Stony Brook University

Low Intensity Vibrations Applied Locally can be Transmitted to the Alveolar Bone Thereby Enhancing the Quality of the Bone in Adult Rats

A Thesis Presented

by

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to

The Graduate School

in Partial Fulfillment of the

Requirements

for the Degree of

Master of Science

in

Biomedical Engineering

Stony Brook University

May 2011

Stony Brook University

The Graduate School

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Abstract of the Thesis

Low Intensity Vibrations Applied Locally can be Transmitted to the Alveolar Bone Thereby Enhancing the Quality of the Bone in Adult Rats

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Oral bone loss is a major concern amongst a large population suffering from estrogen deficiency, osteoporosis and periodontal diseases. The clinical implication of oral bone loss is premature tooth loss. Mechanical loading has proven to be vital in maintaining the quantity and quality of the bone, particularly in impacted bone. Studies have shown that long bones (post-cranial) and alveolar bone (cranial) are both sensitive to mechanical environment through static and dynamic loading. It also has been suggested that whole body vibrations (WBV) at low-magnitude and high frequency are a non-invasive and non-pharmacological method to prevent bone loss, particularly in the long bones. However, not much has been said about the application of these low-magnitude high frequency loads on the alveolar bone. In this pilot study, it is determined if low magnitude of 0.3 g (where g = 9.8ms^{-2}) and high frequency of 50 Hz, localized vibrations can be transmitted to the alveolar bone and also enhance the quantity and quality of the alveolar bone. Twelve-week old, healthy male Sprague Dawley rats were

subjected to localized low intensity vibrations (LIV) for 3 min/day, 5 days/week over 6 weeks. Some studies on long bones suggest 6 weeks of low intensity whole body vibrations (WBV) (0.3 g, 30 – 90 Hz) are sufficient to induce anabolic changes, especially in the trabecular bone. In this pilot study, an actuator was designed to transmit these LIV locally to the mandible (lower jaw) of the rat. However, the magnitude and frequency of the actuator could not be controlled individually as it was connected to a power source. Validation of this actuator confirmed the repeatability and the strength of these LIV over a period of 1-week. At the end of the 6-week experiment, the LIV failed to enhance the quantity of the alveolar bone, however, it was able to increase the mineralization of the bone in the vibrated group by approximately 5%. It was found that the increase in mineralization was not caused by the changes in the chemical composition of the alveolar bone but perhaps attributed to the activation of osteocytes, which caused an increase in the mineralization. Due to the nature of this pilot study, healthy male rats were administered with an extremely short bout of LIV. Studies have shown that bone responsiveness to LIV is dependent parameters such as duration, bout, magnitude and/or frequency. Using the information obtained from this pilot study, we believe, that applying these LIV on an osteoporotic animal model, using different combinations of mechanical loading can provide critical information on the potential treatment modality to prevent oral bone loss caused by diseases such as osteoporosis and periodontitis. Ultimately, from a broader perspective, our application of LIV on the teeth seeks to explore the potential benefits of brushing teeth with an electric toothbrush, to not only clean teeth, but also to maintain healthy teeth caused by LIV, in order to improve the quantity and quality of the alveolar bone.

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Table 7: Chemical parameters obtained using FTIR under Molar 1 is expressed as Mean ± SD. None of the chemical parameters showed significant differences between the Sham and the Vibe groups.

LIST OF ABBREVIATIONS

BMD – bone mineral density

BFR/BS – bone formation rate

BV – bone volume

BV/TV – bone volume fraction

DEXA - dual-emission X-ray absorptiometry

dL.S/BS – double labeled surface

FTIR – Fourier transform infrared spectroscopy

g- force – earth's gravitational force (9.8 ms^{-1})

HA – hydroxyapatite

LIV – low intensity vibrations

LS/BS – labeled surface

µCT – micro computed tomography

MAR – mineral apposition rate

OVX – ovariectomized

PDL – periodontal ligament

PMMA – polymethylmethacrylate

ROI – region of interest

SD – standard deviation

sL.S/BS – single labeled surface

TV – total volume

WBV – whole body vibration

ACKNOWLEDGMENTS

Firstly, I would like to thank my advisor, Dr. Clinton Rubin for giving me an opportunity to work in his lab as a research assistant and for his invaluable advice and guidance through all aspects of my research.

I would also like to thank Dr. Stefan Judex and Dr. Lisa Miller for serving as members of my thesis committee.

Thank you to Dr. Lisa Miller and Alvin Acerbo at National Synchrotron Light Source in Brookhaven National Laboratory for their kind assistance and support during the course of the study.

