

# The Effect of Extracorporeal Shock Wave (ESW) on Cementoblasts In Vitro

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THE EFFECT OF EXTRACORPOREAL SHOCK WAVE (ESW)  
ON CEMENTOBLASTS *IN VITRO*

by

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**ABSTRACT**  
THE EFFECT OF EXTRACORPOREAL SHOCK WAVE (ESW)  
ON CEMENTOBLASTS *IN VITRO*

Joshua Barta, DDS

Marquette University, 2012

Root resorption is an adverse consequence of orthodontic treatment, and currently no effective treatment method exists. Extracorporeal shock wave (ESW) has been applied to enhance angiogenesis, growth and healing of bone, but the effects of ESW on root resorption have not been studied. Cementoblasts are the cells responsible for forming and repairing cementum covering the dental root, and ESW may enhance this reparative process. The purpose of this study was to investigate the effects of ESW on cementoblasts to determine if ESW could potentially be used to treat root resorption. OCCM.30 cementoblasts were prepared in suspension at a density of  $10^7$ /ml and placed in Eppendorf tubes which were held in a specially designed apparatus to focus the shock waves at the cells. Using the focused shock wave stimulator (Storz Medical AG, Switzerland), the cells were subjected to a single dose of 2000 impulses of ESW at their assigned energy level ( $0.1\text{mJ}/\text{mm}^2$ ,  $0.25\text{mJ}/\text{mm}^2$  or  $0.5\text{mJ}/\text{mm}^2$ ). Controls were set under identical conditions without ESW application. Immediately after ESW stimulation, the amount of ATP release was measured since ATP is an early messenger in bone modeling regulation. Cell viability was tested to determine if any dose level caused cell death. After 24 hours of post stimulation incubation, the cells were lysed to test functional protein productions of sclerostin (SOST), a negative regulator of bone formation, receptor activator NF $\kappa$ B ligand (RANKL), a direct stimulator of bone resorption, and osteopontin (OPN), a regulator of osteoclastogenesis. After ESW application, ATP levels in the medium and high-dose groups were found to be significantly increased in a dose-dependent manner. High-dose ESW significantly decreased cell viability. SOST protein was significantly decreased only at the dose level of  $0.25\text{mJ}/\text{mm}^2$  ( $n=3$ ,  $p<0.05$ ). OPN was significantly increased at  $0.1\text{mJ}/\text{mm}^2$  and  $0.5\text{mJ}/\text{mm}^2$  ( $n=3$ ,  $p<0.01$ ), but not at the  $0.25\text{mJ}/\text{mm}^2$  energy level. RANKL was not significantly changed with any of the doses. Our data suggest that ESW at an energy level of  $0.25\text{mJ}/\text{mm}^2$  anabolically modulates bone remodeling by decreasing SOST production but not affecting RANKL and OPN production significantly. These findings suggest that ESW could potentially be used to treat root resorption in orthodontics.

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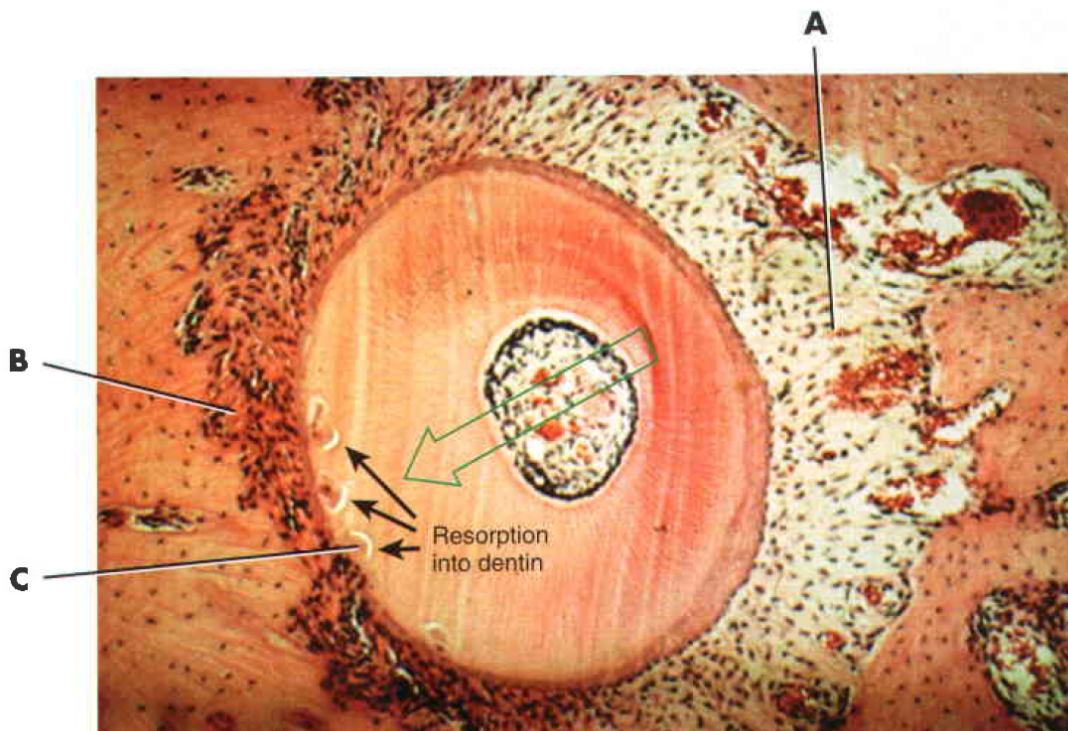
**CHAPTER I**  
**INTRODUCTION**

Root resorption is the adverse loss of cementum and dentin from the root surface of the tooth. Patients undergoing comprehensive orthodontic therapy experience increased incidence and severity of root resorption. This type of root resorption is termed orthodontically induced inflammatory root resorption (OIIRR) and may occur in as many as 90% of orthodontically treated teeth (Weltman et al., 2010). While it is unusual for minor root resorption to create serious clinical problems, severe root resorption can result in decreased periodontal support and reduced crown-to-root ratio. Additionally, root resorption increases the liability of the orthodontist to malpractice claims (El-Bialy et al., 2004; Mizrahi, 2010; Franklin, 2002).

Orthodontic tooth movement occurs as a result of bone resorption and deposition in the compressed and stretched side of the periodontal ligament (PDL), respectively (Krishnan and Davidovitch, 2006). When the PDL is compressed in response to the application of orthodontic force, osteoclasts are activated. The osteoclasts resorb alveolar bone adjacent to the root of the tooth to make tooth movement possible, but these cells also attack cementum as the PDL is remodeled (Brudvik and Rygh, 1995) (Figure 1-1). While cementum is more resistant to resorption than bone, root repair happens regularly during orthodontic tooth movement. Permanent root structure loss occurs when the resorbed cementum and underlying dentin is not fully repaired (Proffit et al., 2007). With increased duration of orthodontic treatment, the potential for loss of root length becomes greater (Weltman et al., 2010; Taithongchai et al., 1996; Killiany, 1999).

Currently, there are no effective treatment methods for root resorption and no high-level evidence has been presented to support any form of treatment (Ahangari et al.,





**Figure 1-1:** Diagram showing the histological cross section of a premolar being moved in the direction of the arrow. A) The PDL is stretched away from the direction of the force. B) The PDL is compressed in the direction of the force and areas of bone resorption can be seen. C) Resorption occurring into the cementum and dentin of the dental root (Proffit et al., 2007)

2010). The purpose of this study was to investigate the effects of extracorporeal shock wave (ESW) on cementoblasts to determine if ESW could potentially be used to treat root resorption. Cementoblasts are the primary cells responsible for the repair of root resorption, and if the cementoblast rate of repair could be increased through ESW stimulation, root resorption could be prevented or reduced accordingly. Cementoblasts were cultured and subjected to three different energy levels of ESW. The changes in the production of several metabolic bone markers were measured after the application of ESW. The results of this study will shed light on the effects of ESW on root resorption.

**CHAPTER II**  
**LITERATURE REVIEW**

### *Root Resorption*

One of the most common and unpredictable complications found during orthodontic treatment is root resorption. Thus, it is important to advise patients of the risks before starting treatment. External root resorption is thought to be caused by a wide range of mechanical and chemical stimuli such as infection, pressure, trauma and orthodontic tooth movement (Ahangari et al., 2010). The amount of root resorption experienced by an individual is also influenced by biological variability and genetic predisposition. External apical root resorption can happen with or without orthodontic treatment, and root resorption is seen in approximately 7 to 13% of individuals who have not received orthodontic treatment (Hartsfield, 2009).

The occurrence of root resorption experienced during orthodontic treatment has been reported to be as high as 90% from histological studies and approximately 73% when radiographic techniques are used for diagnosis (Weltman et al., 2010). Usually, the amount of lost root structure is clinically insignificant. Severe root resorption, which is often defined as the loss of greater than one fourth of the root length, has been reported to range from 1% to 5% of teeth measured (Weltman et al., 2010; Lupi et al., 1996; Killiany, 1999). Severe loss of root structure can decrease the usefulness of these teeth as abutments in the future. Periodontitis can also progress more rapidly in a patient who experiences severe root resorption because 3 mm of lost root length is equivalent to 1 mm of crestal bone loss (Kalkwarf et al., 1986). However, loss of a tooth from root shortening has rarely been reported. Additionally, since the loss of tooth structure is normally towards the narrower apex of the tooth, the loss of 5 mm of root structure still leaves three fourths of the tooth's periodontal attachment intact (Killiany, 1999; Taitongchai et al., 1996).

A positive correlation has been established between root resorption and mechanical loading applied during orthodontic tooth movement (Brezniak and Wasserstein, 1993; Baumrind et al., 1996). While it is accepted that orthodontic treatment does cause root resorption, the exact aspects of treatment that cause root resorption remain unclear. There is no evidence that root resorption is affected by mechanical treatment variables such as archwire sequencing, slot size, elastic use, bracket prescription, or self-ligation (Hartsfield, 2009). Extractions were associated with greater amounts of root resorption, but this was not consistently found in all studies (Jung and Cho, 2011; Sharpe et al., 1987; Krishnan and Davidovitch, 2006; Sameshima and Sinclair 2001b). Presumably, the increased retraction of incisors in premolar extraction cases produced more root resorption. Some studies showed a correlation between increased overbite or overjet with root resorption, but there was a lack of consensus in this regard. When reviewing the published root resorption studies, it was not uncommon to find conflicting results. Most of the research conducted on root resorption has been clinical studies, case reports, or animal studies with few randomized clinical trials.

The teeth exhibiting the greatest amount of root resorption are the maxillary incisors followed by the mandibular incisors (Jung and Cho, 2011; Weltman et al., 2010; Taithongchai et al., 1996; Sameshima and Sinclair, 2001a; Krishnan and Davidovitch, 2006). It is often suggested that teeth with abnormal root shape, such as pointed or dilacerated roots, show increased likelihood of root resorption (Semshima and Sinclair, 2001a; Brin et al., 1993; Mirabella and Artun, 1995; Linge and Linge, 1991). However, a recent systematic review concluded that teeth with unusual root morphology before treatment were only

slightly more likely to have moderate or severe root resorption than those with normal root forms and the difference was not statistically significant (Weltman et al., 2010).

