The Effect Of Focused Extracorporeal Shock Wave (ESW) On Calvaria Bone Ex Vivo

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THE EFFECT OF FOCUSED EXTRACORPOREAL SHOCK WAVE (ESW)
ON CALVARIA BONE *EX VIVO*

by

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ABSTRACT

THE EFFECT OF FOCUSED EXTRACORPOREAL SHOCK WAVE (ESW)

ON CALVARIA BONE *EX VIVO*

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Introduction: Extracorporeal shock wave (ESW) is low frequency high-pressure wave, regularly used to destroy calcifications e.g. kidney stone. Recent evidence shows that ESW produces micro-cracks in the bones of horse legs. As micro-cracks in bone can lead to an increased rate of bone turnover (remodeling), we hypothesize that proper application of ESW will increase bone turnover rate through generating micro-cracks in bone.

Materials and Methods: To test our hypothesis, we used a mouse calvaria bone organ culture model and explored the effects of ESW on bone tissues *ex vivo*. The calvaria bone pieces were collected from 3 days old C57BL/6 neonatal mice and cultured in 10% FBS supplemented DMEM. After dissection, each quadrant (1/4) of calvaria was randomly assigned into one control and three experimental groups which were subjected to a single dose of 2000 shocks of 3 energy levels of ESW i.e. low (0.1mJ/mm², 6 Hz), medium (0.25mJ/mm², 4 Hz), and high (0.5mJ/mm², 3 Hz,) doses, respectively. After ESW stimulation, the bone tissues were further cultured for 1 week and fixed in 10% formalin. The same experiment was repeated three times (n=3). The bone samples were (1) examined by using a customized radiographic analysis program to measure the bone microdensity, and (2) demineralized and processed to undergo H&E staining to observe the cellular changes histologically.

Results: Radiographically the mid dose group exhibited a higher microdensity but not statistically significant ($P > 0.05$) compared to the other two ESW and the control groups. Histologically, some empty osteocytic lacunae exhibited in all the three dose ESW groups, with nearly all osteocytes disappeared in the high dose group. Hypertrophy of osteoblasts was mainly found in the low dose group. No microcracks were observed on all the H&E staining tissue slides. Histological differences cannot be calculated statistically mainly due to the high heterogeneity of the samples.

Conclusion: Although not statistically significant, the mid dose ESW group seems to gain bone microdensity. Due to the lack of statistical differences, definite conclusions cannot be drawn, leaving further endeavors be put on this topic.

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Syrah Quraishi, DDS

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CHAPTER I

INTRODUCTION

Bone tissues are dynamically adapted to the mechanical environment in maintaining its quality and quantity, via two mechanisms – modeling and remodeling. Both of them involve two processes, namely bone resorption by osteoclasts and bone formation by osteoblasts. A large number of cells must be recruited to maintain the structural integrity of bone. Different cells are responsible for the formation, the resorption and the maintenance of the osseous structure.

Bone physiology is controlled by 3 groups of cells,

1. *Osteoblasts*: Bone matrix is secreted by these mononucleated cells, derived from pluripotent stem cells. They are plump, cuboidal cells when active or slightly flattened, lying at the surface of the existing matrix responsible for depositing new layers of bone onto it (Figure 1-1). This newly formed uncalcified matrix called osteoid is composed mainly of collagen and proteoglycans, which act as a scaffold for the deposition of the apatite crystals of bone. Some osteoblasts remain at the surface forming bone, while others become entrapped within the matrix that is secreted (Nanci, 2008).

2. *Osteocytes*: These are the cells that become embedded within the matrix and are derived from the osteoblasts (Figure 1-2). Once formed, they reduce in size and do not secrete matrix proteins in appreciable quantities. The number of cells that become osteocytes depends on the rapidity of bone formation. Embryonic

bone and repair bone have more osteocytes than the lamellar bone. The space
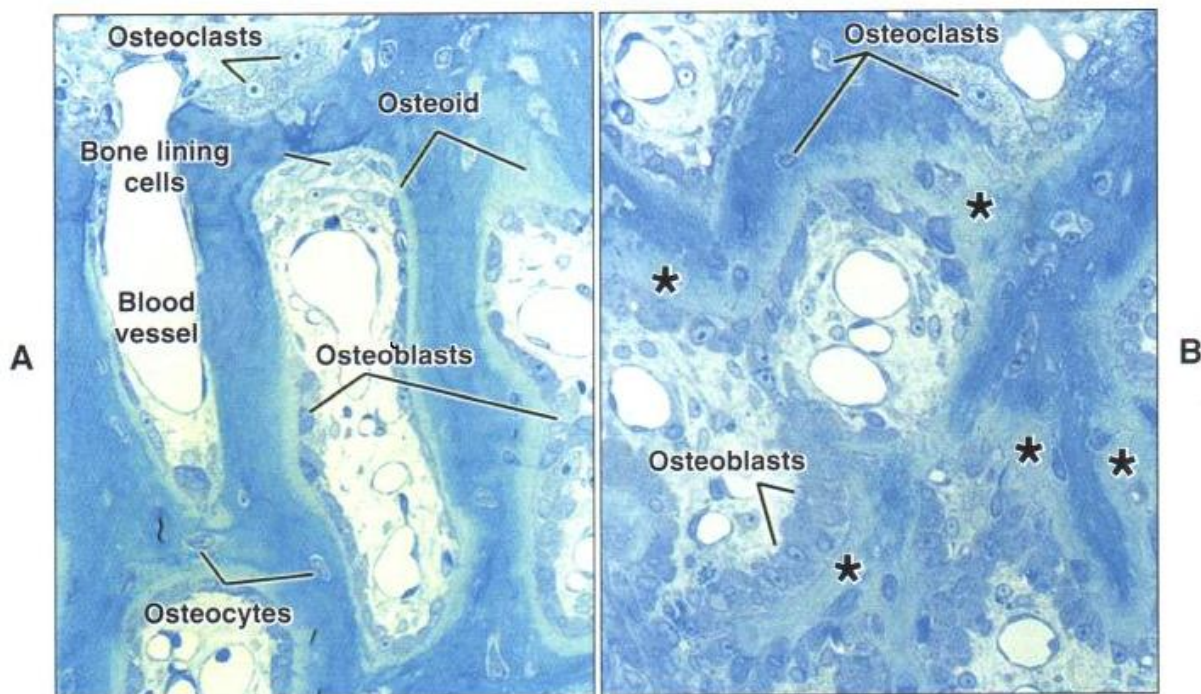
Figure 1-1: A and **B,** Mandibular bone soon after birth. By this time the bone has undergone substantial turnover and appears more compact. Bone-forming surfaces are covered by plump osteoblasts or flattened, less active cells. Quiescent areas are covered by bone lining cells. Osteocytes are present within the calcified matrix and in some cases within osteoid (*asterisks).* Osteoclasts usually are found opposite actively forming bone surfaces (Nanci, 2008).