I would like to thank all the members of Rubin, Judex and Qin labs who helped me through every step of the way from the inception to the completion of this study. A very special thank you to Dr. Ete Chan for her continued effort and great support, I just cannot thank you enough.

Lastly, the support of my family helped me through this and more. Thank you.

CHAPTER 1

INTRODUCTION

Bone

Bone tissue accounts for approximately 98% of the skeleton, which is the permanent supportive framework of the body. The remainder largely consists of cartilage primarily in the form of intervertebral disks in the spine. Osteocytes are the definitive bone cells, which are embedded in concentric layers of bone matrix around a central canal forming a Haversian Canal. The bone matrix consists of the organic component such as collagen fibers and inorganic components such as mineral crystals derived from calcium and phosphorus. Compounding the collagen fibers with the mineral crystals to form a compact, hard unit forms the matrix. The rigidity of bone is provided by the mineralization of the bone. The hydroxyapatite crystals are imperfect crystals of calcium phosphate salt, having substitutions by magnesium, sodium, strontium, carbonate, citrate and fluoride. Furthermore, the osteocytes are embedded in the matrix and regulate the flow of minerals and nutrients between the matrix and the blood. The other bone cells that are involved with the process of bone deposition and bone resorption are osteoblasts and osteoclasts, respectively. Osteoblasts are known as the bone forming cells as they are found on the bone surfaces and in bone cavities. Bone deposition involves the production of collagen and ground substances. As a result, the osteoblast often gets entrapped in the collagen and ground substances to become an osteocyte. Osteoclasts, on the other hand, resorbs bone tissue and are always found in areas of a bone, which are undergoing resorption – removal of bone matrix and the release of minerals into the blood circulation. Resorption of the bone matrix occurs during growth of individual bones and is essential to maintain the shape of the bone [1].

The bone homeostasis is mediated by the coordinated actions of osteoblast-osteoclast coupling. The concept of coupling is based on the idea that osteoblast influences the osteoclast

formation and activity and likewise osteoclasts influence the osteoblast differentiation and activity. Currently, most of the understanding in bone revolves around the influence of osteoblast on osteoclast formation. Firstly, osteoblasts express the majority of the cytokines that regulate osteoclast progenitor differentiation including M-CSF, RANKL and OPG in the bone. During osteoblast differentiation, the level of expression of these cytokines changes with the immature osteoblast producing the highest levels of M-CSF and RANKL. Therefore, the osteoblast begins to mature into a matrix producing bone cell, it signals to local osteoclast precursors with RANKL to differentiate, thereby coupling the new bone formation with the recruitment of new osteoclasts for its subsequent remodeling. Therefore, by coordinating osteoclast differentiation with osteoblast differentiation, the system stays in a state of homeostasis. To summarize the remodeling of the bone tissue, osteoclasts always appear first as they are involved in bone resorption, after which they are followed by osteoblasts, which deposit unmineralized bone matrix, or osteoid, and many of the osteoblasts get entombed in the mineralizing matrix and become into osteocytes [1].

In a growing, healthy individual, bone deposition occurs at a more rapid rate than bone resorption. In young and middle-aged adults, the rates of deposition and resorption are in equilibrium, whereas in old age, bone resorption occurs at a more rapid rate than bone deposition, and there is a net loss of bone [1].

Bones are essentially formed by two processes: intramembranous and endochondral processes. The intramembranous develops during fetal development without the involvement of the cartilage. The intramembranous bones include most of the skull bones. The endochondral bone, on the other hand develops during the fetal development in the presence of cartilage. The endochondral bone includes all the bones of the post-cranial skeleton such as the long bones and some cranial bones. Most bones are formed by one of the two processes mentioned, however, the mandible (lower jaw) is unique in that they originate bone endochondrally and intramembranously [1].

Anatomy of the mandible

The mandible is considered to be the largest and the strongest bone of the face. It serves for the reception of the lower teeth. Growth of the mandible is a result of formation and resorption and remodeling of the bone [2]. The growth of the mandible takes place in a number of directions. Some growth occurs on the superior and inferior borders of the mandible, however, the vertical growth of the mandible is determined by the mandibular condyle. Growth of the condyle is generally in superior and posterior direction, translating the mandible growth in an anterior inferior direction. The posterior vertical portion of the mandible is subjected to resorption on its anterior border and to deposition on its posterior border. This pattern of resorption and deposition lengthens the mandible [3].