The most common conclusion from studies looking at root resorption was a positive correlation with the duration of treatment and the amount of root resorption (Sameshima and Sinclair, 2001b; Weltman et al., 2010; Taithongchai et al., 1996; Killiany, 1999, Jung and Cho, 2011). Unfortunately, no pre-treatment factors have shown a high correlation to the development of root resorption during treatment. Currently, the most accepted predictive factor of severe resorption is the occurrence of mild resorption early in orthodontic treatment (Artun et al., 2005). For this reason, all patients must be informed of the possibility of root resorption before treatment, and progress panoramic radiographs are recommended throughout treatment (Figure 1-2).



**Figure 1-2:** Root resorption from orthodontic treatment. Panoramic radiograph showing severe root resorption of the maxillary incisor roots during orthodontic treatment.

### *Root Resorptive Process and Repair*

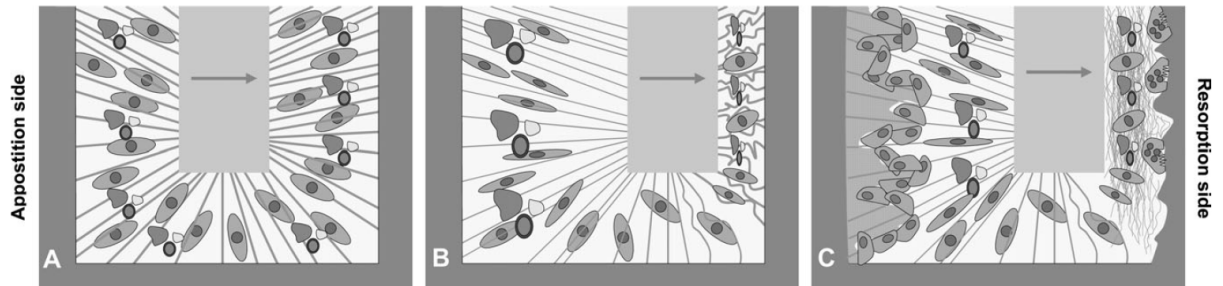
Orthodontic tooth movement is traditionally described using the pressure-tension theory which states that when a force is placed on a tooth, the tooth moves within the PDL space creating a compression side in the direction of the force and a tension side away from the force. The theory suggests that the change in blood flow and subsequent release of chemical messengers induces progenitor cells within the PDL to differentiate into compression-associated osteoclasts and tension-associated osteoblasts, causing bone resorption and apposition, respectively (Masella and Meister, 2006). This hypothesis has been challenged recently, however, because it contradicts current much of orthopedic literature which has shown that mechanical compression stimulates bone formation and tension stimulates resorption (Melsen, 1999). To align the orthodontic and orthopedic theories, it has been hypothesized that on the pressure side, the PDL fibers are unloaded leading to unloading of the alveolar bone which results in resorption. On the tension side, the PDL fibers are stretched, which causes active loading of the alveolar bone leading to apposition (Melsen, 2001; Henneman et al., 2008) (Figure 1-3).

The level of force used to compress the tooth determines the type of resorption that is observed. Light forces will cause direct resorption as described above, while “indirect resorption,” or undermining resorption, is seen when excessive force is applied. Under sustained, heavy force, the blood vessels are totally occluded and the blood supply is cut off to an area of the PDL. Instead of cells being recruited to the area, a sterile necrosis ensues as cell death occurs. Because of its histological appearance after the cells die, the avascular area in the PDL is termed hyalinized. After several days, cells invade the hyalinized area and begin to resorb the underside of the bone immediately adjacent to the necrotic PDL (Proffit

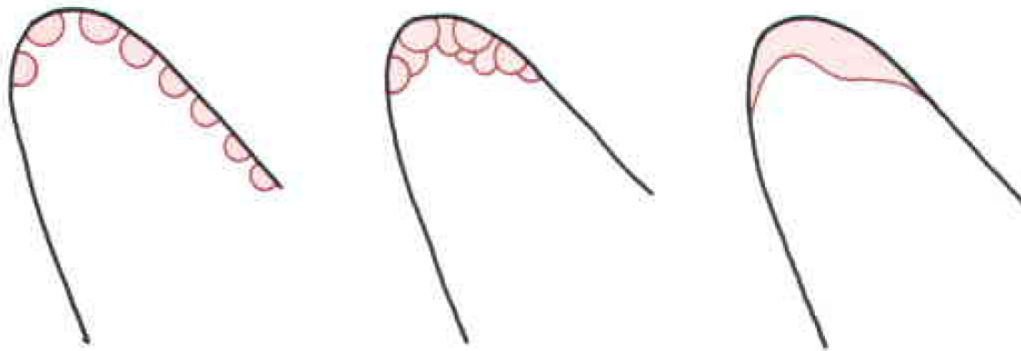
et al., 2007). During orthodontic tooth movement, it is advised to avoid creating areas of undermining resorption as much as possible to allow for more efficient tooth movement and reduce the pain experienced by the patient during treatment.

When high orthodontic forces induce the formation of a hyalinized zone, odontoclasts are recruited to the tooth surface for the removal of necrotic tissue. These cells have almost identical morphologies to osteoclasts, but are generally smaller in size and form smaller resorption lacunae. The odontoclasts, which are sometimes called cementoclasts, mediate root resorption in a process that appears to be quite similar to the cellular mechanisms of osteoclastic bone resorption (Tyrovola et al., 2008). When cementum is resorbed, the dentin is exposed, allowing the multinucleated odontoclasts to degrade the root surface (Reitan, 1974). The odontoclasts create small "inlets," called resorption lacunae, in the root surface, and these cavities can coalesce at the root apex, causing shortening of the root (Figure 1-4). Normally, however, these defects are repaired by cementoblasts.

Cementoblasts are a group of cells lining the dental roots that deposit the organic matrix of cementum onto the root surface, eventually embedding in the mineralized cementum and becoming cementocytes. The cementocytes in the cellular cementum of the dental root maintain the ability for repair after the resorption of root dentin or cementum. As a protective function, the cementoblasts are programmed to maintain a smooth surface of the root (Avery, 2000). When orthodontic forces are applied, cementum is sometimes removed from the root surface by odontoclasts and then restored by cementoblasts in a



**Figure 1-3:** Diagram showing the displacement of the tooth during orthodontic tooth movement. A) The PDL fibers are at equilibrium when no force is applied. B) In the direction of the applied force, the PDL fibers are compressed, unloading the bone. Away from the force, the PDL fibers are stretched causing loading of the bone. C) When the PDL fibers are compressed, the bone is unloaded and resorption takes place in the direction of the force. When the PDL fibers are stretched, the bone is loaded and bone apposition takes place in the opposite direction of the force (Henneman et al., 2008).



**Figure 1-4:** Diagram of resorption lacunae at the root apex. When odontoclasts resorb dentin, resorption lacunae are created. When multiple resorption lacunae are formed at the root apex, the cavities can coalesce and root shortening takes place (Proffit et al., 1997).

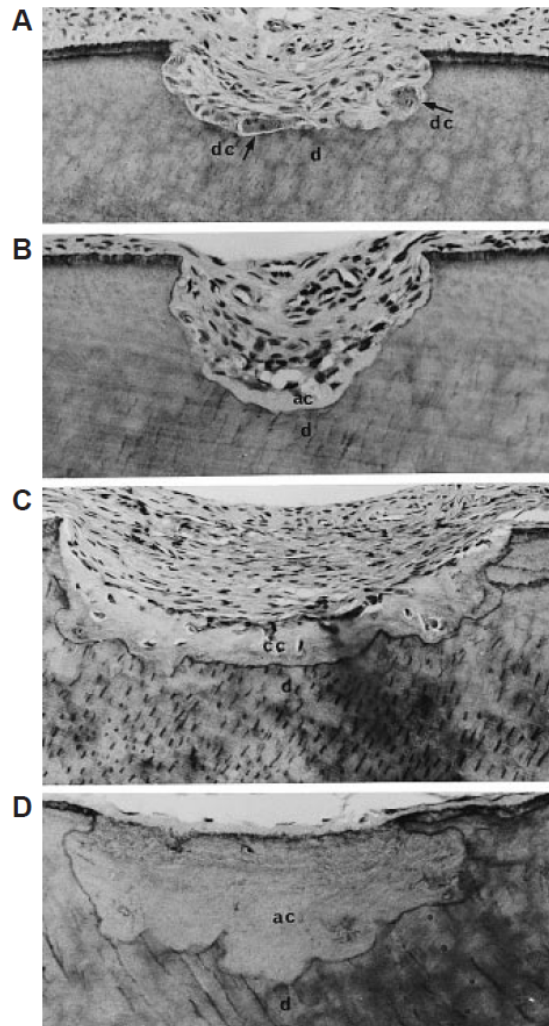


similar process to the way that the alveolar bone is remodeled. Root repair is constantly taking place during orthodontic tooth movement, and permanent loss of root structure only occurs if the lost tooth structure is not fully repaired (Proffit et al., 2007).

When active forces are discontinued or reduced below a certain level, the reparative process commences in the bottom of the resorption cavities created by odontoclasts (Owman-Moll and Kurol, 1998; Brudvick and Rygh, 1995) (Figure 1-5). The process has been shown to start as soon as a week into retention and increase over time (Owman-Moll and Kurol, 1998; Timms and Moss, 1971; Barber and Sims, 1981). Ownman-Moll and Kurol showed that after 2 weeks, 38 percent of root resorptions showed some healing, and this increased to 82 percent after 6 to 7 weeks (1998). After 8 weeks, the repair process appeared to reach a baseline level. Individual variations in healing potential were shown to be large, but Henry and Weinmann found that 72 % of resorptive areas were shown to exhibit full repair in adults (1951).