occupied by the osteocytes is called, osteocytic lacuna. Narrow extensions from each

lacuna, called canaliculae contain radiating osteocytic processes that maintain contact

with neighboring osteocytes and with osteoblasts. They thus form a network that can

sense the biochemical and mechanical environment that maintains bone integrity

especially for the repair of microcracks (Nanci, 2008).
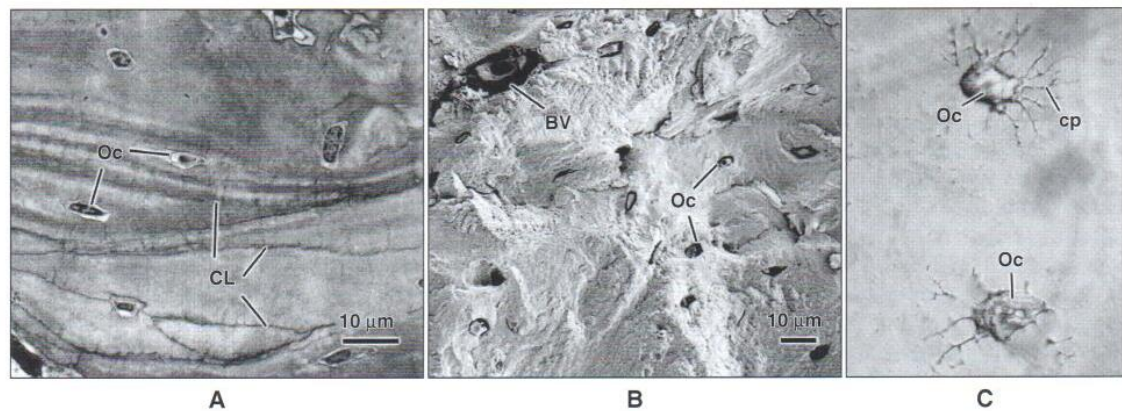
Figure 1-2: Light microscope of rat mandibular bone **(A)** and scanning electron micrograph of rat tibia bone **(B)**. Osteocytes (Oc), residing in lacunae, populate the bone. A, Abundant cement lines (CL) are present in the mandibular bone. **(C),** Osteocytes have an extensive network of cell processes (cp; Nomarski optics). BV, Blood vessel (Nanci, 2008).

3. *Osteoclasts*: These bone resorbing cells are large, multinucleated cells often present in clusters. They are present against the bone surface in hollow depressions called, Howship's lacunae. At the site of attachment to the bone matrix, there is a clear zone and resorptive activity occurs along the characteristic ruffled border. These cells are derived from hematopoetic stem cells that give rise to various lines of macrophage-like cells (Mundy, 1983; Nanci, 2008).

Bone modeling and remodeling are carried out essentially by osteoblasts and osteoclasts and are regulated by systemic hormones, parathyroid hormone, 1,25 dihydroxyvitamin D3 and calcitonin. Some local factors such as, prostaglandin E2,

Interferon-gamma, Interleukin 1, tumor necrosis factor α and transforming growth factors, also play a role in regulating these cellular activities.

Orthodontic tooth movement (OTM) occurs due to prolonged pressure applied on a tooth which results in bone modeling around the tooth. Bone is selectively resorbed in some areas and is deposited in other areas (Proffit *et al.,* 2012). The modeling of bone is a complex series of events orchestrated by the osteoblasts and osteoclasts which determines the modeling rate of OTM. Bone remodeling in the alveolus is influenced by both local factors like teeth and occlusion and systemic factors related to the general metabolism of the bone (Verna et al., 2000). Several studies have shown that bone metabolism plays an important role in tooth movement (Midgett *et al.,* 1981; Goldie and King, 1984; Engström *et al.,* 1988; Hellsing and Hammasström, 1991). It has also been shown that OTM is influenced by pharmacological agents (Yamasaki et al., 1984; Chumbley and Tuncay, 1986; Collins and Sinclair, 1988; Mohammed et al., 1989; Takano Y *et al.,* 1992). It is known that a key component in OTM is alveolar bone remodeling and that remodeling is accelerated during wound healing (Frost, 1994). Corticotomy has been proposed to accelerate tooth movement but has not gained wide acceptance due to its invasiveness (Kole, 1959; Gunderson *et al.,* 1978).

Extracorporeal shock waves (ESW) are high pressure low frequency waves that are generated by a device outside the body and are applied to the tissue in a site-specific manner. It is a minimally invasive approach and was first introduced in medicine more than 20 years ago to disintegrate kidney stones (Capaccio et al., 2009). Over the past 10

years, ESW has been used in various fields of medicine (orthopedics, veterinary medicine, traumatology, treatment of impaired wound healing and burn injuries) to stimulate healing processes (Wang CJ, 2003; Da Costa Gomez TM *et al.,* 2004; Hofmann *et al.,* 2008). In dentistry, ESW has been studied on its effect on periodontitis, peri-implantitis and orthodontic treatment time (Sathishkumar *et al.,* 2008; Li *et al.,* 2010; Hazan-Molina *et al.,* 2011).

A recent study shows that ESW generated microcracks in leg bones of horses (Da Costa Gomez *et al.,* 2004). Microcracks can lead to an increased bone remodeling rate (Verna *et al.,* 2004) which determines the rate of orthodontic tooth movement. Based on these, the aim of our study is to use an *ex vivo* calvaria bone culture model to explore the effect of ESW on bone modeling.

CHAPTER II

LITERATURE REVIEW

Orthodontic Tooth Movement

When orthodontic forces are applied for tooth movement, modeling occurs in

dental and paradental tissues, including gingiva, dental pulp, periodontal ligament (PDL)

and alveolar bone. Orthodontic tooth movement (OTM) occurs as a result of bone

resorption and deposition in the compressed and stretched side of the PDL, respectively

(Krishnan and Davidovitch, 2006). On the compressed or pressure side, the PDL fibers

are unloaded leading to unloading of the alveolar bone, resulting in resorption (Melsen,

2001). On the stretched or tension side, the PDL fibers are stretched, causing active

loading of the alveolar bone leading to apposition (Henneman *et al.,* 2008) (Figure 1-3)
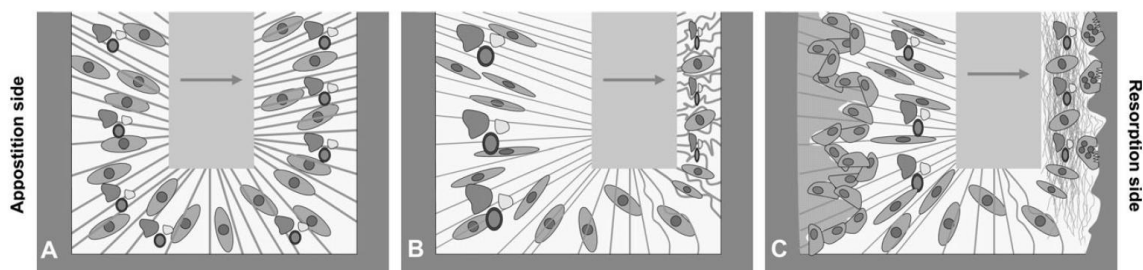
Figure 1-3: Diagram showing the displacement of the tooth during orthodontic tooth movement. A) The PDL fibers are at equilibrium when no force is applied. B) In the direction of the applied force, the PDL fibers are compressed, unloading the bone. Away from the force, the PDL fibers are stretched causing 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).

Bone resorption; the removal of alveolar bone from the path of the moving tooth is needed for orthodontic movement. Osteoclasts are critical for this to occur. 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 1-4). It is noted that when orthodontic forces are applied, osteoclasts appear within days (Tsay PT, 1999). Cementum is, however more resistant to resorption than the alveolar bone and root repair happens regularly during OTM (Proffit *et al.,* 2012). After resorption of alveolar bone by osteoclasts, mononuclear cells from the macrophage linage, are involved in further degradation of collagen, deposition of proteoglycans and release of growth factors to initiate the deposition phase (Raisz, 1999). The end of bone deposition and the start of bone formation occur through a coupling mechanism that ensures an equivalent amount of bone is laid down after the resorptive phase (Hill, 1998).

