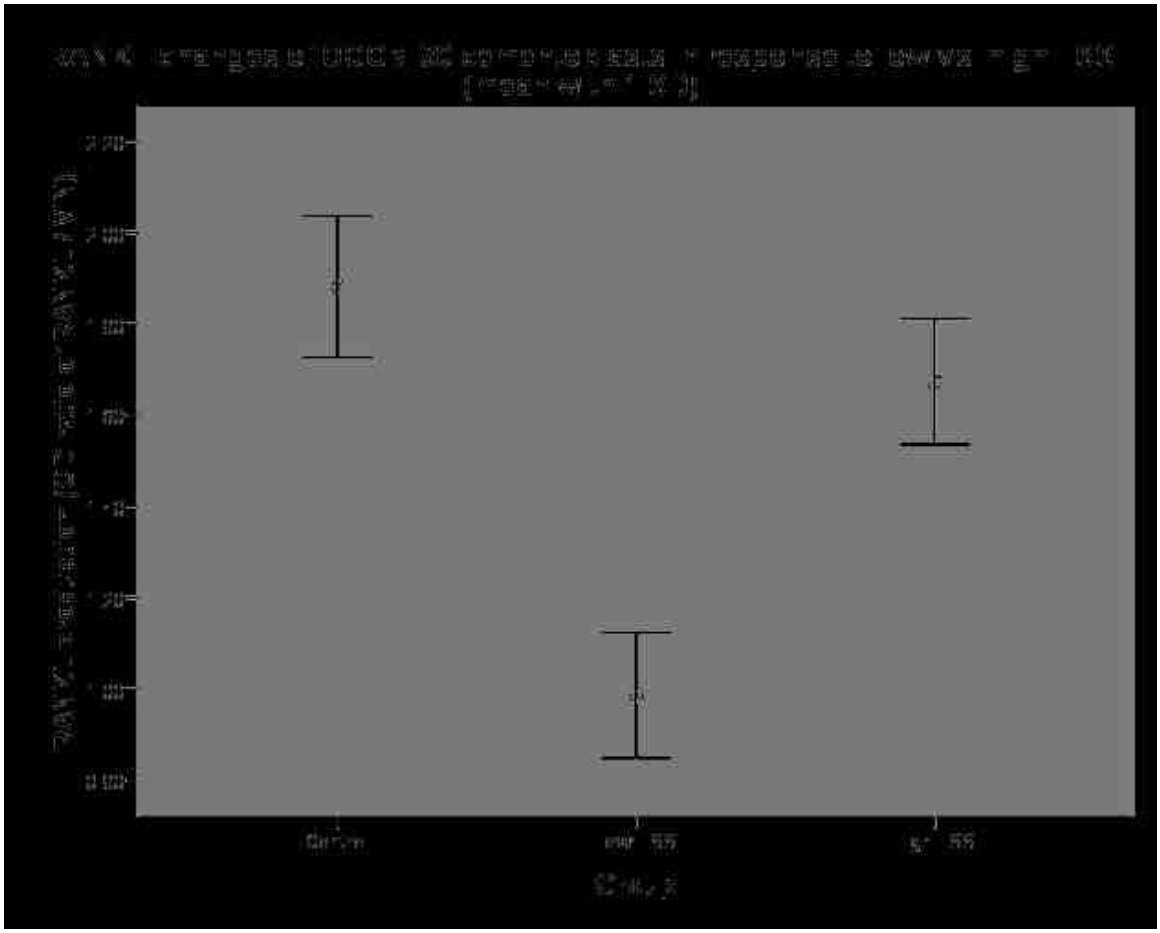

Figure 3-3: Graph showing comparison of RANKL protein production following application of FSS for 1hour. RANKL decreased significantly with Low FSS levels compared to controls ($P<0.05$).

RANKL - ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3.241	2	1.620	8.888	.002
Within Groups	3.828	21	.182		
Total	7.069	23			

RANKL – Tukey's Post Hoc Comparisons

					95% Confidence Interval	
(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Control	Low FSS	.89506*	.21349	.001	.3570	1.4332
	High FSS	.20860	.21349	.599	-.3295	.7467
Low FSS	Control	-.89506*	.21349	.001	-1.4332	-.3570
	High FSS	-.68646*	.24651	.029	-1.3078	-.0651
High FSS	Control	-.20860	.21349	.599	-.7467	.3295
	Low FSS	.68646*	.24651	.029	.0651	1.3078

Table 3-4: Statistical analysis for RANKL protein production. ANOVA and Post-hoc analysis performed by SPSS 17.0 software. Analysis shows a significant ($P<0.05$) difference between Control and Low FSS groups and between Low and High FSS groups.