#### *Molecular Regulation of Root Resorption*

Many layers of networked reactions occur in and around the PDL and alveolar bone cells to change mechanical force into the molecular events making orthodontic tooth movement possible. Fibroblasts, osteoblasts, osteocytes and osteoclasts are part of the complex regulatory network that induces PDL and bone remodeling. Since we have already established that root remodeling takes place during orthodontic tooth movement, odontoclasts, cementoblasts and cementocytes can also be considered to be involved in this cascade of molecular events. Cementum and bone are very similar hard tissues, and



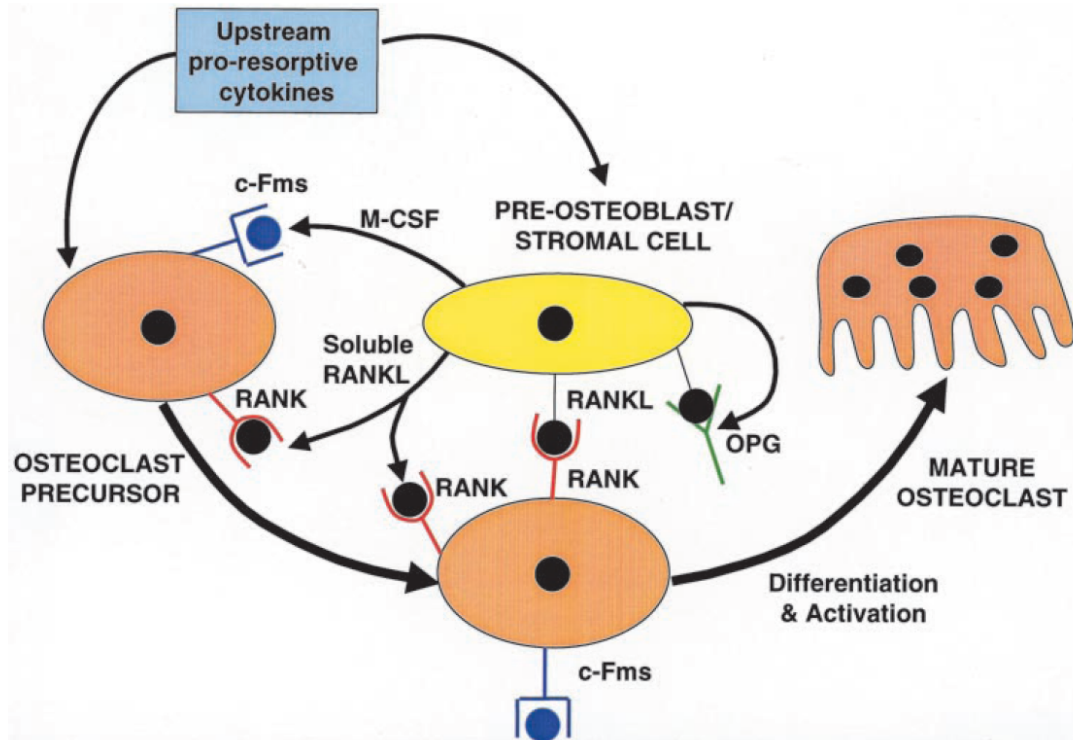
**Figure 1-5:** Diagram showing the histology of varying degrees of repair after root resorption. A) Resorption lacuna with odontoclasts at the bottom of the cavity (Odontoclasts are marked “dc” for dentinoclasts). B) Partial repair of a resorption lacuna by acellular cementum. C) Partial repair of a resorption lacuna by cellular cementum. D) Full anatomic repair of a resorption lacuna by acellular cementum (Owman-Koll and Kurol, 1998).

osteocytes and cementocytes share many morphological biological characteristics.

However, it is still unclear if cementocytes function in the homeostasis of cementum similar to the way osteocytes do in bone. Like osteoblasts, cementoblasts have been shown to express various bone regulatory proteins such as osteopontin (OPN), receptor activator of

NF $\kappa$ B ligand (RANKL), and sclerostin (SOST) (Dalla-Bona et al., 2008; Huang et al., 2009; Jäger et al., 2010).

In bone biology, the OPG/RANKL/RANK system has been established as the method by which osteoblasts modulate osteoclastogenesis (Khosla, 2001; Masella and Meister, 2006). The biological effects of RANKL are produced when it binds to receptor activator of nuclear kappa beta (RANK). The biological effects of osteoprotegerin (OPG) are opposite to the effects of RANKL, because OPG acts as a soluble receptor antagonist which neutralizes RANKL and therefore prevents RANKL-RANK interaction (Tyrovola et al., 2008). Osteoblasts and stromal stem cells (pre-osteoblasts) express RANKL, which binds to RANK on the surface of osteoclast precursor cells and promotes the differentiation, activation and survival of osteoclasts (Hartsfield, 2009). OPG is also secreted by osteoblasts and osteogenic stromal stem cells and it binds to RANKL preventing it from interacting with RANK and thus, decreases bone resorption by blocking the activation of osteoclasts (Figure 1-6). Recently, the OPG/RANKL/RANK system has been applied to explain orthodontic tooth movement. These same proteins have been found to be expressed in the cells of the PDL and participate in the bone modeling making orthodontic tooth movement possible (Ogasawara et al., 2004; Low et al., 2005; Yamaguchi et al., 2006). It is now known that RANKL is expressed in PDL fibroblasts and osteoblasts on the compressed side of the PDL, playing a critical role in the differentiation of osteoclasts in response to mechanical stress (Tyrovola et al., 2008). As expected, the synthesis of OPG is increased on the tensile side during orthodontic tooth movement. It has therefore been concluded that the relative



**Figure 1-6:** Diagram showing the pre-osteoblast/stromal cell regulation of osteoclastogenesis. The pre-osteoblast/stromal cells release RANKL which binds to RANK receptors on the osteoclast precursors leading to the differentiation and activation of mature osteoclasts. OPG is a decoy receptor which blocks the ability of RANKL to bind to RANK, thus preventing the activation of osteoclasts (Khosla, 2001).

expression of OPG and RANKL on the tension and compression sides of the tooth regulates bone remodeling during orthodontic tooth movement.

The coordination of the OPG/RANKL/RANK system seems to contribute not only to alveolar remodeling, but also to resorption during orthodontic tooth movement and physiological root resorption (Tyrovola et al., 2008). It has also been proposed that PDL cells, in cases of severe external apical root resorption, may produce a larger amount of RANKL and up-regulate osteoclastogenesis (Sasaki, 2003). In fact, an increase in RANKL was

seen in samples of gingival crevicular fluid from orthodontic patients that exhibited root resorption (George and Evans, 2009). Therefore, the RANKL to OPG ratio in PDL cells may contribute to root resorption during orthodontic tooth movement (Tyrovola et al., 2008; Al-Qawasmi et al., 2003; Krishnan and Davidovitch, 2006).

Sclerostin, the protein product of the SOST gene, is a cysteine knot-secreted glycoprotein that is a potent inhibitor of bone formation. Loss of the SOST gene in humans causes the high bone mass disorders Van Buchem's disease and sclerosteosis. Modulation of sclerostin levels may be one of the mechanisms by which osteocytes regulate local osteogenesis in response to increased mechanical stimulation (Robling et al., 2008). This is supported by the finding that transgenic mice with over-expression of SOST exhibit low bone mass (Loots et al., 2005). Sclerostin has been found to decrease bone formation by reducing osteoblasts numbers through apoptosis (Masella and Meister, 2006). Originally thought to only be expressed in osteocytes, SOST was recently shown to be expressed in cementocytes (Jäger et al., 2010). This suggests that sclerostin may play a role in orthodontically induced bone modeling and root resorption.

Another molecule involved in osteoclastogenesis that may be linked to root resorption is osteopontin (OPN). OPN is expressed most often by osteoblasts and bone-lining cells on the pressure side of the tooth suggesting that OPN participates in bone resorption. OPN is thought to promote and regulate the adhesion and attachment of osteoclasts to the bone surface during bone resorption (Terai et al., 1999). OPN shows chemotactic activity for osteoclasts and the precursors of osteoclasts, triggering bone remodeling in response to mechanical stress. Since odontoclasts share common morphological and functional characteristics with osteoclasts, OPN may play a role in the

regulation of odontoclast function, and subsequently, root resorption. Two studies with OPN knockout mice have shown that these mice experienced less root resorption due to both a decrease in the number of osteoclasts in the alveolar bone and a decrease in odontoclasts (Fujihara et al., 2006; Chung et al., 2007). One can therefore hypothesize that an increase in OPN would increase the amount of osteoclastogenesis and root resorption seen in a patient.

Adenosine triphosphate (ATP) has been recognized as an important and ubiquitous intracellular and extracellular messenger in various kinds of tissues. Mechanical stress has been shown to induce an increase in ATP release in several cell types (Wongkhantee et al., 2008). ATP is generally seen as an early messenger to modulate the cellular response to mechanical load. The actions of ATP inside the cell are mediated by cAMP, while extracellular ATP binds to P2 purinoceptors on target cells (Hoebertz et al., 2002). As a mediator for activating several signaling pathways, ATP is believed to be one of the regulators of bone homeostasis. ATP has an inhibitory effect on OPG expression and recently, ATP was shown to induce the expression of RANKL in PDL cells (Luckprom et al., 2010). Therefore, ATP could play a role in the regulation of the OPG/RANKL/RANK pathway. Additionally, research by Wongkhantee et al. suggested the ATP may up-regulate OPN expression (2008). This data suggests that ATP plays an important role in bone remodeling and it likely plays a similar role in both orthodontic tooth movement and root resorption.

#### *Treatment of Root Resorption*

The orthodontist must be aware of root resorption, because there are both clinical and legal implications associated with its occurrence. Any evidence of the development of

root resorption during treatment needs to be communicated to the patient. Progress panoramic x-rays should be taken to monitor these patients, and adjustments may need to be made to treatment including using lighter forces, shortening treatment time and reducing treatment goals.

Several ways have been suggested to slow the rate of orthodontic root resorption including the application of drugs, hormones, and growth factors (Krishnan and Davidovitch, 2006). Topical administration of a bisphosphonate was shown to significantly inhibit root resorption in rats, but bisphosphonates inhibit bone resorption and would affect orthodontic tooth movement (Igarashi et al., 1994). Unfortunately, treatment options are generally case-dependent and there is no high level of evidence to support any treatment modality (Ahangari et al., 2010). To date, no randomized controlled trials have been completed that have looked at the effectiveness of different interventions for the management of external root resorption. Weltman et al. suggested that there is some evidence to support a 2 to 3 month treatment pause to decrease further root resorption (2010). Sameshima and Sinclair also found evidence to recommend an inactive phase of 4 to 6 months before the resumption of treatment if root resorption is noted (2001b).

One promising treatment method discussed in the literature is the use of low-intensity pulsed ultrasound (LIPUS) to enhance the repair of root resorption. LIPUS consists of mechanical energy that is transmitted transcutaneously by high frequency acoustic pressure waves. Research has shown that LIPUS can enhance healing of various types of traumatized connective tissues and accelerate bone fracture healing (Heckman et al., 1997; Kristiansen et al., 1997; Mayret et al., 2000). The mechanism of this process is not well

understood, but it is believed to be mediated by micromechanical stimuli and increased local angiogenesis (Oyonarte et al., 2009).

Root resorption that occurs as a result of orthodontic treatment has all of the features of an inflammatory reaction, thus the term “orthodontically induced inflammatory root resorption.” When ultrasound was found to have an anti-inflammatory action, LIPUS was suggested as a possible method of reducing root resorption. In addition to possessing anti-inflammatory properties, LIPUS has also been proven to stimulate the production of growth factors, up-regulate bone proteins and enhance dental tissue formation (El-Bialy et al., 2004). Liu et al. suggested ultrasound might be useful to protect against root resorption when they found that ultrasound significantly up-regulated the expression of OPG and down-regulated RANKL expression (2011). Similarly, Dalla-Bona et al conducted another study using the same cementoblast cell line that was used in this study (OCCM.30 cells) and found that ultrasound induced an increase in cementoblastic OPG synthesis, while RANKL protein levels were unaffected (2008).

Ultrasound has been shown to increase the healing of root resorption in rats (Liu et al., 2012) and minimize root resorption in replanted teeth in rats (Rego et al., 2011). Furthermore, a clinical study conducted by El-Bialy et al. suggested that LIPUS minimized root resorption and accelerated healing by reparative cementum in humans during orthodontic treatment (2004). The results demonstrated a reduction of root resorption and acceleration in healing of already resorbed sites over a 4 week period of application. While these results were promising, it still is unclear whether ultrasound repairs root resorption or just prevents it by decreasing the osteoclastogenesis process.