Bone formation is a complex series of events involving differentiation of osteoblast precursor cells from mesenchymal cells, maturation of osteoblasts, matrix formation and mineralization (Mundy, 1987). The osteoblasts gradually flatten and become quiescent lining cells and some are embedded in the newly formed matrix and are now called osteocytes. Osteocytes are the longest lived bone cell. They live for decades within their mineralized environment. The osteoid osteocyte performs two major functions simultaneously. It regulates mineralization and form connective dendritic processes (Bonewald, 2011). These cells are critical in maintaining fluid flow through bone and any changes in fluid flow are transmitted to osteoblasts and
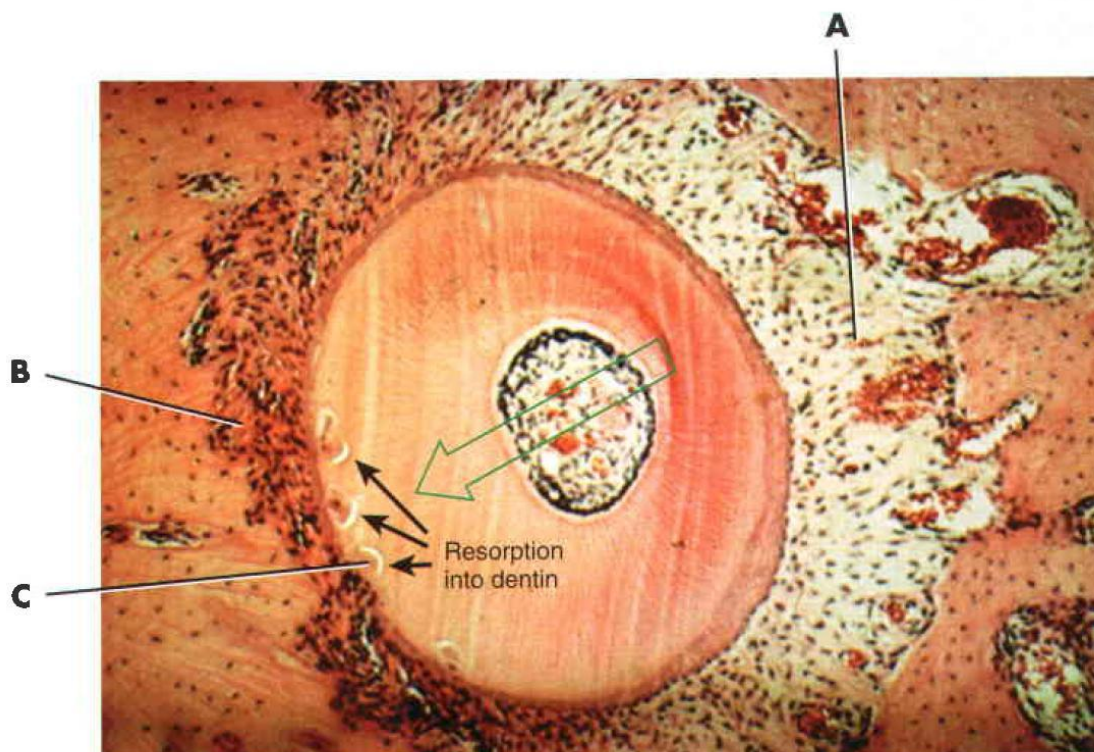
Figure 1-4: Diagram showing the histological cross section of a premolar being moved in the direction of the arrow. A) The PDL is stretched away from the direction of the force. B) The PDL is compressed in the direction of the force and areas of bone resorption can be seen. C) Resorption occurring into the cementum and dentin of the dental root (Proffit *et al.,* 2012)

osteoclasts which then again carry out modeling and remodeling activities (Bozal CB, 2001).

The amount of orthodontic force used to compress the tooth determines the type of resorption that is observed. Light forces ensure survival of cells within the PDL and the activity of osteoclasts and osteoblasts is synchronized (Melsen, 1999). In this direct or frontal resorption of the tooth socket, the PDL width is maintained and the tooth moves with bone along with its alveolus. When excessive orthodontic force is

applied, indirect or undermining resorption is seen and there is little formative activity that takes place at PDL tension sites and only minor displacement of the tooth occurs. Under heavy sustained forces, the blood vessels are totally occluded and the supply is cut off to an area of the PDL causing necrosis of the cellular elements within the PDL as cell death occurs. The histological appearance of this avascular area in the PDL is referred to as hyalinized. After several days, cellular elements invade the hyalinized area and start to resorb the underside of the bone immediately adjacent to the necrotic PDL area (Proffit *et al.,* 2012). When the hyalinized tissue is removed, the tooth begins its displacement and becomes mobile due to the widened PDL. In orthodontic tooth movement, it is ideal to avoid creating areas of PDL necrosis and undermining resorption to allow for efficient tooth movement and reduce the pain experienced by the patient during treatment.

Accelerated Tooth Movement

Remodeling of alveolar bone is crucial for orthodontic tooth movement and the remodeling of bone is accelerated during wound healing (Frost, 1994). Hullihan, a pioneer American oral surgeon in the late nineteenth century proposed to move teeth after making cuts in alveolar bone (Proffit *et al.,* 2012). This approach was not widely accepted due to infections and bone loss in this pre-antibiotic era. Later, Köle a German surgeon (1959) and Gunderson *et al.* (1978) proposed that cuts between teeth could result in faster tooth movement. However the theory was again not widely accepted due to its invasive nature. The idea of corticotomy gained some acceptance in the late

1990's due to better understanding of the mechanism. Corticotomy is considered a demineralization and remineralization process that allows a regional acceleration of bone remodeling leading to faster tooth movement (Liu, 2009). However, the cost of the surgery, morbidity and inconvenience to the patient remain concerns. The widespread remodeling of alveolar bone is still recommended to move teeth more physiologically using lighter forces (Proffit *et al.,* 2012).

Recently, three other methods have been proposed to accelerate tooth movement i.e. vibration of the teeth, use of phototherapy (LASER) to the alveolar process and the application of ultrasound to teeth and adjacent bone (Proffit *et al.,* 2012).

Microcracks in bone

Microcracks and microdamage have been associated with bone remodeling (Verna *et al.,* 2004). Frost (1960) was the first to describe microdamage in bone. Microdamage is the result of fatigue, creep and other mechanical processes that alter the microstructure permanently (Martin, 2003). It was proposed that physiologic strains continue to produce fatigue damage in bone which weakens bone and is associated with activation of osteocyte apoptosis and remodeling. Apoptosis of osteocytes is observed in rats during experimental tooth movement. This apoptosis is essential for damage repair and normal skeletal replacement (Hamaya *et al.,* 2002). Remodeling is the means of removal of the damage caused by microdamage (Noble, 2003). Four types of microdamage has been described: (1) microcracks found in cortical bone that extend

shock waves can travel through fluid and soft tissue and change their physical properties

by attenuation when they travel through a medium and by reflection and refraction

when entering another medium making it possible to focus these waves within the

tissue. When these waves meet an interface of two different media, one part of the

shock wave will be reflected and the other part will be transmitted and at that interface,

high pressure, shear forces and the most biologic effects occur (Li *et al.,* 2010). ESW are

generated under water and transferred to the subject by means of a contact medium.

This ensures minimal loss due to attenuation and reflection at the interface and the

energy can be focused on the treatment area (Coombs *et al.,* 2000).