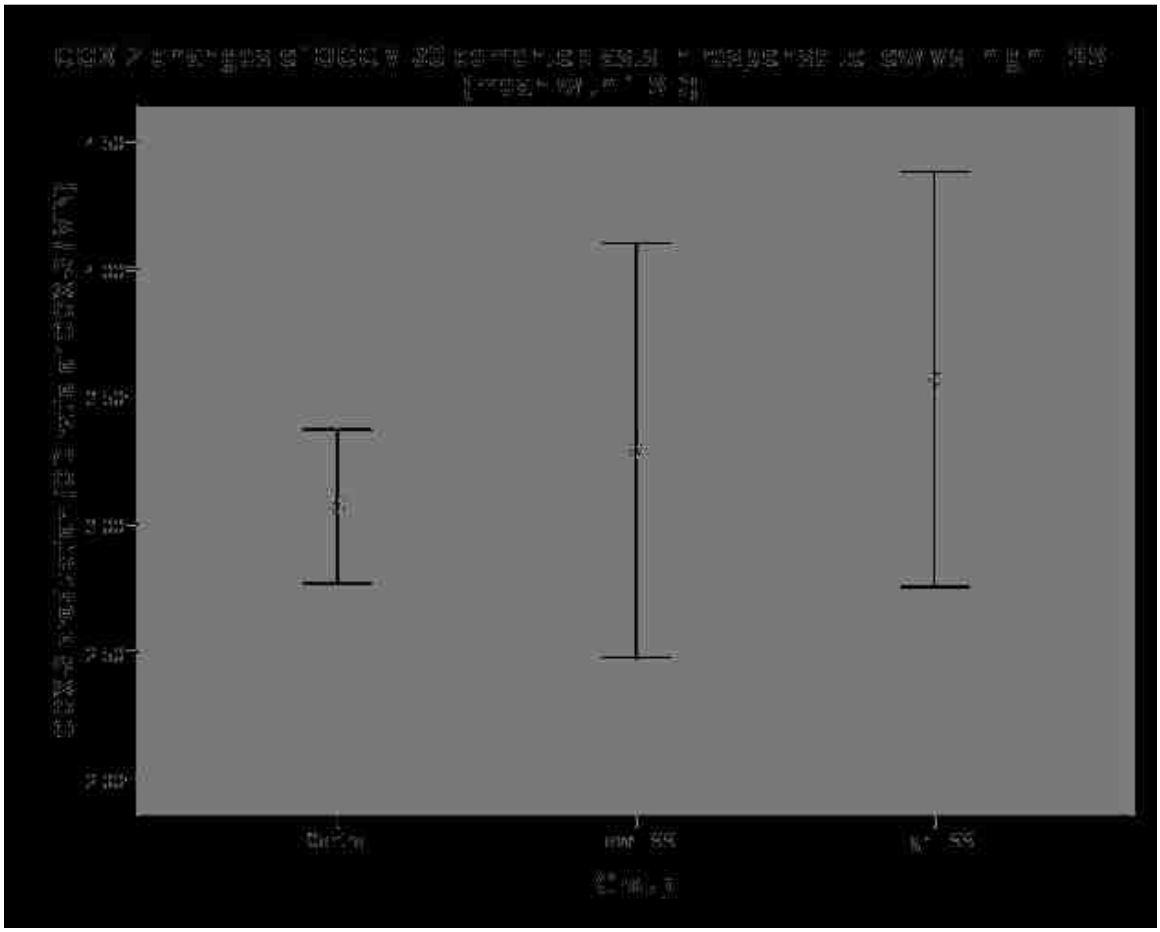

Figure 3-4: Graph showing comparison of COX-2 protein production following application of FSS for 1hour. No significant difference was found between groups.

COX-2 - ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1.003	2	.502	.242	.787
Within Groups	43.591	21	2.076		
Total	44.595	23			

COX-2 Tukey's Post hoc Comparisons

					95% Confidence Interval	
(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Control	Low FSS	-.21798	.72038	.951	-2.0337	1.5978
	High FSS	-.49776	.72038	.771	-2.3135	1.3180
Low FSS	Control	.21798	.72038	.951	-1.5978	2.0337
	High FSS	-.27979	.83182	.940	-2.3764	1.8169
High FSS	Control	.49776	.72038	.771	-1.3180	2.3135
	Low FSS	.27979	.83182	.940	-1.8169	2.3764

Table 3-5: Statistical analysis for COX-2 protein production. ANOVA and Post-hoc analysis performed by SPSS 17.0 software. Analysis shows there was no significant ($P<0.05$) difference found between control, Low FSS or High FSS groups.