### *Extracorporeal Shock Wave Therapy*

Extracorporeal shock wave (ESW) therapy and LIPUS are both forms of sound wave treatment, but ESW differs in that shock waves have lower frequency, minimal tissue absorption and no thermal effect (Li et al., 2010). Shock waves are single high-amplitude sound waves generated by electrohydraulic, electromagnetic, or piezoelectric methods that propagate in tissue with a sudden rise from ambient pressure to its maximum pressure at the wave front, followed by lower tensile amplitude (Gerdesmeyer et al., 2002). These waves propagate through water or soft tissue just as ultrasound does.

ESW was introduced into medical use over 20 years ago to be used for the disintegration of kidney stones. Later, ESW came into regular use as a minimally invasive method to break down salivary duct stones in the management of sialolithiasis (Capaccio et al., 2009) (Figure 1-7). The potential repair shown by ESW treatment has led to its application in musculoskeletal disorders such as plantar fasciitis, lateral epicondylitis, calcifying tendinitis and avascular necrosis of the femoral head (Zelle et al., 2010). Recent research has begun to shed light on how ESW stimulates bone healing. ESW produces maturation of human bone osteoblasts (Hofmann et al., 2008) and stimulates osteoblasts in cell cultures via increased release of alkaline phosphatase and osteocalcin (Martini et al., 2003). Angiogenesis is also stimulated by ESW, presumably in response to the increased expression of early angiogenesis-related growth factors, such as endothelial nitric oxide synthase (eNOS), vascular endothelial growth factor (VEGF), and proliferating cell nuclear



**Figure 1-7:** Diagram showing the procedures of ESW application for the treatment of sialolithiasis. Tooth guards and ear plugs are inserted on the side to be treated. Ultrasound jelly is applied to the side of the face. The focused ESW handpiece is placed over the affected area, and the prescribed impulses are applied (Capaccio et al., 2009).

antigen (PCNA) (Wang, 2003). ESW is believed to accelerate healing by modifying the local intracellular and extracellular biological environment and stimulating the in-growth of new blood vessels.

The routine use of ESW on patients seems to be safe and without serious risks. The side effects from treatment by ESW are dependent on the energy and impulses used, and it has been observed that shock waves at low levels create low side effects on the way through muscles, fat and connective tissue. If excessive shock wave energy is applied to bone, induction of trabecular and cortical fracture can occur (Da Costa Gómez et al., 2004). Local hematomas, petechial hemorrhage and local swelling have also been reported, but they disappeared within a few days without any complications (Shrivastava and Kailash, 2005). The only contradictions for treating a patient with ESW are pregnancy and the presence of a cardiac pacemaker (Capaccio et al., 2009). Another advantage of ESW treatment is low invasiveness since the patient avoids any surgical procedure.

In dentistry, ESW has begun to be explored for the regeneration of periodontal defects (Sathishkumar et al., 2008), a treatment for peri-implantitis (Li et al., 2010), and the acceleration of periodontal remodeling to shorten orthodontic treatment time (Hazan-Molina et al., 2011). LIPUS and ESW have been shown to elicit stimulatory effects on human periosteal cells in vitro (Tam et al., 2008). Both methods of transcutaneous mechanical stimulation also have been proven to modulate bone remodeling and promote healing (Zelle et al., 2010; Mayr et al., 2000). LIPUS is being considered as a possible form of treatment for root resorption, and similarly, ESW should also be studied to find out if the cementum responds in a similar manner to this form of mechanical stimulation.

Tamma et al. applied shock waves to murine osteoblasts and found a decreased ratio of RANKL/OPG production suggesting an inhibition of osteoclastogenesis (2009). The research conducted on ESW to date appears to suggest that the effects of ESW are produced by stimulating the differentiation of osteoblasts (Sathishkumar et al., 2008) and reducing osteoclastogenesis (Tamma et al., 2009). Assuming that cementoblasts on the root have the ability to regulate the activity of odontoclasts the way that osteoblasts regulate osteoclasts, one could hypothesize that ESW may reduce that amount of root resorption by reducing osteoclastogenesis and stimulating the anabolic process of osteoblasts.

### *Cementoblasts*

Cementum is the hard tissue that covers the entire surface of the dental root. A thin layer of cementum, approximately 20  $\mu\text{m}$  thick, covers the cervical half of the root, while the cementum becomes the thickest at the apex of the root approaching 200  $\mu\text{m}$ . Cementoblasts form cementum by incrementally depositing cementoid, a collagenous matrix, which becomes secondarily mineralized to form mature cementum. Once formed, the cementum seals the tubules of the root dentin and serves as an attachment for periodontal fibers to hold the tooth in the alveolus (Avery, 2000). Once the cementum surrounds the cementocytes, they reside in lacunae and communication via a canalicular network.

Although the composition of cementum resembles bone, there are distinct structural and functional differences between these two mineralized tissues. Cementum does not have the lamellar organization found in bone, is avascular, is non-innervated, does not contain bone marrow and does not undergo physiological remodeling (Jäger et al.,

2010). Cementum seems to be excluded from remodeling activities associated with maintenance of calcium homeostasis. Even though the cementum has a greater ability than bone to resist resorption, orthodontic force can sometimes cause resorption of root cementum, which may then proceed into the dentin (Krishnan and Davidovitch, 2006). When resorption occurs, repair cementum is deposited in the defects by cementoblasts.

In the past, the lack of availability of a cementoblast cell line has made it difficult to study these cells in culture. With the recent development of an immortalized murine cell line, OCCM.30, we were able to test the effects of ESW stimulation on these cementoblasts to see how they respond. Cementoblasts have already been shown to express molecules that are critical in bone remodeling including SOST, RANKL and OPN proteins (Dalla-Bona et al., 2008; Huang et al., 2009; Jäger et al., 2010). It has been shown that cementoblasts are sensitive to mechanical strain and ultrasound, and this study will determine if the cementoblasts respond to ESW stimulation (Huang et al., 2009, Dalla-Bona et al., 2008). This study will also evaluate any change in the expression of the previously mentioned proteins to determine if ESW could affect the remodeling of cementum by cementoblasts.

### *Hypothesis*

It has been shown that ESW can reduce osteoclastogenesis and stimulate the maturation of osteoblasts. As discussed previously, this is believed to be the mechanism by which ESW produces anabolic effects on bone. Additionally, bone metabolism is thought to be regulated in the same way that alveolar remodeling and root resorption are regulated. Therefore, our working hypothesis is that when ESW is applied to OCCM.30 cementoblasts,

it will produce an anabolic response evidenced by a decrease in the markers for bone resorption such as RANKL, OPN and a decrease in the bone formation inhibitor SOST.

**CHAPTER III**  
**MATERIALS AND METHODS**

### *Cell Culture*

An immortalized murine cementoblastic cell line of OCCM.30 cells were provided by Dr. MJ Somerman (University of Washington). The OCCM.30 cells were cultured in  $\alpha$ -minimal essential medium ( $\alpha$ -MEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin. Cells were cultured in T75 cell culture flasks maintained at 37°C with 5% CO<sub>2</sub> in a humidified incubator. Cells were routinely divided and passaged at confluence. Passages 10-20 were used for experimentation. Prior to ESW application, 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. The cementoblasts were then prepared in suspension at a density of 10<sup>7</sup>/ml. One ml of cell suspension was placed into 1.5 ml Eppendorf tubes for ESW experimentation. All cell culture supplies were purchased from Sigma (St. Louis, MO) unless otherwise noted.

### *Protocols*

The cells were divided into four groups including control, low, medium and high dose of ESW. Each group of cells received one episode of ESW treatment consisting of 2000 impulses at their assigned energy level. The three energy levels of ESW used for experimentation were low (0.1 mJ/mm<sup>2</sup>, 6.0 Hz), medium (0.25 mJ/mm<sup>2</sup>, 4.0 Hz), and high (0.50 mJ/mm<sup>2</sup>, 3.0 Hz). Immediately after ESW treatment, the cell suspension from each tube was divided into two portions; 40% (0.4ml out of 1 ml) of the cell suspension was used to test ATP release and cell viability, while the other 60% (0.6ml out of 1 ml) was further cultured for 24 hours to test functional protein productions.



### *Extracorporeal Shock Wave Application*

The ESW system used to apply the shock waves was the Extracorporeal Pulse Activation Treatment System (Duolith SD1<sup>®</sup>, Storz Medical AG, Postfach, Switzerland) (Figure 2-1). This system utilizes high-energy, focused, cylindrical-source, electromagnetic shock wave technology that is applied with a corded, Focused Shock Wave (F-SW) handpiece (Figure 2-2). It has a short pulse length and is concentrated on areas of a few millimeters in diameter. The F-SW handpiece used in this experiment was equipped with the stand-off device I, which is able to provide a therapeutically effective penetration depth up to 105 mm. Its focal zone is 30 mm in diameter and its depth of focal zone ranges from 15 to 45 mm. The effective distance from the surface of the handpiece to the center of the focal zone is approximately 30 mm. The F-SW handpiece was attached to the bottom of a specially designed holder (Figure 2-3). The top of this holder contained a slot that held a single Eppendorf tube so that the tube would be at the center point of the focal zone. The holder was then filled with water, which has been shown to be an ideal medium for transmission of shock waves (Shrivastava and Kailash, 2005). The water was filled to the level of the suspension in the tubes but not high enough to completely immerse the tubes. Each tube then received one administration of 2000 impulses at their assigned dosage during the entire experiment. The control group was placed in identical conditions without ESW stimulation.