Developed in the 1970's, the application of ESW was used in medicine for

disintegration of kidney stones and has become the standard nonsurgical method for

treating uroliths. It has been used in the management of gall stones and sialolithiasis

and is considered a safe and minimally invasive method to break down salivary duct

stones (Capaccio *et al.,* 2009) (Figure 1-5). The success with ESW in lithotripsy led to its

application in orthopedics but not to disintegrate tissues rather than induce

neovascularization to promote tissue regeneration and improve blood supply in both

humans and animals. The convenient and cost-effective use of ESW has led to its

application in the healing of non-union long bone fractures, calcifying tendonitis of the

shoulder, lateral epicondylitis of the elbow, proximal plantar fasciitis, avascular necrosis

of femoral head, patellar tendonitis, osteochondritis dessicans and non-calcifying

tendonitis of the shoulder (Wang, 2003). It has also been shown that ESW stimulates the

Figure 1-5: 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 hand piece is placed over the affected area, and the prescribed impulses are applied (Capaccio *et al.,* 2009).

early expression of angiogenesis-related growth factors, including eNOS (endothelial nitric oxide synthase), VEGF (vessel endothelial growth factor) and PCNA (proliferating cell nuclear antigen), resulting in neovascularization and improving blood supply and also accelerates healing by modifying the local intracellular and extracellular biological environment. There is up-regulation of growth factors and activation of osteoblasts and fibroblasts to accelerate injury repair (Wang *et al.,* 2008). Recent research has shown that ESW stimulates bone healing by inducing periosteal detachment and hemorrhage resulting in new bone formation (Da Costa Gomez *et al.,* 2004). Shock waves can cause microfracture and hematoma formation that can lead to maturation of human osteoblasts, increased callus formation and bone healing (Hoffman *et al.,* 2008).

ESW is minimally invasive and is advantageous to the patient due to avoidance of any surgical procedure. Its use is considered safe and without any serious risks. Any side effects from treatment depend on the energy and impulses used since bone responds in a dose dependent manner to the shock waves. It has been reported that when excessive amounts of shock wave energy is applied to bone, induction of trabecular and cortical fracture can occur whereas lower energy waves can stimulate osteogenesis (Da Costa Gomez *et al.,* 2004). Petechiation of the skin, local hematomas and swelling have been reported but they tend to disappear within a few days without any complications (Shrivastava and Kailash, 2005). Some of the contraindications for treating a patient with ESW are pregnancy and the use of a cardiac pacemaker (Capaccio *et al.,* 2009) and ESW must also be never focused on gas-filled cavities like lung or intestine due to considerable tissue damage at the interface (McClure, 2004).

In dentistry, ESW was used to determine its effect on the healing of periodontal tissue and alveolar bone resorption resulting from periodontitis (Sathishkumar *et al.,* 2008). It was also used as an adjuvant treatment for peri-implantitis (Li *et al.,* 2010). Hazan-Molina *et al.* found that the application of shock waves in a rat model after induction of orthodontic force influences the expression of IL-1β and VEGF resulting in enhanced periodontal remodeling (Hazan-Molina *et al.,* 2011, 2012).

Hypothesis

Since micro-cracks in bone can lead to a higher bone turnover (remodeling) rate, we hypothesize that proper focused application of ESW will increase bone turnover rate through generating micro-cracks in bone. To test our hypothesis, we used a mouse calvaria bone organ culture model to explore the cellular mechanism and the proper dose of ESW on bone *ex vivo*.

CHAPTER III

MATERIALS AND METHODS

Tissue (Calvarial bone) Culture

Calvarial bones were harvested from 3 day old male neonatal mice (provided by Dr. Stephen Downs, Department of Biological Science, MU). Briefly, the fresh sacrificed neonatal mice were placed on surgical table. Steps: (1) Scalp was incised and calvarial bone was exposed. (2) The calvarial bone was removed using surgical blade and scissors, and placed in a culture dish containing α-minimal essential medium (α-MEM) supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin). (3) The calvarial bones were cleaned by removing the soft tissue and washed 2 times with the culture medium mentioned above (Figure 2-1). (4) Each of the cleaned calvarial bone was cut along the sagittal suture and along a transverse line half way perpendicular to the suture (Figure 2-2). (5) All the bone cuts from all the mice at the time of the experiment were pooled together and rinsed again with culture medium. At the time of experiment, each one of the bone cuts was randomly assigned to a designated group and cultured in a 24-well culture plate (1 bone cut/well) filled with 2 ml/well of complete growth medium (α-MEM with 10% fetal bovine serum (FBS)) at 37°C with 5% CO_2 in a humidified incubator (Figure 2-3). All cell culture supplies were purchased from Sigma (St. Louis, MO) unless otherwise noted.

Experimental Protocols

The bone cuts were randomly assigned into four groups as (1) control (n = 4-6), (2) low dose (n = 4-6), (3) medium dose (n = 4-6), and (4) high dose (n = 4-6) of ESW. Each group of bone cuts received one episode of ESW treatment consisting of 2000
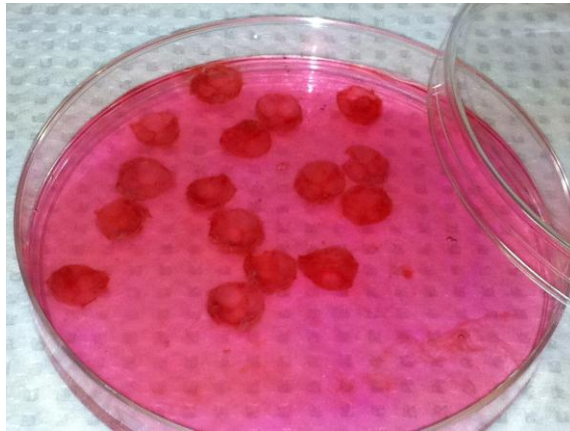
Figure 2-1: Calvarial bone cultured in α-MEM supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin)
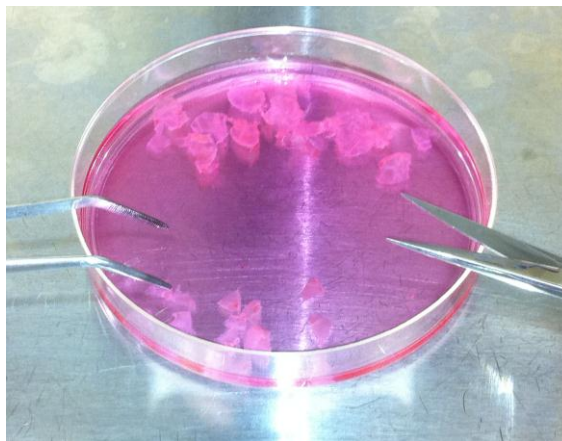
Figure 2-2: The mouse calvaria dissected sagittally and transversally into four equal parts
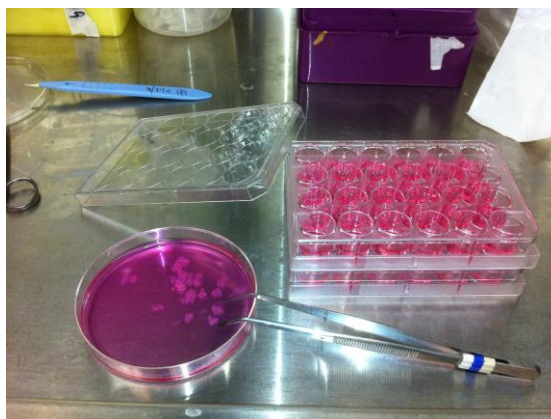
Figure 2-3: The bone cuts cultured in a 24-well culture plate (1 bone cut/well) filled with 2 ml/well of complete growth medium (α-MEM with 10%FBS)

impulses at their assigned energy level. The three energy levels of ESW used for experimentation were low (0.1mJ/mm², 6.0 Hz), medium (0.25mJ/mm², 4.0 Hz), and high (0.5mJ/mm², 3.0 Hz,). After ESW treatment, the tissue sample from each treatment group was re-placed in the 24-well plates with complete culture medium for 6 days.