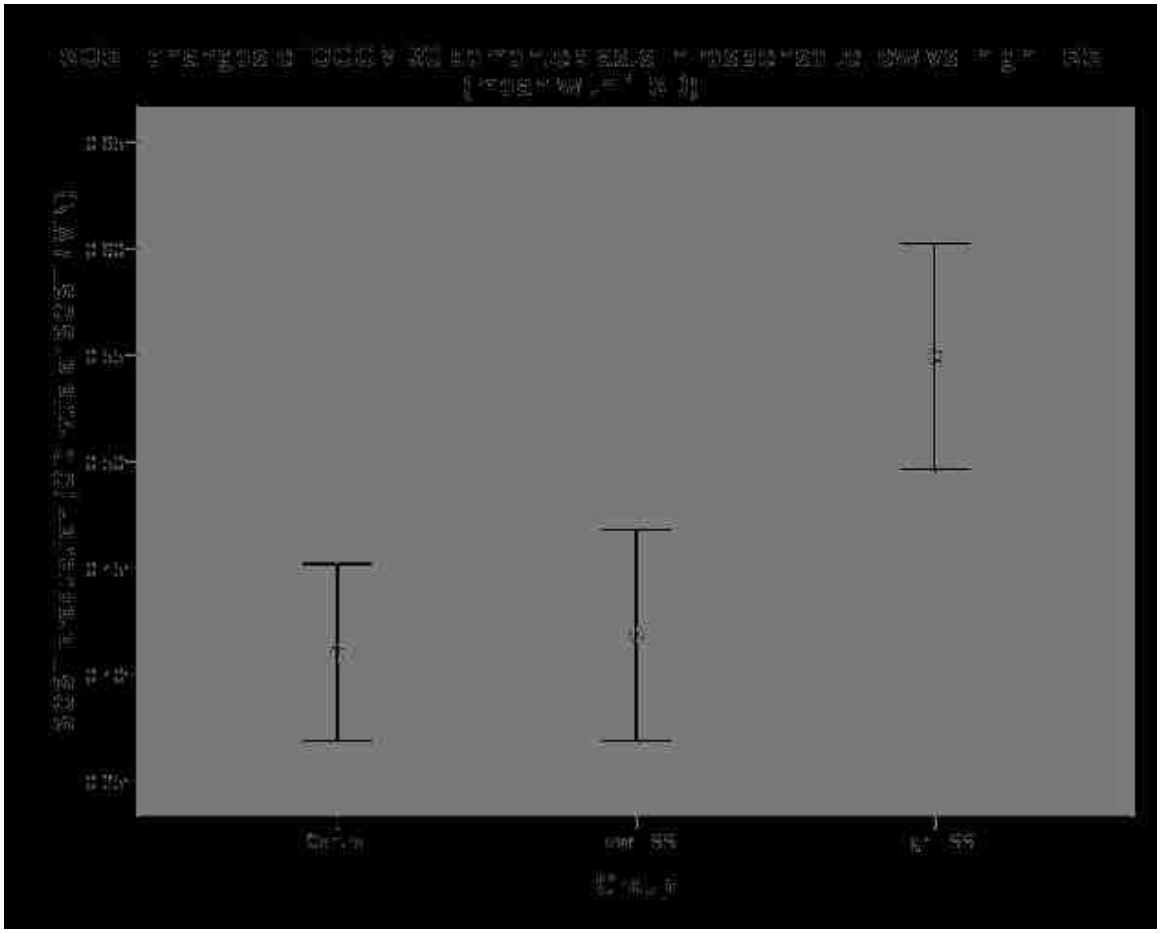

Figure 3-5: Graph showing comparison of SOST protein production following application of FSS for 1hour. No significant difference was found between groups.

SOST - ANOVA

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	.084	2	.042	2.587	.099
Within Groups	.340	21	.016		
Total	.424	23			

SOST – Tukey's Post hoc Comparisons

					95% Confidence Interval	
(I) Group	(J) Group	Mean Difference (I-J)	Std. Error	Sig.	Lower Bound	Upper Bound
Control	Low FSS	-.00792	.06365	.991	-.1683	.1525
	High FSS	-.13894	.06365	.098	-.2994	.0215
Low FSS	Control	.00792	.06365	.991	-.1525	.1683
	High FSS	-.13102	.07349	.200	-.3163	.0542
High FSS	Control	.13894	.06365	.098	-.0215	.2994
	Low FSS	.13102	.07349	.200	-.0542	.3163

Figure 3-6: Statistical analysis for SOST protein production. ANOVA and Post-hoc analysis performed by SPSS 17.0 software. Analysis shows there was no significant ($P<0.05$) difference found between control, Low FSS and High FSS groups.