### *Detection of ATP*

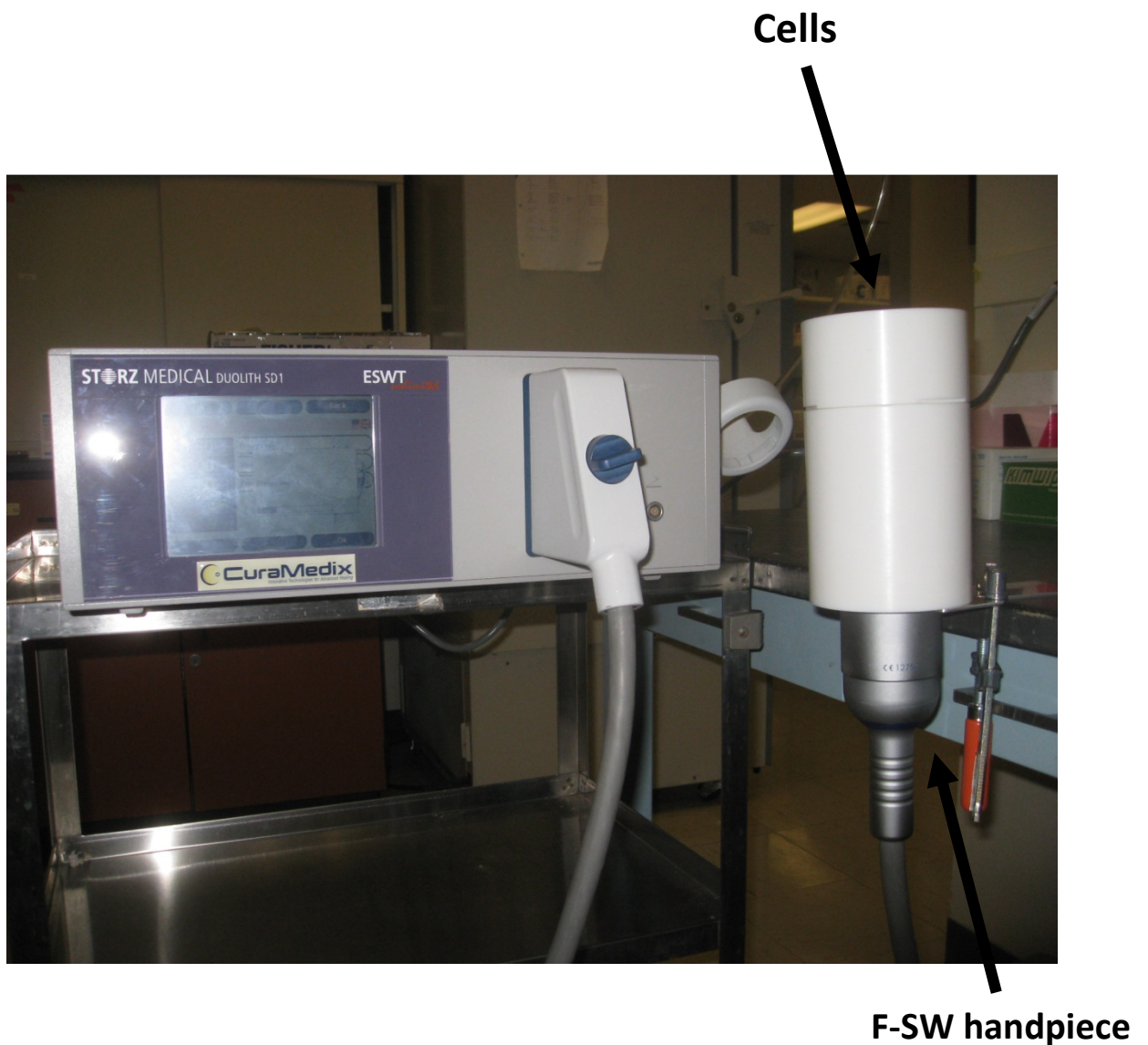
The 0.4 ml of cell suspension used for ATP testing was spun at 1000 rpm for 5 minutes to separate the cells and any cellular debris from the suspension. The supernatant



**Figure 2-1:** Storz Medical Duolith<sup>®</sup> SD1 shock wave therapy system. The model pictured is the table top version which was used during experimentation.



**Figure 2-2:** F-SW handpiece with stand-off device I for 30 mm depth of focus.



**Figure 2-3:** Picture of the Duolith<sup>®</sup> SD1 system used for experimentation. The F-SW handpiece was attached to the bottom of the specially designed holder. Eppendorf tubes with cell suspensions were placed into the top of the holder.

was removed from the tube and used to test the ATP released. 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 Luminometer detection system (Zylux Corp, Huntsville AL). Experimental samples were compared to  $\alpha$ -MEM containing 0.2% FBS as a control. Samples were run in duplicates.

#### *Cell Viability Assay*

In parallel with the ATP assay, the cell precipitate was lysed by adding 100  $\mu$ l of sample buffer. Previously we determined the linear relationship between viable cell numbers and the total protein of the cells. Total protein of the whole cell lysate was then quantified using the amido black method to determine cell viability.

#### *Protein Production*

Following 24 hours of post-ESW incubation, protein samples were centrifuged at 14,000 rpm for 10 min. Proteins were separated by gel electrophoresis by loading 50  $\mu$ g of whole cell lysate and 5  $\mu$ l pre-stained molecular weight marker (Bio-Rad Laboratories, Hercules, CA ) and running through a 10% sodium dodecyl sulfate polyacrylamide 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) and 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 documented 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 loading control).

#### *Statistical Analysis*

SPSS software (version 17.0) 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 test to determine where the significance lies between the different groups. Values were graphed as mean  $\pm$  standard deviation of the individual groups. Statistical significance was determined at  $p < 0.05$ .

**CHAPTER IV**

**RESULTS**

***Cell Viability***

ESW treatment did not affect cell viability at the low and medium doses. There was not a significant change in the total protein of the cells that were viable in the low and medium groups after the application of ESW. The high-dose group, however, showed a significant decrease in cell viability compared to the control (Figure 3-1, Table 3-1).

***ATP Release***

ESW induced ATP release from the cementoblasts. As the dose of ESW was increased, the release of ATP increased (Figure 3-2). The increase in ATP was significant in the medium and high-dose groups, but not in the low-dose group. The most significant increase in ATP release compared to the control group was observed in the high-dose group ( $p = 0.000$ ) (Table 3-2).

***Protein Production******OPN Production***

ESW increased OPN production from the cementoblasts with all of the applied doses (Figure 3-3). Compared to the control, OPN production was significantly increased at the low and high-dose groups. Although a small increase was seen in OPN production in the medium-dose group, the change was not significant (ANOVA,  $*p < 0.04$ ) (Table 3-3).

*RANKL Production*

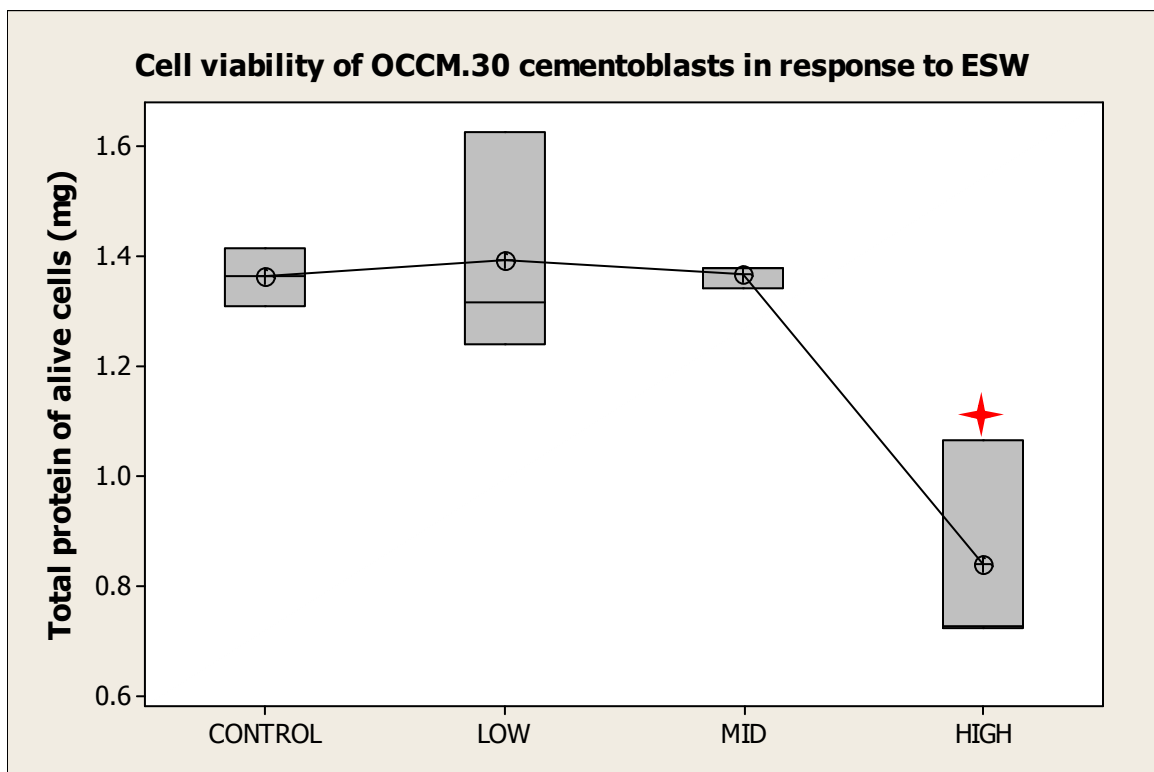
ESW increased the production of RANKL from the cementoblasts (Figure 3-4). The increase in RANKL, while much greater in the low and high-dose groups, was not found to be significant in any of the groups (Table 3-4). Statistically, the medium dose produced a nearly unaltered level of RANKL when compared to the control.

*SOST Production*

ESW decreased SOST production in all groups of cementoblasts (Figure 3-5). The medium dose significantly decreased SOST production compared to the control group, while the low and high doses did not (Table 3-5).



| SAMPLE#     | CONTROL           | LOW             | MID             | HIGH            |
|-------------|-------------------|-----------------|-----------------|-----------------|
| 1           | 1.416             | 1.317           | 1.379           | 0.726           |
| 2           | 1.311             | 1.626           | 1.343           | 0.722           |
| 3           | 1.364             | 1.239           | 1.377           | 1.067           |
| <b>Mean</b> | <b>1.36366667</b> | <b>1.394</b>    | <b>1.366333</b> | <b>0.838333</b> |
| <b>SD</b>   | <b>0.05250079</b> | <b>0.204668</b> | <b>0.020232</b> | <b>0.198041</b> |



**Figure 3-1:** Cell viability was unchanged after application of the low and medium doses of ESW. Significant cell death was seen with the high dose.

### Cell Viability – ANOVA

|                       | Sum of Squares | df | Mean Squares | F     | Sig.         |
|-----------------------|----------------|----|--------------|-------|--------------|
| <b>Between Groups</b> | 0.6489         | 3  | 0.2163       | 10.27 | <b>0.004</b> |
| <b>Within Groups</b>  | 0.1685         | 8  | 0.0211       |       |              |
| <b>Total</b>          | 0.8175         | 11 |              |       |              |