The mandible is a unique mammalian structure during its development as it involves cells that originate from the neural crest, which populate the first pharyngeal arch. The endochondral differentiation of the neural crest cells occurs to create osteoblasts that form the intramembranous bone. The neural crest cells also differentiate to form Meckel's cartilage in the mandible. Therefore, the mandible ossifies through both endochondral ossification and also through the intramembranous process [4]. In the growth physiology, long bones and mandible undergo different ossification processes, however, they undergo the same mechanism of osteoblast differentiation to form osteoid and eventually mineralized bone [2].

Mineralization of Bone

Calcium hydroxyapatite is the main component of mineral formation in bone in humans. Calcification of an osteoid (bone mineralization) is an orderly process as the hydroxyapatite is embedded in the empty zones of the collagen molecule and does not disrupt the spatial organization of collagen. The mineral is initially deposited as an amorphous calcium phosphate. This initial solid phase is randomly oriented. Subsequently a serious of solid phase transformations occurs that leads to a crystalline hydroxyapatite as the final stable solid phase. In mature bone, there is a high possibility of crystalline calcium carbonate containing

hydroxyapatite being deposited rather than an amorphous calcium phosphate or hydroxyapatite [5].

Mineralization is potentially regulated by genetic, hormonal and mechanical factors. Other exogenous factors that affect mineralization include aluminum intoxication, fluoride intoxication and phosphate deficiency [5].

DEXA technology is at present the most commonly used method to study the bone mineralization from early childhood into adulthood. DEXA provides an estimate of total-body bone mineral content (g) and the total bone area (cm^2). The ratio of total body bone mineral to total bone area is used to estimate bone mineral density (g/cm^2). More recently, magnetic resonance imaging, MRI, is used to visualize and quantify trabecular bone and aid in the understanding of the architecture of skeletal tissue [1].

Alveolar Bone

Alveolar bone, also known as alveolar process, is the bone that contains the tooth sockets on bones that bear teeth (**Fig. 1)** such as the mandible and maxilla. On the mandible, the alveolar process is a ridge on the inferior surface, while on the maxilla it is a ridge on the superior surface [6]. The mandible contains a region of cortical bone, which forms the mandibular base and trabecular bone, which is commonly known as alveolar bone. However, the alveolar bone cannot be clearly demarcated from the mandibular base or the cortical bone [2].

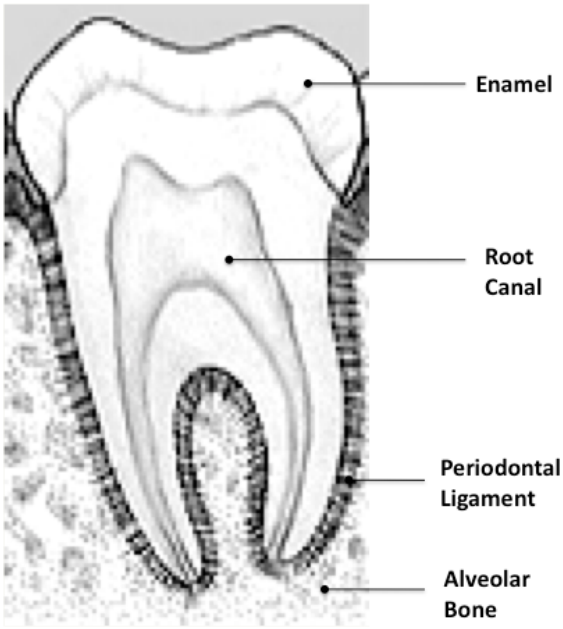


Figure 1: Schematic of a tooth, showing the enamel, root canal, periodontal ligament and alveolar bone.

Development of Alveolar Bone

The osteoblasts of the alveolar bone arise from the same population of cells, which produce odontoblasts that deposit dentine. Alveolar bone will not form until the dental follicle mesenchyme is present, which means that if teeth are not present, neither is alveolar bone [7]. In order to achieve tooth movement, remodeling of the alveolar bone surrounding the dental roots is required. Like any other bones, alveolar bone remodeling involves a complex network of cells, cell interaction and cell-matrix interactions controlled by hormones, growth factors and cytokines. During tooth movement, the alveolar bone is removed from the path of the moving tooth by bone resorption (osteoclasts). At the same time, bone apposition (osteoblast) takes place in the tension sites to fill the space caused by the drifting of the tooth [8]. Alveolar bone is among the most physiologically active bone in mammals, perhaps the bone that can be resorbed the fastest [9]. However, bone resorption is a much faster process than bone apposition as it can take up to 3 months to replace bone resorbed in only 2 to 3 weeks in humans [8]. Similar to humans, alveolar bone remodeling in rats is associated with the continuous drift of the teeth throughout the life of the animal [9].