Focused Extracorporeal Shock Wave Application

The focused ESW system used to apply the shock waves was the Extracorporeal Pulse Activation Treatment System (Duolith SD1®, Storz Medical AG, Postfach, Switzerland) (Figure 2-4). This system utilizes high-energy, focused, cylindrical-source, electromagnetic shock wave technology that is applied with a corded, Focused Shock Wave (F-SW) hand piece (Figure 2-5). This is the apparatus specially and only designed for the *in vitro* study using ESW. It has a short pulse length and is concentrated on areas of a few millimeters in diameter. The F-SW hand piece 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 hand

piece to the center of the focal zone is approximately 30 mm. The F-SW hand piece was

attached to the bottom of a specially designed holder (Figure 2-6). 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.
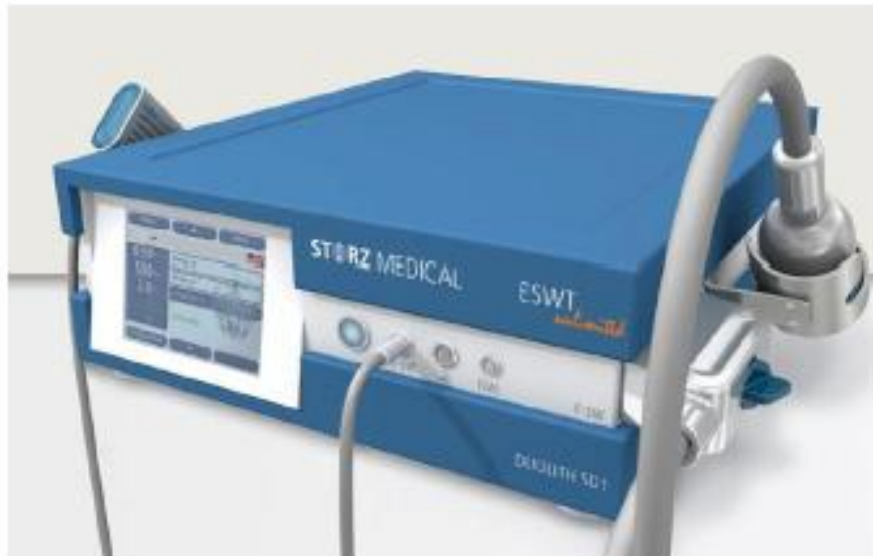
Figure 2-4: Storz Medical Duolith®SD1 shock wave therapy system. The model pictured
is the table top version which was used during experimentation.

2-5: F-SW hand piece with stand-off device I for 30 mm depth of focus.

Calvaria Bone

Figure 2-6: Picture of the Duolith® SD1 system used for experimentation. The F-SW hand piece was attached to the bottom of the specially designed holder. Eppendorf tubes with cell suspensions were placed into the top of the holder.

Radiographic examination of the bone cuts

All the tissue samples from each experiment group were placed on a radio-sensor on a table surface and an x-ray unit was placed perpendicular to the film (Figure 2-7). Each slide was radiographed with an exposure time of 0.05 sec at a setting of 70 KvP and 8 mA. To get an equal exposure to X-ray, the following procedures were

followed. (1) All the samples (n = 4-6) from the same treatment group were radiographed together, to avoid the variation between bone cuts; (2) the X-ray tube was oriented 10 cm perpendicular to the table surface where the samples were placed on a radio-sensor; (3) a gradient aluminum wedge was placed aside as a standard control of method (Figure 2-7). The radiographs were analyzed for bone microdensity as the primary outcome parameter of the study.

Figure 2-7: Radiographic measurement of the bone microdensity

The method followed in this study was adopted from a previous publication in which bone microdensity was measured (Rothe, 2006). An application to measure differences between samples in the radiographs was developed using a densitometer (Delphi XE2 Embarcadero, San Francisco, CA). The application displays the radiograph as an image with width of 900 pixels and height of 641 pixels (Figure 2-8). A separate marker image, with 75 pixel width and height, is used to indicate the location from which to record the grayscale level of pixels from the radiograph image. The marker

image has a transparent background and contains 4, 25 pixel by 25 pixel yellow squares.

Two squares are centered, one between rows 1 to 25 and other between rows 51 to 75.

The other 2 squares about the outer edges between rows 26 to 50. Using the keyboard

arrow keys, the user positions the marker image over a sample in such a way so that the

4 yellow squares are completely within the sample. When the user presses the "Record

Measurements for this marker" button, the application adds a line to a measurement

file including the name of the radiograph file, a date/time stamp, the upper left

coordinate of the marker image, and 2500 integers which are the grayscale value for

each pixel in the radiograph image covered by the 4 yellow squares in the marker image.

The average pixels for all samples recorded in one radiograph were computed to

represent the bone density level for that radiograph/sample.
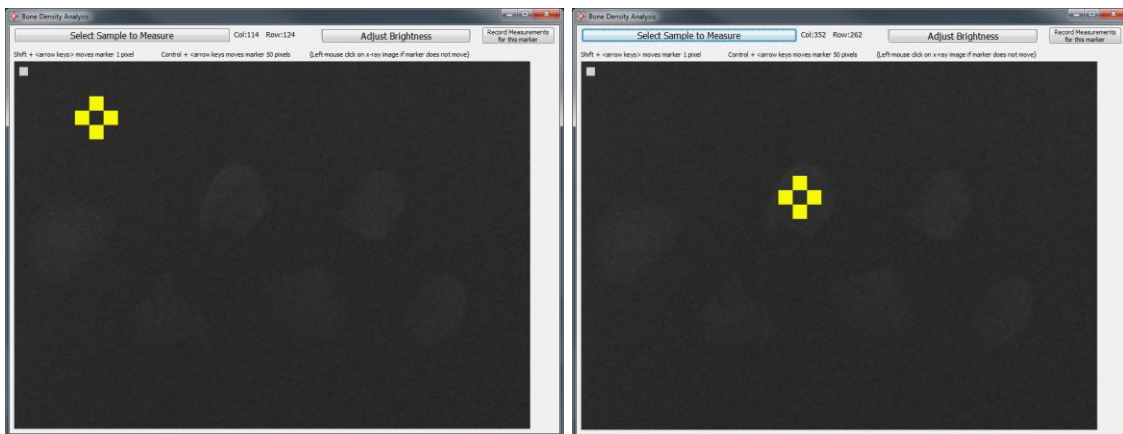
Figure 2-8: Procedure steps of measuring bone microdensity for the bone cut samples.

Histological Examination of the bone cuts

After radiographic examination, the bone cuts were treated for histological study in order to find the histological changes of bone cells at the tissue level. Steps: (1) demineralization: all the bone cuts were treated with 4% ethylenediaminetetraacetic acid (EDTA) for 4 weeks, with the treatment medium changed every week, until fully demineralized. (2) The demineralized bone cuts were dehydrated and embedded in paraffin blocks for tissue sectioning. When embedded, all the bone cuts were oriented along the same line in order to get a consistent series of sections of bone samples. For each paraffin block, three series of cuts were made as one in the middle of the block, and two side cuts series 100 μm away from the middle one. (3) Hematoxylin and eosin (H&E) staining was performed for all bone sections. During microscopic analysis to observe the histological changes, digital images of the microscopic histology were made with image analysis software (Image Pro Plus Software v 5.1, Media Cybernetics, Silver Spring, MD) running on a Windows XP workstation. A video camera (Model DFC 280, Leica Microsystems, Cambridge, UK) was coupled to the microscope (Zeiss Universal, Oberkochen, Germany) camera tube and used to acquire digital images of the microscopic appearance of the stained sections. A variety of magnifications were used. All of the pictures taken were labeled with the magnification used.