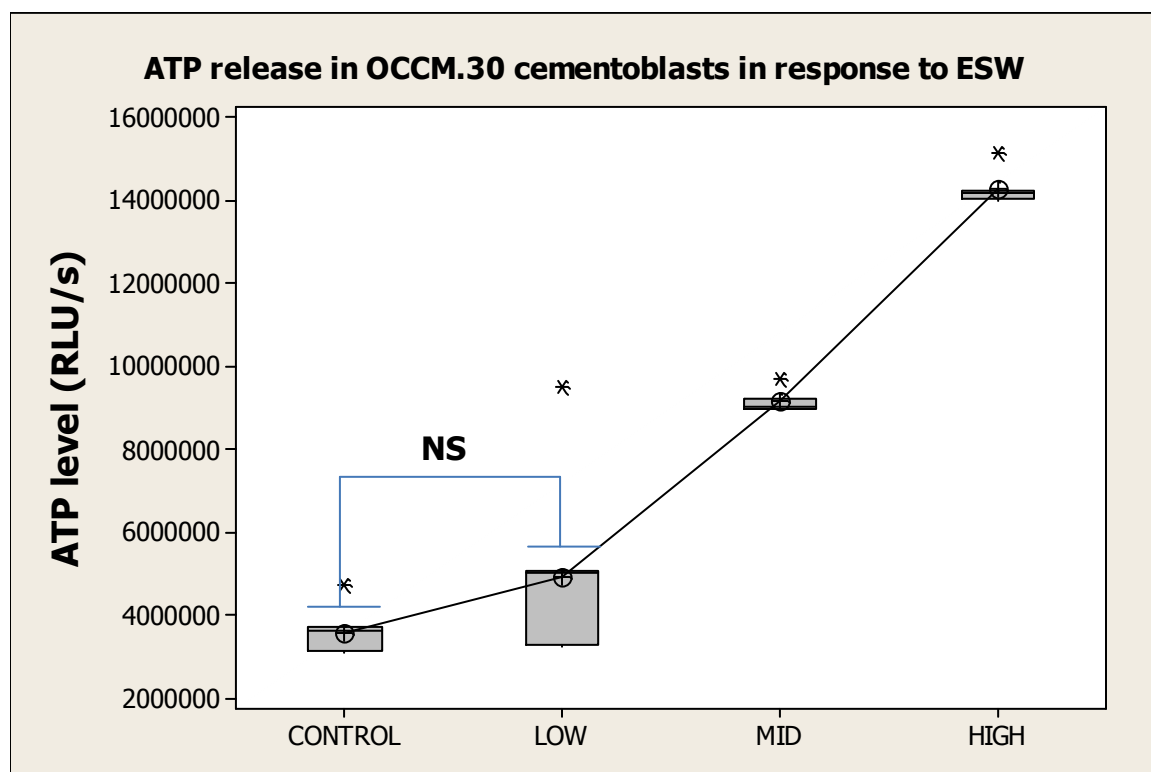
### Cell Viability – Post Hoc Comparisons

| (I) VAR00001 | (J) VAR00001 | Mean Difference (I-J) | Std. Error | Sig.        | 95% Confidence Interval |             |
|--------------|--------------|-----------------------|------------|-------------|-------------------------|-------------|
|              |              |                       |            |             | Lower Bound             | Upper Bound |
| Control      | LOW          | -.03033               | .11852     | .994        | -.4099                  | .3492       |
|              | MID          | -.00267               | .11852     | 1.000       | -.3822                  | .3769       |
|              | HIGH         | .52533*               | .11852     | <b>.009</b> | .1458                   | .9049       |
| LOW          | Control      | .03033                | .11852     | .994        | -.3492                  | .4099       |
|              | MID          | .02767                | .11852     | .995        | -.3519                  | .4072       |
|              | HIGH         | .55567*               | .11852     | <b>.007</b> | .1761                   | .9352       |
| MID          | Control      | .00267                | .11852     | 1.000       | -.3769                  | .3822       |
|              | LOW          | -.02767               | .11852     | .995        | -.4072                  | .3519       |
|              | HIGH         | .52800*               | .11852     | <b>.009</b> | .1485                   | .9075       |
| HIGH         | Control      | -.52533*              | .11852     | <b>.009</b> | -.9049                  | -.1458      |
|              | LOW          | -.55567*              | .11852     | <b>.007</b> | -.9352                  | -.1761      |
|              | MID          | -.52800*              | .11852     | <b>.009</b> | -.9075                  | -.1485      |

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

**Table 3-1:** Statistical analysis of cell viability. ANOVA and Post-hoc analysis performed by SPSS 17.0 software. Analysis shows that both the low and medium-dose groups have similar cell viability when compared to the control, while the high-dose group showed a significant decrease in cell viability.

| SAMPLE#     | CONTROL           | LOW             | MID             | HIGH            |
|-------------|-------------------|-----------------|-----------------|-----------------|
| 1           | 3723098           | 5066501         | 9094292         | 14223524        |
| 2           | 3623786           | 5043251         | 9252124         | 14030246        |
| 3           | 3703831           | 5037131         | 9716656         | 14091180        |
| 4           | 3127314           | 3321577         | 9060150         | 14178000        |
| 5           | 3221606           | 3265177         | 8995754         | 14207814        |
| 6           | 3178470           | 3355668         | 9021094         | 14264778        |
| <b>Mean</b> | <b>3429684.17</b> | <b>4181551</b>  | <b>9190012</b>  | <b>14165924</b> |
| <b>SD</b>   | <b>281693.622</b> | <b>950690.3</b> | <b>273315.8</b> | <b>88301.37</b> |



**Figure 3-2:** ATP release increased with ESW application. As the dose level increased, the release of ATP was increased.

## ATP – ANOVA

|                       | Sum of Squares | df | Mean Squares | F      | Sig.         |
|-----------------------|----------------|----|--------------|--------|--------------|
| <b>Between Groups</b> | 4.88488E+14    | 3  | 1.62829E+14  | 121.18 | <b>0.000</b> |
| <b>Within Groups</b>  | 3.22487E+13    | 24 | 1.34370E+12  |        |              |
| <b>Total</b>          | 5.20736E+14    | 27 |              |        |              |

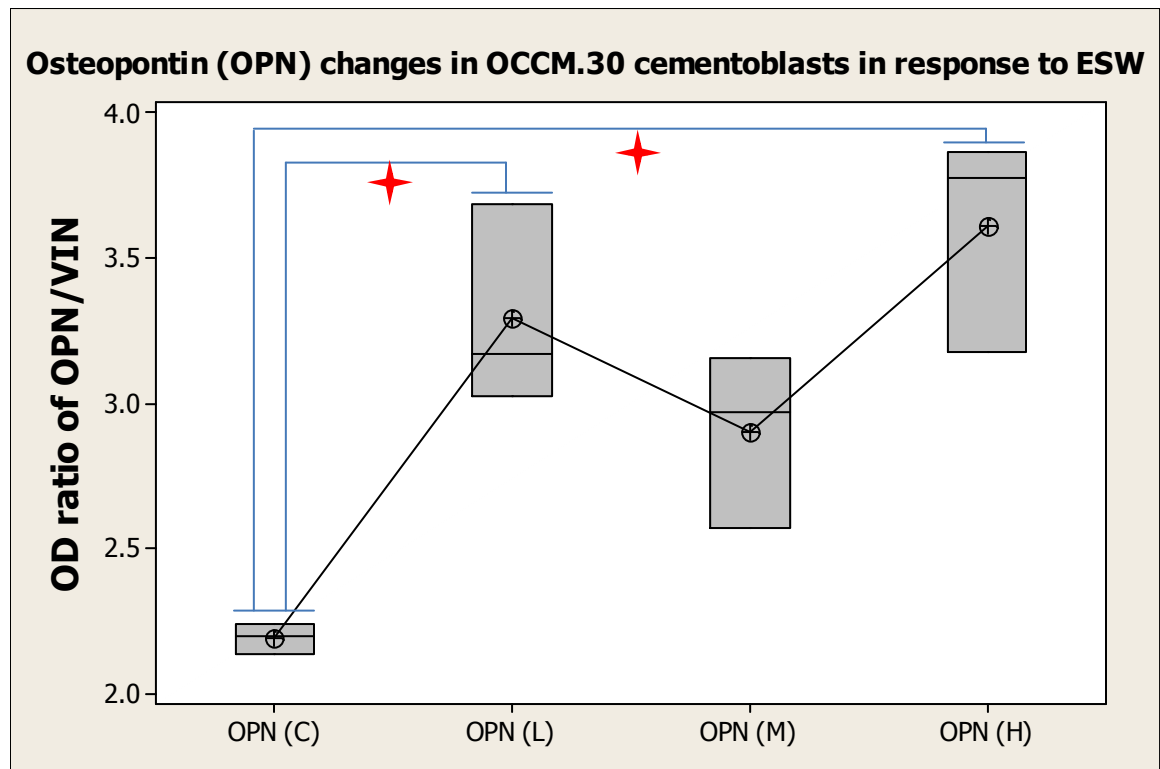
## ATP - Post Hoc Comparisons

| (I) VAR6 | (J) VAR6 | Mean Difference (I-J) | Std. Error   | Sig.        | 95% Confidence Interval |               |
|----------|----------|-----------------------|--------------|-------------|-------------------------|---------------|
|          |          |                       |              |             | Lower Bound             | Upper Bound   |
| Control  | LOW      | -1330313.28571        | 619607.22020 | .167        | -3039567.3116           | 378940.7402   |
|          | MID      | -5553757.71429*       | 619607.22020 | <b>.000</b> | -7263011.7402           | -3844503.6884 |
|          | HIGH     | -10698404.57143*      | 619607.22020 | <b>.000</b> | -12407658.5973          | -8989150.5455 |
| LOW      | Control  | 1330313.28571         | 619607.22020 | .167        | -378940.7402            | 3039567.3116  |
|          | MID      | -4223444.42857*       | 619607.22020 | <b>.000</b> | -5932698.4545           | -2514190.4027 |
|          | HIGH     | -9368091.28571*       | 619607.22020 | <b>.000</b> | -11077345.3116          | -7658837.2598 |
| MID      | Control  | 5553757.71429*        | 619607.22020 | <b>.000</b> | 3844503.6884            | 7263011.7402  |
|          | LOW      | 4223444.42857*        | 619607.22020 | <b>.000</b> | 2514190.4027            | 5932698.4545  |
|          | HIGH     | -5144646.85714*       | 619607.22020 | <b>.000</b> | -6853900.8831           | -3435392.8312 |
| HIGH     | Control  | 10698404.57143*       | 619607.22020 | <b>.000</b> | 8989150.5455            | 12407658.5973 |
|          | LOW      | 9368091.28571*        | 619607.22020 | <b>.000</b> | 7658837.2598            | 11077345.3116 |
|          | MID      | 5144646.85714*        | 619607.22020 | <b>.000</b> | 3435392.8312            | 6853900.8831  |

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

**Table 3-2:** Statistical analysis of ATP release. ANOVA and Post-hoc analysis performed by SPSS 17.0 software. Analysis shows that the increase in ATP release was significant in both the medium and high-dose groups, while it was not significant in the low-dose group.

| SAMPLE#     | CONTROL           | LOW             | MID             | HIGH            |
|-------------|-------------------|-----------------|-----------------|-----------------|
| 1           | 2.20082744        | 3.024325        | 2.569341        | 3.780964        |
| 2           | 2.13693026        | 3.688779        | 2.971915        | 3.182373        |
| 3           | 2.24067002        | 3.173725        | 3.160656        | 3.868326        |
| <b>Mean</b> | <b>2.19280924</b> | <b>3.295609</b> | <b>2.900637</b> | <b>3.610554</b> |
| <b>SD</b>   | <b>0.05233262</b> | <b>0.348592</b> | <b>0.302033</b> | <b>0.37338</b>  |



**Figure 3-3:** OPN production increased in all groups after the application of ESW.

## OPN – ANOVA

|                       | Sum of Squares | df | Mean Squares | F      | Sig.        |
|-----------------------|----------------|----|--------------|--------|-------------|
| <b>Between Groups</b> | 3.365          | 3  | 1.122        | 12.642 | <b>0.02</b> |
| <b>Within Groups</b>  | .710           | 8  | .089         |        |             |
| <b>Total</b>          | 4.075          | 11 |              |        |             |