The supporting tissues of the tooth, which include the periodontal ligament (PDL) and alveolar bone (**Fig. 1**), have a remarkable plasticity that permits physiologic tooth movement and accommodates to the constant minor movements that the tooth makes during mastication. This plasticity of the PDL and alveolar bone permits orthodontic tooth movement. Light forces, equivalent to physiologic forces can be used to bring about tooth movement without causing supporting tissue damage. Differentiation of osteoclasts occurs and they resorb the alveolar bone on the pressure side. At the same time remodeling of collagen fibers in the PDL occurs to accommodate the new tooth position. On the tension side, bone formation occurs along side with remodeling of collagen fiber bundle. Morphologically, as a tooth migrates, one side of the socket is in continuous bone formation, while the opposite side shows cyclic resorption and formation within small foci. However, unlike physiologic tooth movement, in which bone resorption of the alveolar wall occurs on its PDL aspects, orthodontic tooth movement also

causes some undermining resorption, causing the alveolar bone to remodel from its endosteal face [10].

A study indicated that the alveolar bone remodeling occurs as the tooth migrates at a rate of 6.7 μm per day. The total duration of each remodeling cycle is about 6 days in the alveolar bone of an adult rat. This time is a lot shorter compared to 60-120 days in the adult human trabecular bone [9]. Therefore, rat alveolar bone remodeling offers an ideal biological system to accurately measure the cellular activities involved in bone modeling and remodeling.

Causes for Alveolar Bone Loss

Periodontal diseases

According to NIH and the Surgeon General's Report on Oral Health, 42% of Americans over the age of 65 are toothless, meaning having lost at least one tooth. Also, oral-bone loss and subsequent tooth loss costs an estimated $5-6 billion/year for just the cosmetic and functional surgical treatments related to bone restoration and tooth salvation [11]. The most common cause of alveolar bone loss is attributed to chronic periodontitis. It is considered the most common oral disease of adulthood, however, early features of the disease are sometimes apparent in teenagers and young adults [12]. It affects approximately 35% of dentate adults in the United States between ages 30 – 90, and approximately 13% have a moderate to severe form of the disease [13].

Periodontitis is an inflammatory disease characterized by loss of connective tissue and loss of alveolar bone. Periodontitis is considered a "silent" disease, not causing symptoms until late in the disease process when tooth loss may occur. While the etiologic agent in periodontitis is a pathogenic bacterial plaque in a susceptible patient, periodontitis and osteoporosis have several risk factors in common. Risk factors include an increased prevalence with increasing age, smoking, influence of other diseases and even hereditary [14].

The treatment of periodontitis is to mainly control the infection. The number and types of treatment will vary depending on the extent of the diseases. Medications include antimicrobial mouth rinse, antibiotic gels, antibiotic microspheres, enzyme suppressant and oral antibiotics. However, for chronic cases of periodontitis surgery such as flap surgery and bone and tissue grafts is required to promote bone formation and growth [15]. In addition oral bisphosphonates have also shown to be effective in controlling and reducing the occurrences of tooth loss in patients with moderate to severe periodontal diseases over a 24 months period [16]. However, cases of osteonecrosis of the jaws have been observed in some patients receiving bisphosphonates for at least 36 months [17]. In saying that, a study revealed that osteonecrosis of the jaws occurred mostly in subjects that received intravenous bisphosphonate instead of oral bisphosphonates [16].

Osteoporosis

The clinical significance of osteoporosis lies in the susceptibility to cause fractures when the bone density is reduced [18]. The risk of fractures caused by osteoporosis is greater in women than in men and it varies appreciably between countries [19, 20]. A few prospective studies that exist to date suggest that alveolar bone loss is more prominent in women suffering from osteoporosis or low estrogen levels [21]. In the US alone, osteoporosis causes 1.3 million fractures per year, thereby imposing a tremendous burden on the health care cost [22, 23]. The National Osteoporosis Foundation reported the expected cases of osteoporosis is estimated to increase from 10 million people in 2000 to 14 million people in 2020 [24]. As of 2005, more then 2 million incidents of fractures caused by osteoporosis have been estimated to cost $17 billion [25]. Osteoporosis, most frequently encountered in postmenopausal women, is not exclusive to women as it also occurs, albeit to a lesser extent in men [26] and younger individuals [27]. In the USA, it has been estimated that 30% of postmenopausal women are osteoporotic and by the age of 80 they are expected to rise to 70% [28]. According to the World Health Organization (WHO), a bone is considered osteoporotic if the bone mass in more than 2.5 SD below the mean peak

bone mass in young healthy adults [29]. It has been projected that by 2025, annual fractures and costs are expected to rise by almost 50% [24].