Statistical Analysis

For the radiographic data, all the values were graphed as mean ± standard deviation (SD) of the individual groups. SPSS software (version 17.0) was used to

complete the statistical analysis of the bone microdensity measurements. 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. Statistical significance was determined at $p < 0.05$.

CHAPTER IV

RESULTS

Bone Microdensity Measurement

The raw data of the representative images of the bone cuts (Figure 3-1) is presented in Table 3-1
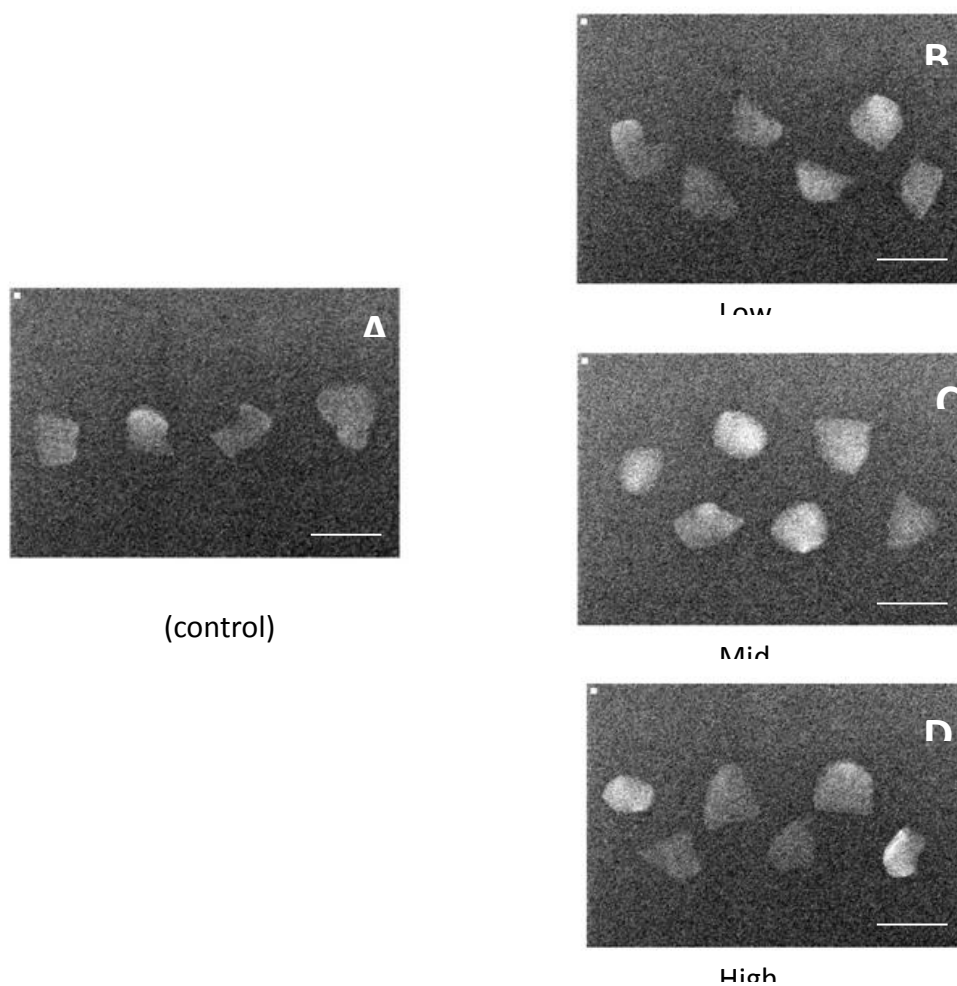
Figure 3-1: Radiographic images of the bone cuts from the 3 experimental groups and control

Table 3-1: Radiographic measurements of bone microdensity

Treatment	Sample group #	Microdensity (average grayscale intensity/2500 pixels)
Control	1	44.7213
	2	46.5304
	3	46.34947
Low dose	1	45.6469
	2	46.96936
	3	45.45567
Mid dose	1	46.3911
	2	48.55553
	3	47.10045
High dose	1	44.8108
	2	46.8668
	3	45.29153

Table 3-2: Descriptive statistics for radiographic measurements of bone microdensity

Table of Descriptive Statistics

Microdensity

	N	Mean	Std. Deviation	Std. Error	95% Confidence Interval for Mean Lower Bound	95% Confidence Interval for Mean Upper Bound	Minimum	Maximum
1.00	3	45.87	.997	.576	43.39	48.34	45	47
2.00	3	46.03	.822	.475	43.98	48.07	45	47
3.00	3	47.35	1.103	.637	44.61	50.09	46	49
4.00	3	45.66	1.075	.621	42.98	48.33	45	47
Total	12	46.22	1.102	.318	45.52	46.92	45	49

Table 3-3: ANOVA analysis for radiographic measurements of bone microdensity

ANOVA

Microdensity

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5.264	3	1.755	1.735	.237
Within Groups	8.090	8	1.011		
Total	13.353	11			

Table 3-4: Tukey adjustment for radiographic measurements of bone microdensity

Multiple Comparisons

Microdensity
Tukey HSD

(I) group	(J) group	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
1.00	2.00	-.160	.821	.997	-2.79	2.47
	3.00	-1.482	.821	.337	-4.11	1.15
	4.00	.210	.821	.994	-2.42	2.84
2.00	1.00	.160	.821	.997	-2.47	2.79
	3.00	-1.322	.821	.425	-3.95	1.31
	4.00	.370	.821	.968	-2.26	3.00
3.00	1.00	1.482	.821	.337	-1.15	4.11
	2.00	1.322	.821	.425	-1.31	3.95
	4.00	1.693	.821	.244	-.94	4.32
4.00	1.00	-.210	.821	.994	-2.84	2.42
	2.00	-.370	.821	.968	-3.00	2.26
	3.00	-1.693	.821	.244	-4.32	.94

Note: 1-control (no ESW); 2-low dose ESW; 3-mid dose ESW; 4-high dose ESW

Microdensity of calvarial bone cuts after expsoure to focused ESW
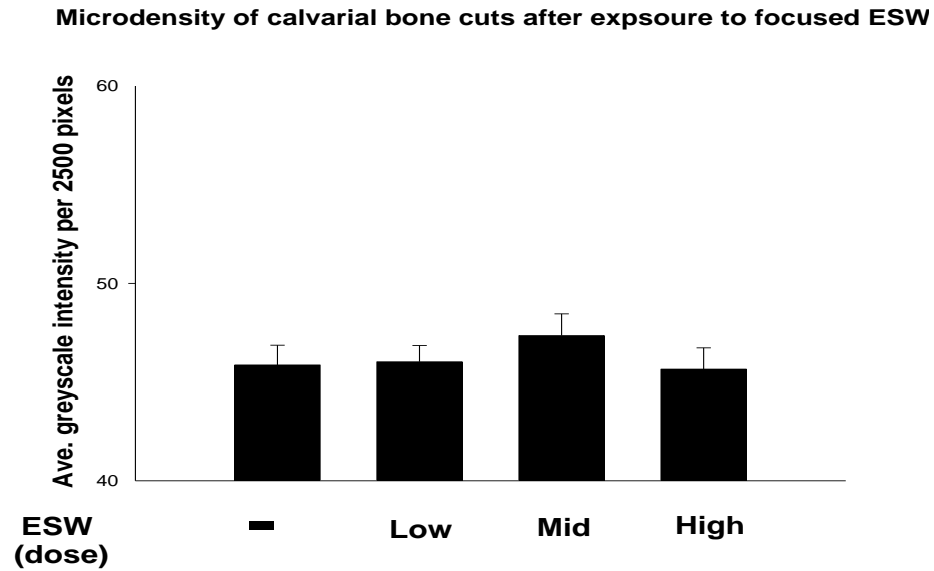
Figure 3-2: Microdensity of calvarial bone cuts.