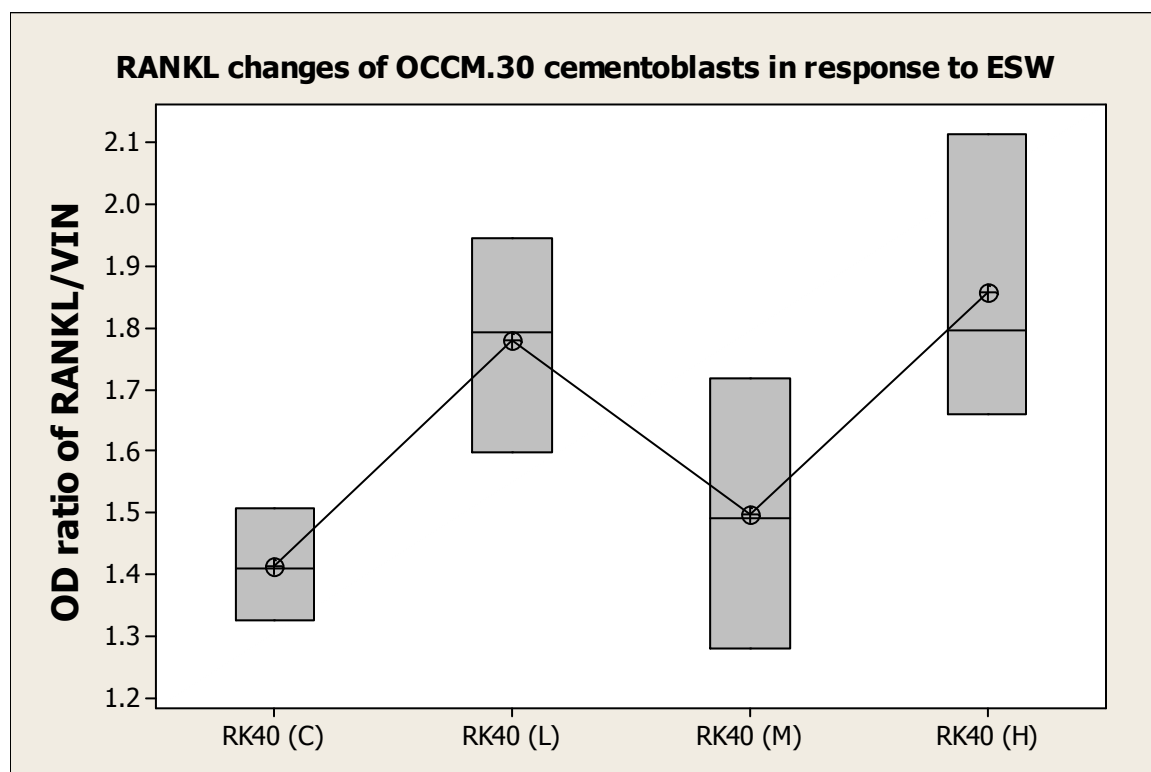
## OPN – Post Hoc Comparisons

| (I) VAR00001 | (J) VAR00001 | Mean Difference (I-J) | Std. Error | Sig.        | 95% Confidence Interval |             |
|--------------|--------------|-----------------------|------------|-------------|-------------------------|-------------|
|              |              |                       |            |             | Lower Bound             | Upper Bound |
| Control      | LOW          | -1.10280 <sup>*</sup> | .24320     | <b>.008</b> | -1.8816                 | -.3240      |
|              | MID          | -.70783               | .24320     | .075        | -1.4867                 | .0710       |
|              | HIGH         | -1.41775 <sup>*</sup> | .24320     | <b>.002</b> | -2.1966                 | -.6389      |
| LOW          | Control      | 1.10280 <sup>*</sup>  | .24320     | <b>.008</b> | .3240                   | 1.8816      |
|              | MID          | .39497                | .24320     | .418        | -.3839                  | 1.1738      |
|              | HIGH         | -.31494               | .24320     | .591        | -1.0938                 | .4639       |
| MID          | Control      | .70783                | .24320     | .075        | -.0710                  | 1.4867      |
|              | LOW          | -.39497               | .24320     | .418        | -1.1738                 | .3839       |
|              | HIGH         | -.70992               | .24320     | .075        | -1.4887                 | .0689       |
| HIGH         | Control      | 1.41775 <sup>*</sup>  | .24320     | <b>.002</b> | .6389                   | 2.1966      |
|              | LOW          | .31494                | .24320     | .591        | -.4639                  | 1.0938      |
|              | MID          | .70992                | .24320     | .075        | -.0689                  | 1.4887      |

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

**Table 3-3:** Statistical analysis of OPN production. ANOVA and Post-hoc analysis performed by SPSS 17.0 software. Analysis shows that while OPN increased in all groups, the increase was only significant in the low and high-dose groups.

| SAMPLE#     | CONTROL           | LOW             | MID             | HIGH            |
|-------------|-------------------|-----------------|-----------------|-----------------|
| 1           | 1.50663515        | 1.598076        | 1.491152        | 1.797349        |
| 2           | 1.32666566        | 1.945426        | 1.719012        | 2.112389        |
| 3           | 1.41055696        | 1.791687        | 1.279718        | 1.661363        |
| <b>Mean</b> | <b>1.41461926</b> | <b>1.778396</b> | <b>1.496628</b> | <b>1.857034</b> |
| <b>SD</b>   | <b>0.09005349</b> | <b>0.174056</b> | <b>0.219698</b> | <b>0.231361</b> |



**Figure 3-4:** RANKL production increased in all groups after the application of ESW, but the increase seen in the medium-dose group was very small.

## RANKL – ANOVA

|                       | Sum of Squares | df | Mean Squares | F    | Sig.  |
|-----------------------|----------------|----|--------------|------|-------|
| <b>Between Groups</b> | 0.4127         | 3  | 0.1376       | 3.92 | 0.054 |
| <b>Within Groups</b>  | 0.2804         | 8  | 0.0351       |      |       |
| <b>Total</b>          | 0.6931         | 11 |              |      |       |

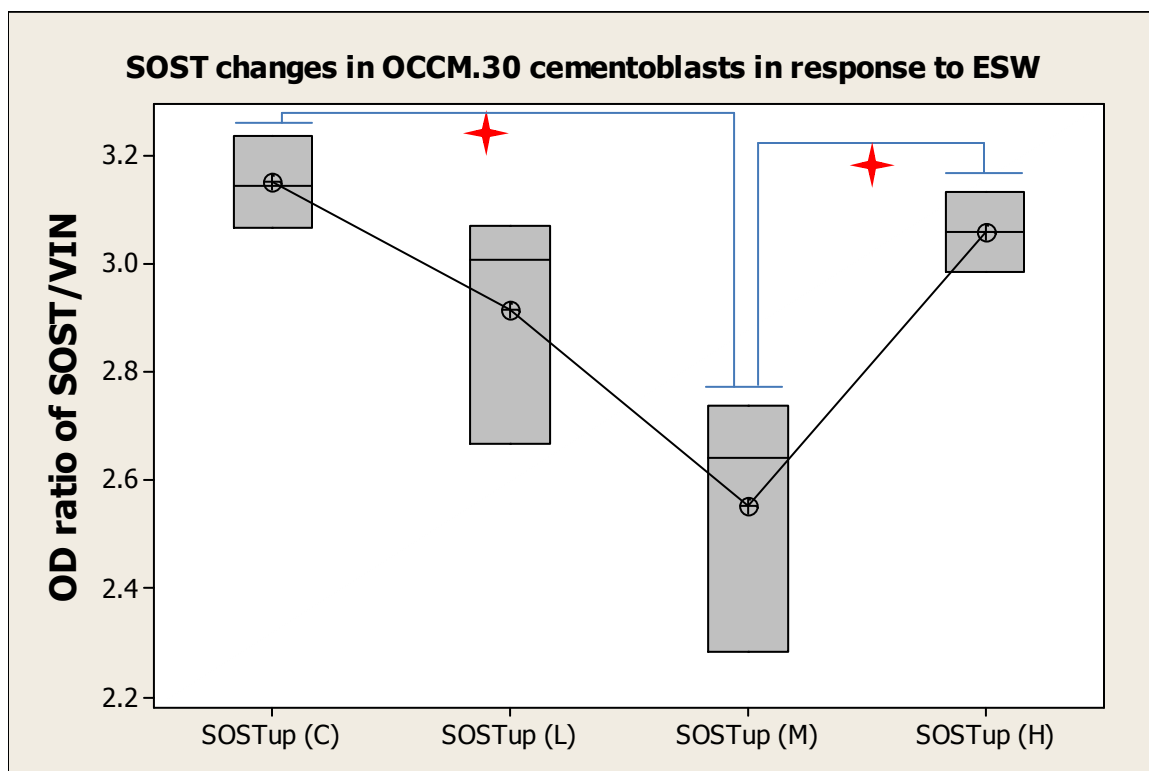
## RANKL – Post Hoc Comparisons

| (I) VAR00001 | (J) VAR00001 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval |             |
|--------------|--------------|-----------------------|------------|------|-------------------------|-------------|
|              |              |                       |            |      | Lower Bound             | Upper Bound |
| Control      | LOW          | -.36378               | .15286     | .159 | -.8533                  | .1257       |
|              | MID          | -.08201               | .15286     | .948 | -.5715                  | .4075       |
|              | HIGH         | -.44241               | .15286     | .077 | -.9319                  | .0471       |
| LOW          | Control      | .36378                | .15286     | .159 | -.1257                  | .8533       |
|              | MID          | .28177                | .15286     | .322 | -.2077                  | .7713       |
|              | HIGH         | -.07864               | .15286     | .953 | -.5682                  | .4109       |
| MID          | Control      | .08201                | .15286     | .948 | -.4075                  | .5715       |
|              | LOW          | -.28177               | .15286     | .322 | -.7713                  | .2077       |
|              | HIGH         | -.36041               | .15286     | .164 | -.8499                  | .1291       |
| HIGH         | Control      | .44241                | .15286     | .077 | -.0471                  | .9319       |
|              | LOW          | .07864                | .15286     | .953 | -.4109                  | .5682       |
|              | MID          | .36041                | .15286     | .164 | -.1291                  | .8499       |

**Table 3-4:** Statistical analysis of RANKL production. ANOVA and Post-hoc analysis performed by SPSS 17.0 software. Analysis shows that the increase in RANKL production was not significant in any of the groups. While the change was nearly significant in the low and high-dose groups, the medium-dose group was nearly unchanged.



| SAMPLE#     | CONTROL           | LOW             | MID             | HIGH            |
|-------------|-------------------|-----------------|-----------------|-----------------|
| 1           | 3.06639281        | 3.069941        | 2.640428        | 3.056942        |
| 2           | 3.23675865        | 2.66652         | 2.73636         | 2.98472         |
| 3           | 3.14233642        | 3.007215        | 2.283719        | 3.130958        |
| <b>Mean</b> | <b>3.14849596</b> | <b>2.914559</b> | <b>2.553502</b> | <b>3.05754</b>  |
| <b>SD</b>   | <b>0.08534978</b> | <b>0.217086</b> | <b>0.238512</b> | <b>0.073121</b> |



**Figure 3-5:** SOST production decreased in all groups of cementoblasts after application of ESW.

## SOST – ANOVA

|                       | Sum of Squares | df | Mean Squares | F    | Sig.         |
|-----------------------|----------------|----|--------------|------|--------------|
| <b>Between Groups</b> | 0.6164         | 3  | 0.2055       | 7.05 | <b>0.012</b> |
| <b>Within Groups</b>  | 0.2333         | 8  | 0.0292       |      |              |
| <b>Total</b>          | 0.8497         | 11 |              |      |              |