All of the diseases of bone occur as a consequence of the effect of cellular events in the normal bone remodeling cycle. In the cases of osteoporosis and alveolar bone loss, there is an imbalance in the osteoclast and osteoblast activity. Therefore, when the osteoblast activity does not completely repair the defects left by the resorption of the osteoclasts, thinning of bone occurs [30].

Osteoporosis is a serious health and economic issue and appropriate measures have been taken in order to decrease the occurrences by pharmacological and non-pharmacological intervention [31]. Since osteoporosis is characterized by deterioration of the bone structure and a reduction in bone mass such that the bone is susceptible to fracture with very little impact. Therefore, the ideal treatment for osteoporosis would be with agents that improve the bone strength thereby improving bone fracture rates [32]. Several antiresorptive therapies like estrogen replacement therapy (ERT), various bisphosphonates such as alendronate, the SERM raloxifene, calcitonin and sodium fluoride, including calcium and vitamin D have been approved modalities for the treatment and prevention of bone loss. However, intermittent administration of parathyroid hormone (PTH) is considered an ideal therapy for osteoporosis [31] as it is considered anabolic to bone. All of these agents main effect is to reduce the bone turnover, however, by reducing the bone turnover, they effectively prolong the secondary mineralization phase of the bone remodeling cycle by suppressing the osteoclast activity, thereby improving bone strength. The balance of bone formation over bone resorption has proven to be positive with antiresorptive agent treatment, as seen by a significant increase in the bone mineral density (BMD) within the first 2 to 4 years of therapy. However, this effect has shown to plateau as well [32]. On the other hand, parathyroid hormone (PTH) is the principal regulator of calcium homeostasis in mammals. PTH is released when serum calcium levels are low and are suppressed when the calcium levels are high and they also regulate the bone metabolism. Most importantly, PTH has anabolic properties on bone, meaning it promotes bone formation followed by some bone resorption. Several theories on the mechanism of PTH have been

postulated to explain the cause of anabolism, however, the most likely theory suggests that the rapid anabolic action is the result of a combination of factors that includes increasing the lifespan and activity of the osteoblast [32]. Studies have demonstrated statistically significant increases in bone mass and BMD of healthy adult rats when subjected to PTH for only 10 days [33] and increase in BMD in postmenopausal women with osteoporosis [34].

It is well established that skeletal unloading such as that occurs in spinal cord injury, prolonged bed rest, limb immobilization and microgravity results in generalized skeletal loss and loss of mineralization of the skeleton, particularly in bones that bear weight under normal conditions. This bone loss is primarily due to elevated bone resorption caused by osteoclast activity and decreased osteoblast mediated bone formation [35, 36]. The effect of additional loading on the skeleton is not only variable but also not very well understood. The bones response to exercise varies as a function of skeletal age, diet, hormonal level and also the nature of the activity [35]. Weight-bearing activities and muscle strength/endurance exercise programs are also said to play an important role in suppressing bone loss. The two main determinants of bone mineral density are the magnitudes of peak bone mass achieved and amount of bone lost. Therefore, peak bone mass is a major determinant of bone mass later in life and increased amounts of peak bone mass may decrease the risk of osteoporotic fractures. Genetic factors play an important role in determining the peak bone mass. The other factors include environmental factors such as nutrition and exercise. Post menopausal bone mass is related to physical activity and also calcium intake. Therefore, exercise plays an important role in the development of peak bone mass [36]. Research suggests that exercise is a viable strategy for the management of osteoporosis. However, the impact is greater when used in conjunction with other treatment modalities. Postmenopausal women who exercise and are treated with estrogen have shown to have a greater increase in the bone mineral density than in women without the estrogen treatment [37]. Studies have suggested that bone remodeling is influenced by mechanical stress. There is a positive relationship between decreased risk of osteoporosis and increase in exercise. In particular, exercise has long been shown to enhance bone mineral accrual during growth. In a study conducted on young female, the bone mineral density of young gymnasts was shown to have a 30-85% increase over that measured in

sedentary children [66]. Therefore it can be concluded, that bone loss with aging, estrogen deficiency or osteoporosis may be mediated by an increase in skeletal loading. It is clear from the research that weight-bearing activities are an important component of management of osteoporosis [36].