CHAPTER 4

DISCUSSION

This project aimed to uncover the effects of different levels of mechanical stress on cementoblasts *in vitro* by examining the changes in various mineralized tissue biomarkers. A specific apparatus, designed and fabricated to apply a fluid shear stress to the OCCM-30 cementoblast cells, was used for mechanically loading the cells. Cells were subjected to two different levels of FSS and then examined for the changes in ATP release and protein production. Our results confirm that OCCM-30 cementoblasts do express proteins involved in bone remodeling and root resorption such as OPN, RANKL, COX-2 and SOST. All proteins under examination were positively identified by western blot analysis. This confirms other recent findings in the literature showing that cementoblasts are positive for these regulatory proteins (Dalla Bonna 2008; Huang 2009; Jager 2010; Liu In Press).

Our data show that OCCM-30 cementoblasts are responsive to FSS as evidenced by changes in both ATP release and protein production following application of one hour of FSS followed by one hour of post incubation. This is similar to the results from Liu et al that showed an increase in ATP release and protein production of OCCM-30 cells following application of FSS (In Press). Huang et al also confirmed that cementoblasts are mechanosensitive *in vitro* (2008). They subjected OCCM-30 cells to a compressive force (similar to that seen on the resorptive side of tooth movement) and found an increase in OPN, thereby promoting osteoclastogenesis. These studies support the idea that cementoblasts, similar to osteoblasts and osteocytes, play a role in bone modeling during orthodontic tooth movement as well as root resorption and repair.

The cellular changes following FSS can be categorized into early versus late responses. ATP release has been shown to occur rapidly within one minute after the onset of FSS (Liu 2008). Our results were similar with a significant increase in ATP release following one minute of FSS at both low and high levels. The importance of ATP in extracellular signaling has been demonstrated. Its regulatory action in bone remodeling is mediated through the P2 family of receptors expressed on target cells (Hoebertz 2002). Extracellular ATP released from osteoblasts or cementoblasts can inhibit OPG induction while upregulating RANKL and OPN consequently stimulating osteoclastogenesis (Hoebertz 2002; Buckley 2002; Wongkhantee 2008). In contrast, increased levels of ATP can also promote osteoblast survival and proliferation making the overall effect of ATP release difficult to define (Gallagher 2004). Being such an important molecule in both cell signaling and metabolism, ATP most likely has multiple roles in bone regulation. Our data may suggest the idea that ATP released from cementoblasts in response to FSS plays a role in cementoblast proliferation and possibly root repair.

While ATP is released as an early response to FSS, protein production in response to FSS is more delayed. Extracellular ATP, released from cementoblasts, acts as a signaling molecule to mediate the expression of downstream regulatory proteins. Of the four regulatory bone proteins examined, both OPN and RANKL changed significantly and were found to decrease in response to a low, physiological level of FSS (12 dynes/cm^2) for one hour. OPN is a protein involved in attachment of osteoclasts to bone to begin the resorptive process (Terai 1999). This finding may suggest that FSS at physiological levels

actually provokes a reparative or protective mechanism against root resorption by decreasing available OPN for osteoclast attachment. RANKL is needed for the differentiation, activation and survival of osteoclasts (Khosla 2001). A down regulation of RANKL is suggestive of a shift away from osteoclastogenesis and possibly toward bone apposition or root repair. These findings support the idea that the physiological level of FSS has an anabolic effect of bone remodeling. Tan et al. showed that osteocytes subjected to fluid flow actually inhibited osteoclast formation (2007). Similar studies subjecting MLO-Y4 osteocytes to FSS also found that bone resorption was inhibited and that the RANKL/OPG ratio was decreased (You 2008). Ultrasound, another form of mechanical stress, also has an anabolic effect on bone regulation and is known to accelerate fracture healing (Gallagher 2004). OCCM-30 cementoblasts subjected to ultrasound application showed an increase in cell proliferation, protein production of OPG and alkaline phosphase, a crucial enzyme in bone calcification (Dalla Bona 2006; 2008).