## SOST – Post Hoc Comparisons

| Dependent Variable | (I) VAR1 | (J) VAR1 | Mean Difference (I-J) | Std. Error | Sig.        | 95% Confidence Interval |             |
|--------------------|----------|----------|-----------------------|------------|-------------|-------------------------|-------------|
|                    |          |          |                       |            |             | Lower Bound             | Upper Bound |
| VAR2               | Control  | LOW      | .23394                | .13943     | .393        | -.2126                  | .6804       |
|                    |          | MID      | .59499*               | .13943     | <b>.012</b> | .1485                   | 1.0415      |
|                    |          | HIGH     | .09096                | .13943     | .912        | -.3555                  | .5375       |
|                    | LOW      | Control  | -.23394               | .13943     | .393        | -.6804                  | .2126       |
|                    |          | MID      | .36106                | .13943     | .119        | -.0854                  | .8076       |
|                    |          | HIGH     | -.14298               | .13943     | .740        | -.5895                  | .3035       |
|                    | MID      | Control  | -.59499*              | .13943     | <b>.012</b> | -1.0415                 | -.1485      |
|                    |          | LOW      | -.36106               | .13943     | .119        | -.8076                  | .0854       |
|                    |          | HIGH     | -.50404*              | .13943     | <b>.028</b> | -.9505                  | -.0575      |
|                    | HIGH     | Control  | -.09096               | .13943     | .912        | -.5375                  | .3555       |
|                    |          | LOW      | .14298                | .13943     | .740        | -.3035                  | .5895       |
|                    |          | MID      | .50404*               | .13943     | <b>.028</b> | .0575                   | .9505       |

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

**Table 3-5:** Statistical analysis of SOST production. ANOVA and Post-hoc analysis performed by SPSS 17.0 software. Analysis shows that the decrease seen in SOST production was significant only in the medium-dose group when compared to the control.

**CHAPTER V**

**DISCUSSION**

The aim of this project was to determine the effects of different levels of ESW stimulation on cementoblasts. One of the primary issues to be investigated before ESW application could be used as a treatment for root resorption is whether the cells remain viable after ESW stimulation. Obviously, if cell death was induced by shock waves, then ESW would not improve the ability of the cementum to repair itself and could not be considered as a treatment modality. One of the reasons that ESW has begun to be used in the treatment of segmental bone defects is that cell proliferation is seen in the callus after ESW treatment (Chen et al., 2004). High energy ESW has been well reported to have a necrotic effect on cells, while lower doses have been shown to maintain cell viability or slightly increase it (Tamma et al., 2009). Dalla-Bona et al. studied the same cell line used in this study and found that the mechanical stimulation by ultrasound actually caused an increase in cell number and collagen synthesis (2007). A recent study conducted by Lyon et al. at Children's Hospital in Wisconsin using the same shock wave device as this study found that a dose of  $0.25 \text{ mJ/mm}^2$  significantly improved the cell viability of chondrocytes, while a higher dose of  $0.55 \text{ mJ/mm}^2$  decreased cell viability (In Press). This agreed with our results that showed that ESW at the low and medium dose maintained cell viability. Cell death was significant after application of the high energy level which agrees with the previously mentioned necrotic effect of high energy ESW on cells. When looking at the rest of the results in this study, it must be taken into account that the release of ATP and protein production may be affected by the increased cell death seen in the high-dose group.

The results of this study indicated that the cementoblasts were responsive to the mechanical stimulation produced by the shock waves, and established that OCCM.30 cementoblasts express proteins involved in bone modeling, specifically OPN, RANKL and

SOST. These proteins were positively identified by western blot analysis confirming the findings by other studies that cementoblasts do express SOST, RANKL and OPN protein (Dalla-Bona et al., 2008; Huang et al., 2009; Jäger et al., 2010). Further, the results for each measurement revealed a dose-dependent response of the cells to ESW. The dose-dependent effect of ESW has been the subject of several investigations, and it has been found that there is a minimum energy threshold necessary to effect bone cell growth (Zelle et al., 2010). Other studies have confirmed the dose-dependent effect of ESW on cells and shown that the number of impulses is not as important as the energy level of the shock waves (Tamma et al., 2009). Three energy levels were chosen based on effective doses that were reported in other cell studies, and these energy levels were found to elicit a change in the cementoblast release of ATP and expression of bone regulatory proteins.

The reaction of the cementoblasts to ESW can be divided into an early and a late response. ATP release happens very rapidly and is considered to be an early messenger in signaling pathways. Mechanical stress has been shown to induce an increase in ATP release in many other cell types, but the reaction of cementoblasts to ESW has not been examined. In agreement with these studies, our results showed an increase in ATP release after the application of mechanical stress in the form of shock waves (Wongkhantee et al., 2008). The high-dose group, which exhibited increased cell death, actually showed the greatest increase in ATP release. This could be explained by the fact that ATP is not exclusive to bone remodeling and serves as an intracellular and extracellular messenger for several signaling pathways. The increase in ATP could be in response to the increased cell signaling of apoptosis in the high-dose group. The reason we were interested in the expression of ATP is because it may play a role in the regulation of the OPG/RANKL/RANK pathway and help us

understand the effect of ESW on cementoblasts. It has been suggested that ATP may decrease OPG expression and up-regulate both OPN and RANKL expression leading to bone resorption (Wongkhantee et al., 2008; Luckprom et al., 2010). Therefore, an increase in ATP could indicate increased osteoclastogenesis and root resorption. Our results showed an increase in ATP release at all dose levels with greater release as the energy level was increased. This may indicate that ESW induced the cementoblasts to create a resorptive state or the ATP may have been released due to another cellular signaling pathway that was up-regulated in response to the shock waves. The result alone does not allow us to make a statement about the effect of ESW on root resorption. It does, however, indicate that cementoblasts respond to shock waves and makes further investigation necessary.

To examine the late response of cementoblasts to ESW, the production of certain proteins was measured. Three proteins were examined, because they all play an important role in the regulation of bone remodeling. OPN promotes the adhesion of osteoclasts to the bone surface increasing bone resorption. SOST inhibits bone formation by reducing the number of osteoblasts. RANKL regulates osteoclast formation and activation leading to increased bone resorption. Therefore, in a resorptive or catabolic state, increases in the expression of OPN, SOST and RANKL should be seen. In our study, the low-dose and high-dose group showed increases in both OPN (significant) and RANKL (nearly significant) production suggesting an increase in bone resorption. Interestingly, all groups showed decreases in SOST protein, while one may have expected SOST to increase in the low and high dose groups to correspond with the changes seen in OPN and RANKL expression. However, the decrease of SOST expression in these two groups was not significant, so SOST expression was essentially unchanged in the low and high-dose groups. Conversely, the

expression of SOST was significantly decreased in the medium-dose group suggesting the stimulation of bone formation at this energy level. The expression of both OPN and RANKL was not significantly increased in the medium-dose group allowing us to say that the expression of these proteins was maintained at the same level as the control. Since these proteins were not significantly elevated and SOST was significantly decreased in the medium-dose group, one could expect to see reduced bone resorption. These results suggest that the medium dose could promote cementoblast anabolic activity while decreasing the further breakdown of cementum. The protein expression seen in the low and high-dose groups suggests that these dose levels would increase remodeling of adjacent bone and cementum, encouraging a catabolic state.

The one proven risk factor of root resorption is increased duration of orthodontic treatment (Weltman et al., 2010; Taithongchai et al., 1996; Killiany, 1999). Therefore, patients who are in orthodontic treatment for longer periods of time could benefit most from a treatment to reduce root resorption. Mechanical stimulation of cementoblasts may be able to augment the repair process by changing the expression of proteins that regulate bone remodeling. Although the reaction of the cementoblasts to the medium energy level suggested the creation of an anabolic environment, the levels of OPG were not measured. RANKL was unchanged by the medium-dose group, but we do not know if OPG expression changed. If OPG was measured and a decreased RANKL/OPG ratio was seen, then we could more definitively state that the medium dose could inhibit osteoclastogenesis. Additionally, the increase of ATP release seen at the medium energy level seems to contradict the protein results. In order to confirm our hypothesis, we had expected to see a decrease in ATP and a decrease in the three proteins measured. As stated earlier, however, the ATP

release could be related to another cellular signaling pathway. ESW at the medium dose (0.25mJ/mm<sup>2</sup>) does appear to modulate cementoblasts anabolically, but further investigation is needed to confirm that ESW does decrease bone resorption. If future studies find similar effects on cementoblasts, then ESW could be considered as a potential treatment for root resorption.

### *Limitations*

This study sheds light on the cellular response of cementoblasts to ESW. Cell studies are usually the first step in order to determine the effects of new treatment modalities. This study would need to be followed by animal studies and eventual clinical studies to establish whether ESW could be used to treat root resorption.

One major limitation to all cell studies is the fact that the cells are studied in isolation. In this case, the cementoblasts are not in their natural environment. When cells are studied in isolation, their complex interactions with other types of cells and the surrounding matrix are difficult to replicate. The shock waves would normally pass through tissue before reaching the targeted cells in a clinical setting. In this study, however, shock waves were focused directly at the cells passing only through the culture medium and the water in the holder. Although this design was not ideal, water was used as a transition medium for the shock waves because of its similarity in acoustic impedance to the tissue. Additionally, the cells were in suspension when exposed to ESW. Except for blood cells, most cells, including cementoblasts, are naturally adherent cells.



Another limitation to this study is the failure to measure OPG expression. Therefore, we were not able to determine if there was an alteration in the ratio of RANKL/OPG expression in cementoblasts after ESW application.

The cementoblasts used in this study are murine cementoblasts. Cementum deposition was found to be different between humans and animals, such as rats and dogs, when observed with a scanning electron microscope (Boyde and Jones, 1968). Also, it is known that the growth pattern of cementum in lower animals involves continuous eruption, with cementum being formed throughout their lifetimes. Higher animals, such as monkeys, are more useful for studying root resorption, but are obviously very expensive to obtain. (El-Bialy et al., 2004).

#### *Future Studies*

We are planning to work with the company who provided the ESW equipment used in this study to design a new ESW apparatus that could be used to directly stimulate cell culture dishes. The cells would not have to be placed in suspension, and the experiment design could mimic the cells environment more accurately.

To compliment this study, we also applied shock waves to murine calvaria to determine the effect of ESW on bone tissue. The organ culture experiment can reveal more about the effect of ESW on the tissue level.

Eventual animal studies may shed more light on how ESW affects root resorption. There are already studies that looked at the effect of LIPUS on root resorption in rats, but there are no similar studies focusing on ESW treatment.

### *Conclusions*

Our results show that OCCM.30 cementoblast cells are responsive to the application of focused shock waves. This study confirmed that cementoblasts do express OPN, RANKL and SOST proteins and ESW can affect the production of these regulatory bone markers in a dose-dependent manner. We concluded that ESW at the medium dose ( $0.25\text{mJ}/\text{mm}^2$ ) modulates cementoblasts anabolically by decreasing SOST production while keeping RANKL production unaltered. This suggests the potential application of ESW to treat root resorption.

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