Studies in the past have also shown that low magnitude mechanical signals are anabolic to long bones and are a potential non-pharmacological intervention to treat osteoporosis [38]. Furthermore, studies have demonstrated that mechanical loading, originating from either muscular function or gravity, is an important factor in bone homeostasis and in maintaining structure and mass of the bone throughout lifetime. Unlike the long bones, the alveolar bone is completely dependent on masticatory function, since in the absence of teeth or denture-transmitted mechanical stimulation it undergoes disuse atrophy. Therefore, the sensitivity to and dependence on mechanical stimulation are possibly the main reasons for a uniquely high responsiveness of periosteal and endosteal alveolar bone surfaces to mechanical stresses and also a high level of remodeling activity under quiescent physiological conditions. Therefore, it is clear mechanical signals are also anabolic to alveolar bones cellular responses [39].

Bones Sensitivity to Mechanical Loading

For the prevention and treatment of osteoporosis, physical exercise is highly recommended [40, 41]. In humans, the practical goal of an exercise intervention is not merely to increase bone mass, but also to reduce the incidence of fractures. The etiology of osteoporotic fractures includes both low bone mass and falls. Falls account for over 90% of hip fractures and over 50% of vertebral fractures. Therefore, developing exercise intervention that serve to improve bone mass and prevent falls is necessary to reduce the risk of fractures. The animal studies in the past have clearly suggested that bone responds preferentially to certain forms of mechanical loading. It has been long been known that factors such as high-magnitude loads that induce relatively large bone strains are more osteogenic than low-magnitude loads [42]. Early work using the turkey ulna clearly showed that bone formation increased with larger strain magnitudes, until it reached a saturation point where the effect of the strain reached a plateau

[43, 44]. This effect was referred to as “mechanostat” theory that describes a control system in which a minimum effective strain is necessary for the maintenance of the bone [45]. The bone responds variably depending on the extent of loading. A strain magnitude of < 50 – 200 microstrain has no effect on the bone remodeling as the mechanical stimulus is not felt by the bone. Studies conducted during bed rest and flight space reveal that this results in a net loss of bone over times [46, 47]. Strains in the physiological loading zone of approximately 200 - 2000 microstrain maintain remodeling at a steady state, that in turn maintains bone strength [48]. If the loading-induced local strain exceeds 1500 – 2000 microstrain, the bone is in a state of overuse. When the strain magnitude increases 2000 - 3000 microstrain, the bone enters the overload zone where modeling is stimulated and new bone is added in response to the mechanical demand. However, when the strain magnitude exceeds 4000 microstrain, bone suffers microdamage [49]. Several studies in the past have demonstrated the sensitivity of bone to mechanical loading by the interrelationship of load magnitude, cycle number and strain frequency. At higher frequencies, much lower strains are necessary to maintain bone mass. It has been predicted that the strain magnitudes required for maintaining bone mass can be much lower than 70 microstrain at very high loading frequency. This can be interpreted, as the bone requires extremely low-level magnitudes, to stimulate the bone formation if delivered at a suitably high frequency [38, 50, 51]. Studies have shown that the strain on the bone surface induced by low-level mechanical vibrations at 0.3 g is less than 10 microstrain, which are far below than what might cause damage to the bone [52]. From an *in vivo* study conducted on specimens of Galago crassicaudatus, to assess the bite force and the associated bite strain on objects of different hardness, the maximum values of bone strain and bit force were recorded at 435 microstrain, which is still lower than what might potentially cause damage [53].

Both the long bone and alveolar bone are very sensitive to altered mechanical environment through static and dynamic loading [54]. Studies have investigated that whole body vibrations (WBV) at low-magnitude and high frequency are a non-invasive and non-pharmacological methods to prevent post-ovariectomy bone loss in an animal models [55] and also in postmenopausal women [56]. Recent studies have indicated a high sensitivity of bone to low level mechanical signals of less than 0.3 g (where $g=9.8ms^{-2}$) coupled with high frequency in

the range of 20-90 Hz when low-magnitude, high frequency WBV are employed [43, 57]. These mechanical signals are far below that what might potentially cause damage to the bone and are therefore safe. Clinical trials of these WBV have shown that the low-level, high frequency mechanical signals are readily transmitted to the lower appendicular and axial skeleton of an individual standing on the vibrating plate. There is growing evidence that these low-level, high frequency mechanical signals are anabolic to bone and thereby a potential non-invasive and non-pharmacological treatment for osteoporosis [58]. In animal models such as rodents and larger mammals, these signals promote bone formation [59], enhance bone morphology [57] and also increase bone strength [60] with as little as 10 min per day. However, studies have indicated that these signals are anabolic to the trabecular bone and not so much in the cortical bone due to the less metabolic activity in the cortical bone [61].