Histological Observations

The histological observations made on the four groups are as follows.

1. **Control Group (no ESW)**

   Intramembranous bone formation of calvaria in the control group was observed. Normal osteocyte lacunae were filled with osteocytes. Osteoblasts were located along the surface of intramembranous ossification. No identifiable osteoclasts were seen and no microcracks were observed (Figure 3-2).

2. **Low dose ESW group**

   Intramembranous bone formation of calvaria in the low dose group was observed. Some regions showed filled osteocyte lacunae. Some cartilage resembling suture was observed along the length and some areas showed only cartilage. Hypertrophied

Figure 3-2: Intramembranous bone formation of calvaria in control (no ESW) group. As found, normal osteocyte lacunae were filled with osteocytes. Osteoblasts were located along surface of intramembranous ossification. No identifiable osteoclasts and no microcracks were observed. Magnification: x79 (A) and x200 (B).

osteoblasts were observed in some areas and other areas had few flattened bone-lining cells (osteoblasts). No osteoclasts or microcracks were observed in the low dose group (Figure 3-3).

Figure 3-3: Intramembranous bone formation of calvaria in low dose ESW group. As shown, some regions show filled osteocyte lacunae. Some cartilage resembling suture along the length – some areas just cartilage. Hypertrophied osteoblasts. Few flatte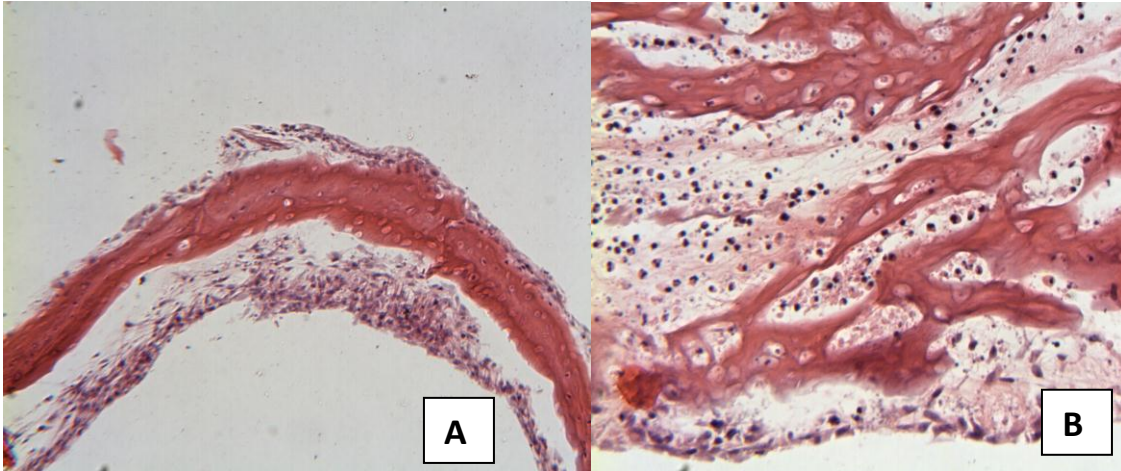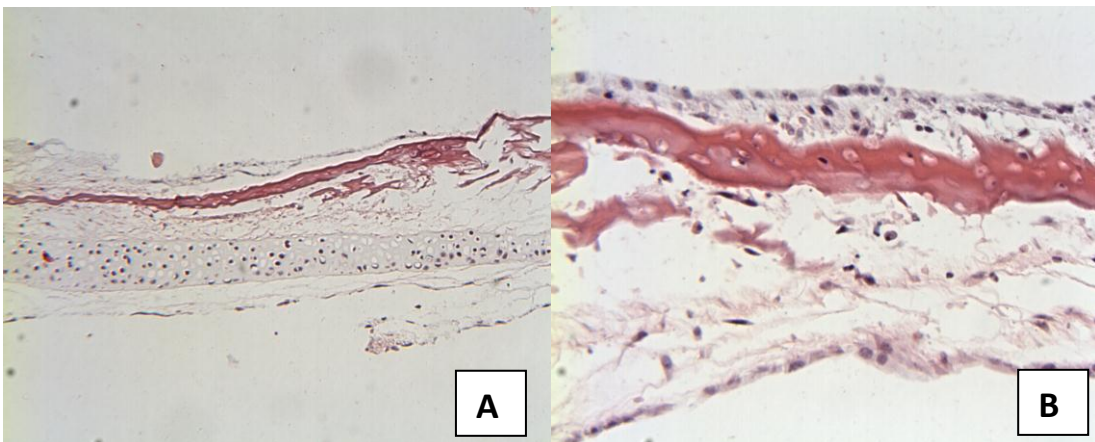ned bone-lining cells (osteoblasts) along other surfaces. No osteoclasts, no microcracks. Magnification: x79 (A) and x200 (B).

3. **Mid dose ESW group**

    There was intramembranous bone formation of calvaria in the mid dose ESW
    group. In some regions, there were filled osteocyte lacunae while other regions
    showed empty osteocyte lacunae. There were mixed regions of hypertrophied
    osteoblasts in intramembranous ossification along some surfaces and flattened
    bone-lining cells (osteoblasts) along other surfaces. No osteoclasts or
    microcracks were observed (Figure 3-4).
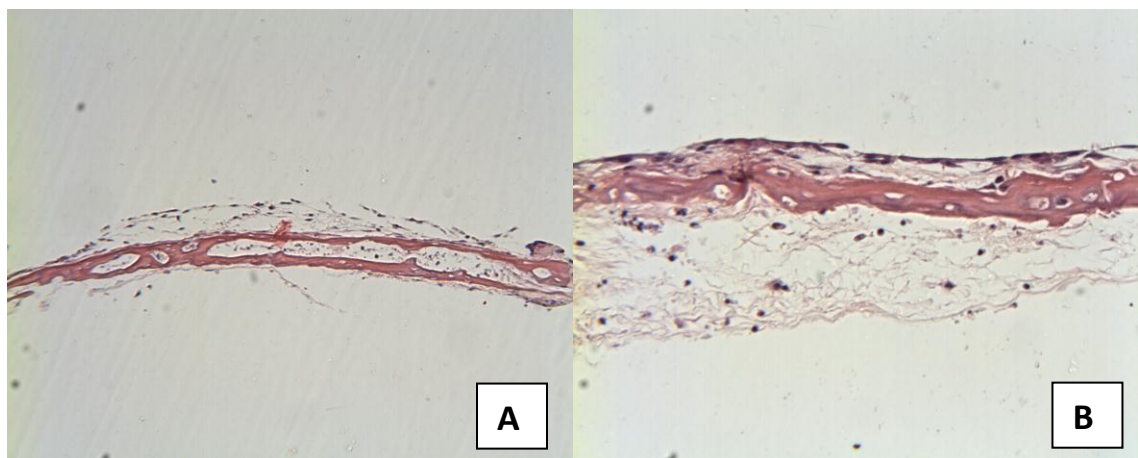
Figure 3-4: Intramembranous bone formation of calvaria in mid dose ESW group. As
shown, some regions show filled osteocyte lacunae, other regions show empty
osteocyte lacunae. Mixed regions of hypertrophied osteoblasts in intramembranous
ossification along some surfaces. Flattened bone-lining cells (osteoblasts) along other
surfaces. No osteoclasts and no microcracks were observed. Magnification: x79 (A) and
x200 (B).