We did not see, in contrast, an increase in OPN or RANKL with a higher level of FSS application which would be suggestive of active resorption. This finding could probably be explained if our high level of mechanical loading (18.5 dynes/cm^2) was not high enough to simulate heavy force levels. Weinbaum showed that FSS is physiological between 8-30 dynes/cm^2 in long bones (1994). Although the actual level of physiological FSS in the PDL is not known at this time, if the level is proportional to the amount in long bones, the 18.5 dynes/cm^2 used in our study may not have exceeded the physiological range the cementoblasts are subjected to along the root surface. The physical

parameters of our FSS apparatus precluded us from increasing the level of FSS any further. The amount of force is proportional to the height of the column. In our current set-up, it was not possible to increase the column height any further and remain within the confines of the heated and aired box needed for cell survival. If FSS levels could be increased 10-fold or more, simulating heavier forces, an increase in catabolic biomarkers may be seen.

Our results did not show any significant changes in either COX-2 or SOST production following one hour of low or high levels of FSS. COX-2 has been identified previously in cementoblasts and may play an important role in cementoclastogenesis (Oka 2007). Our experimental conditions, however, did not elicit a significant response. The levels of FSS used in our study may not have been enough to stimulate COX-2 production as would occur in bone and root resorption. COX-2 production was evident in all groups, but it remained unchanged. This explanation may apply to the results seen for SOST protein levels as well. We did confirm the expression of SOST protein in cementoblasts as shown by Jager et al (2010), but we were unable to elicit a change in protein levels following FSS application. There was a slight increase noted for the high level FSS group, but it was not significant. This may suggest that under higher levels of FSS, bone formation is inhibited. More studies are needed to explore this possibility.

Limitations

The focus of this study was to characterize the cellular response of cementoblasts to FSS to provide further understanding into their role in orthodontic root resorption.

While cellular studies are essential to understand the molecular processes that occur, they must be balanced with tissue, whole animal and clinical studies to provide the whole picture. In cell culture, cells exist in isolation. This is an inherent limitation to all *in vitro* studies. Without the complex environment that occurs *in vivo*, the intricate signaling mechanisms and pathways that contribute to cellular differentiation and molecular regulation cannot be completed fully. Another limitation to our study was the lack of a working OPG antibody to measure changes in OPG production. At present, an OPG antibody for western analysis has not produced a successful and reliable signal in our lab. Adding OPG to the proteins studied would allow us to compare not only RANKL individually, but also the RANKL/OPG ratio, which is a common method to show in which direction the balance between resorption and apposition may shift (Low 2005; Tyrovolo 2008; Yamaguchi 2009; George and Evans 2009). In addition, our study was limited by the physical constraints of our FSS apparatus as explained earlier. Our current system used the tallest column possible to create the highest force levels available but we were still within the defined FSS range of 8-30 dynes/cm^2 as determined for long bones (Weinbaum 1994). FSS levels of 18.5 dynes/cm^2 may not be high enough to emulate heavy force levels seen clinically. Results of the current investigation to determine the physiological level of FSS in the PDL space will provide insight into appropriate FSS levels for future studies and direct the design of a new apparatus if needed.

Future Studies

To complement this study, several future experiments are planned. RNA collected from this experiment will be used to analyze gene expression using real-time polymerase chain reaction (PCR). This will provide more information on the early response of cementoblasts to FSS when compared to protein production. Also repeating the experiment with an increased post-incubation time (for instance 6 hours) may allow more time for capturing delayed signaling pathways and protein production. During this experiment, MLO-Y4 osteocytes were also subjected to FSS according to the same protocol. Results were not analyzed as they were outside of the scope and resources of this study. Comparison, however, between OCCM-30 cementoblasts and MLO-Y4 osteocytes, will provide novel insight into how these cells regulate bone and root resorption in response to FSS. Lastly, to further test the role of cementoblasts, subjected to FSS, in root resorption, the collected flow media from the current study will be added to incubate RAW 264.7 pre-osteoclastic cells to determine the possible ultimate regulation of cementoblasts on osteoclastic cell formation and activity.

CHAPTER 5

CONCLUSIONS

Our results confirm that OCCM-30 cementoblast cells do express regulatory bone marker proteins such as OPN, RANKL, COX-2 and SOST and that these cells are indeed responsive to FSS as evidenced by the changes in both ATP release and protein production. Results showed that at lower levels, FSS may have an anabolic effect on cementoblast cells by decreasing both OPN and RANKL protein production. These results directly support the first part of our hypothesis which stated that an equivalent physiological level of FSS applied to OCCM-30 cementoblast cells will produce anabolic responses. In contrast, our results did not support the second part of our hypothesis which proposed that higher levels of FSS may produce catabolic responses, promoting osteoclastogenesis. In summary, our results suggest that cementoblasts play a role in the modeling of cementum following orthodontic tooth movement and may actively participate in the root resorption and repair process.

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