Various theories on the mechanisms of tooth eruption exist. These theories may be attributed to the immense success of orthodontics in moving teeth with force application. Unlike tooth eruption, orthodontic tooth movement is a process that combines both pathologic and physiologic responses to externally applied forces. Orthodontic tooth movement is accompanied by minor irreversible injury to the tooth-supporting structures such as the periodontal ligament (PDL) and alveolar bone. This is superimposed by the physiologic adaptation of alveolar bone to mechanical strains. Therefore, there has been a growing need to understand relevant inflammatory mechanisms along with skeletal mechanotransduction for a thorough understanding of orthodontic tooth movement.

The orthodontic tooth movement consists of three distinct phases: an initial tooth displacement, a delay phase where no visible movement occurs and a period of linear tooth movement. The applied forces create strains in the tooth-supporting tissues causing compressive and tensile forces on the alveolar bone. The tensile forces causes bone resorption while the compressive causes bone formation. Tensile forces in orthodontia have been characterized as being osteogenic and the nature of the applied load determines the osteoblast recruitment. Static loads do not seem to play an important role in skeletal osteogenesis. Instead,

osteogenesis is driven by dynamic loading, where the most important characteristics are strains rates, frequency and the duration of the dynamic loading [62].

Amongst the bone cells present, osteocytes are considered the most sensitive cell to mechanotransduction in the bone. Osteocytes have several characteristics that make them most sensitive to mechanical signals. They are located throughout the bone tissue and have cellular processes that are easily able to deform; osteocyte cell processes are bathed in pericanalicular fluid that is in a confined space and therefore very susceptible to slight changes in fluid flow brought about by mechanical perturbations and the osteocytic processes are connected to each other through gap junctions that facilitate the transmission of signals throughout the tissue [63, 64]. Therefore, teeth movement occurs through alveolar bone, whether through the normal process of tooth eruption or by strains generated by orthodontic appliances. Both eruption and orthodontics accomplish this through similar biological processes, osteoclastogenesis and osteogenesis [64].

Current study

Previous studies have worked to evaluate the bone's response to low level mechanical signals as the basis of a non-pharmacologic intervention for osteoporosis, but have focused on the post-cranial skeleton [38, 57, 65]. Furthermore, the sensitivity of both the long bones [61] and the alveolar bone [54] to mechanical signals has been address in the literature. These low-magnitude, high frequency mechanical signals are anabolic to bone and thereby promote bone formation [43]. The process of ossification in the cranial skeleton very different to that of the postcranial skeleton, however, the mechanism of bone cells: osteoclasts, osteoblasts and osteocytes are identical [2]. It has been well established that mechanical loading is crucial to maintain the integrity of bone as disuse results in bone loss in both cranial and postcranial skeleton. Studies on mechanical loading of the skeleton have proven to improve bone quality and quantity and also minimize falls, which could potentially cause osteoporotic fractures [35]. Furthermore, low-level mechanical signals on the postcranial skeleton have shown to have a positive effect on the bone formation and mineralization [57]. However, it is unknown to what

degree these low-level mechanical signals can influence the formation, resorption and/or mineralization of alveolar bone.

From a preliminary study conducted on mice, it was determined that WBV does not transmit the mechanical signals all the way to the skull as most of it is potentially absorbed by body, joint movement and long bones. Therefore, the premise of our study is to determine if low-level mechanical signals, which are administered locally, can influence the bone resorption, formation and remodeling activities, thereby altering the mechanical properties of the alveolar bone. If these mechanical signals are beneficial to the mandibular morphology and/or alveolar bone, it may serve as a foundation to explore in people to slow tooth loss. Because these signals are well below than what might potentially cause microdamage, low-level mechanical signals of about 0.3 g, coupled with high frequency can be explored as a potential non-invasive, non-pharmacological intervention to prevent alveolar bone loss, thereby slowing tooth loss caused by osteoporosis and also periodontal diseases. To examine this hypothesis, we developed an actuator capable of delivering low magnitude high frequency signals to the mandible of an anesthetized animal. Using this actuator tested our **global** hypothesis that low intensity vibrations are able to enhance parameters of quantity and quality of the alveolar bone. Based on our global hypothesis, we explored the following hypotheses and specific aims:

Hypothesis 1

Mechanical signals are transmitted to the alveolar bone when low-intensity vibrations (LIV) are applied to the mandible.

Specific Aim 1

A mechanical loading device will be designed and validated to generate low magnitude mechanical signals to the mandible in the range of 0.2 g - 0.4 g peak (where g=9.8ms^{-2}) and high frequency signals in the range of 50 Hz – 90 Hz. This actuator will be designed to be placed in the jaws of male rats to locally stimulate the alveolar bone.