4. **High dose ESW group**

    Intramembranous bone formation of calvaria in the high dose ESW group was
    observed. Many regions showed empty osteocyte lacunae. No osteoblasts were
    seen and there was no intramembranous ossification along the surface. In some
    samples, karyorrhexis and karyopyknosis was observed. In all the samples, no
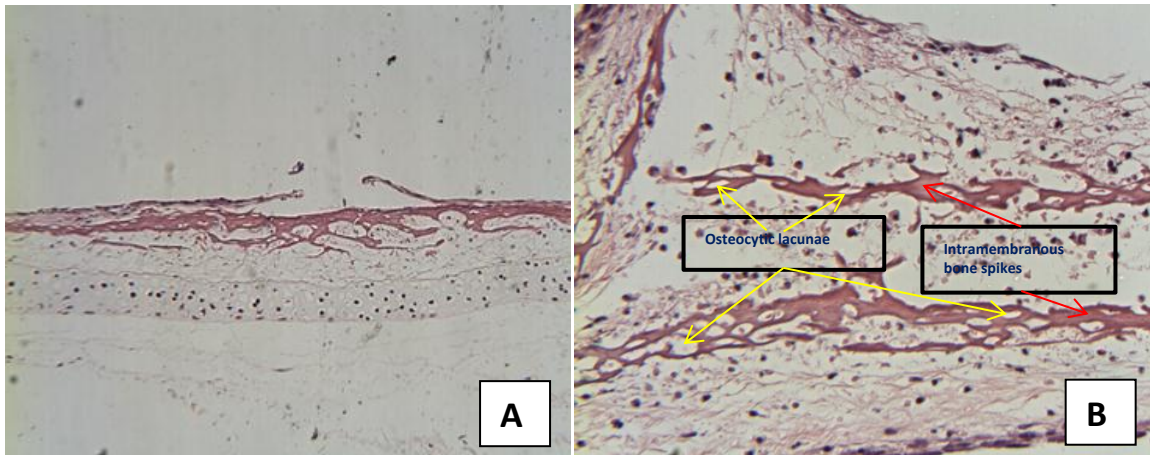   osteoclasts or microcracks were observed (Figure 3-5).

Figure 3-5: Intramembranous bone formation of calvaria in high dose ESW group. As shown, many regions with empty osteocyte lacunae. No osteoblasts, no intramembranous ossification along surface, no osteoclasts, no microcracks. Magnification: x79 (A) and x200 (B).

CHAPTER V

DISCUSSION

Long treatment time is one of the biggest challenges in clinical orthodontics, with negative consequences such as root resorption and white spot lesions. The reduction of treatment time has become a priority for many orthodontic clinicians and the focus of researchers. Orthodontic tooth movement (OTM) is a mechanically induced bone modeling process wherein bone resorbed on the pressure side of periodontal ligament and deposited on the tension side. Decortication (surgically drilling and/or cutting alveolar bone between dental roots) has been used to move teeth faster through wound healing (an accelerated bone remodeling process), which however is a surgical procedure and not well accepted by patients with questionable results in the literature (Proffit *et al.,* 2012). Therefore if a non-surgical approach can be found to accelerate bone modeling to help move teeth faster, it will be a benefit to patient care. As shown in previous studies, ESW can generate microcracks which have been shown to be able to increase the rate of bone remodeling. This led us to speculate whether the ESW can be used to modulate alveolar bone remodeling rate (possibly through microcracks), in turn to help move teeth faster. Since ESW is non-invasive with few side-effects, a proven effect on bone remodeling may be a viable alternative to various forms of invasive decortication procedures being used in a limited population of orthodontic patients.

The main aim of this project was to determine the effects of different levels of ESW stimulation on mouse calvaria bone organ culture model. Secondary aims were to explore the cellular mechanism and the proper dose of ESW on bone *ex vivo*. In the current literature several studies have reported negative or no effect of ESW on bone. Augat *et al.* applied 500 shock waves at a relatively low energy (14kV) to sheep calcaneous *in vivo* and found no new bone formation at the periosteal surface even (1995). From this study it was concluded that there was insufficient energy to reach the threshold needed for bone stimulation. High energy ESW has been well reported in the

literature 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). In one study, human osteoblastic cells in a culture were treated with 0.15 or 0.31 mJ/mm^2 for 500 or 1000 pulses and found that low levels of ESW resulted in cytostimulation and no biologic adverse effects (Martini *et al.,* 2003). Lyon et al. conducted a study using the same shock wave device as this study and found that a dose of 0.25mJ/mm^2 significantly improved the cell viability of chondrocytes, while a higher dose of 0.55mJ/mm^2 decreased cell viability (personal communication). Using cell culture model and the same experimental set up as used in this study, Barta showed that the mid dose of ESW showed a significant anabolic (forming bone) effect, comparing to low (0.10mJ/mm^2) and high (0.50mJ/mm^2) doses (Barta, 2012). This agrees with our results showing that although not statistically significant (P = 0.237) the mid dose group showed a relatively higher bone microdensity than the control, low and high ESW groups. The high dose group (0.50mJ/mm^2) had evidence of cell death. This group exhibited no osteoblasts, and no intramembranous bone formation with evidence of karyopyknosis and karyorrhexis. Therefore, the high dose is not appropriate due to the adverse events.

The type of ESW energy generator can also affect its application. The experimental set up (ESW machine) used in our study was specially designed to generate a focused (with a focal point) ESW. This is especially important and meaningful because a serious concern is that when applied intra-orally (if in the future), many of the oral and dental tissues approximate to interdental alveolar bone will be potentially exposed to ESW if its energy is not focused. In order to achieve the desired outcome, it is necessary that the shock wave is precisely focused on the fracture or osteotomy (Gerdesmeyer and Hausch, 2001). For this reason, in our study we used focused ESW that can target the interdental alveolar bone without damaging the surrounding mineralized tissues like enamel.

Limitations

This study was performed in an *ex vivo* model with no blood flow or intact innervation. A special attention needs to be paid when interpreting the *ex vivo* data to clinical application.

A limitation in this study is the heterogeneity of the bone cuts of calvaria. Due to the lack of neonatal mice, we cut each calvaria into four equal (approximate) quadrants, and pooled all the bone cuts together. This introduced variation from cut to cut, although random assignment into the experimental groups was done. If halves of the calvaria along sagittal suture can be used as individual samples, the standard deviation of the results can be reasonably reduced.

Contrary to our expectation, no microcracks were found in our study. This can be due to several reasons. The mineralization degree of our collected calvaria is relatively low (3 days after birth) when used as a model to target bone remodeling. However, the viability of the tissue organ culture can also be a challenge if older calvaria are used. Another possible reason is we did not use the authentic method of Basic Fuchsin staining to reveal microcracks. H&E staining may be enough for relatively bigger cracks but may not be sufficient for micro-cracks.

Conclusions

Based on the radiographic and histological results, our hypothesis that proper application of ESW will increase bone turnover rate through generating micro-cracks in bone cannot be proved in this study.

Future Studies

Future endeavors will be required to overcome the shortcomings of this study and advance the study on this topic.

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