Hypothesis 2

Low intensity vibrations (LIV) will enhance the bone quantity of the alveolar bone when localized vibrations are applied to the mandible.

Specific Aim 2

Investigate the short-term effects of LIV on the morphological changes to determine the quantity of the alveolar bone in 12-week old male Sprague Dawley rats when subjected to LIV (50 Hz, 0.3g) for 6 weeks (3min/d, 5d/wk). We will assess the bone morphology and the quantity of the alveolar bone using μCT and dynamic histomorphometry.

Hypothesis 3

Low intensity vibrations (LIV) will enhance the bone quality of the alveolar bone when localized vibrations are applied to the mandible.

Specific Aim 3

Investigate the short-term effects of LIV on the quality of the alveolar bone in 12 weeks male Sprague Dawley rats when subjected to LIV (60 Hz, 0.3g) for 6 weeks (3min/d, 5d/wk). We will assess the quality of the alveolar bone using μCT and Fourier transform infrared spectroscopy (FTIR) to understand the degree of mineralization and the changes in chemical composition caused by LIV.

Ultimately, from a broader perspective we hope to determine if there may be some benefit of brushing teeth, perhaps optimized as based on the mechanical parameters enabled by using an electric toothbrush, to maintain healthy teeth caused not only due to cleaning teeth but also due to the low magnitude high frequency mechanical signals to improve the quality of the alveolar bone.

CHAPTER 2

Mechanical signals are transmitted to the alveolar bone when low intensity vibrations (LIV) are applied to the mandible

Abstract

The skeletal system, specifically cranial and post-cranial skeleton, is sensitive to mechanical loading. Several studies have indicated the efficacy of low-level mechanical signals to enhance the quality and quantity of the post-cranial skeleton when subjected to whole body vibrations. Clinically, these signals may someday represent a potential treatment for osteoporosis. However, not much is known about the responsiveness of these signals on the alveolar bone, which supports the teeth. In this pilot study, the transmissibility of these mechanical signals to the alveolar bone was assessed when applied to the mandible. Twelve-week old male Sprague Dawley rats were employed in this study and were divided into two groups (n=10): Sham control and Vibe group. An actuator and a set-up was designed that could transmit the low intensity vibrations (LIV, 0.3 g, 50 Hz) through the mandible. An electric toothbrush was connected to the power source that served as an actuator. The animals were anesthetized and placed on bedding while being locally vibrated at the mandible. An accelerometer, which was placed used the head of the rat, embedded in the bedding recorded the mechanical signals at the skull. The Vibe group was mechanically stimulated (0.3 g, 50 Hz) for 3 min/day for 5 days/week over 6 weeks. While the Sham was not vibrated, the entire mandible set-up and procedure was simulated but without the power turned on to the device. A validation study confirmed the feasibility and repeatability of this study over a period of 1-week at the skull of the rat. Subsequent studies (Chapters 3 & 4) will be performed to assess the ability of the LIV signal to enhance the quantity and quality of the alveolar bone. If successful, it can provide as a promising modality to prevent alveolar bone loss caused by estrogen depletion, osteoporosis and periodontal diseases, which have serious clinical implication of early tooth loss.

Introduction

Approximately 36 million women in the United States are in the postmenopausal phase of life. Estrogen deprivation arising from menopause increases the risk of developing osteoporosis and oral diseases [67]. Approximately 40% of women and 15% of men worldwide can expect to suffer an osteoporotic fracture during their lifetime. Literature review has consistently reported a positive association between osteoporosis or low BMD at various skeletal sites, including low BMD of the jaw, greater alveolar bone loss and fewer teeth retained [68, 69]. A few studies that exist to date suggest that alveolar bone loss is more extensive in women with osteoporosis and greater rates of bone loss at systemic sites are related to tooth loss [70, 71]. Osteoporosis is a metabolic disorder characterized by low bone mineral density (BMD) and micro architectural deterioration leading to susceptibility to bone fracture [72]. Studies also show that postmenopausal women with osteoporosis and periodontitis are likely to exhibit a loss of dentoalveolar bone height and a decreased BMD of the alveolar bone. As a result, these women are at a high risk of experiencing early loss of teeth caused by decreased BMD [70, 73, 74]. In a 2 year prospective study conducted, the changes in alveolar bone height between women with spinal osteoporosis and those with normal BMD found that the osteoporotic group developed more sites with significant alveolar bone loss than the normal BMD group [70, 71]. Like most of the studies of BMD and oral bone loss or tooth loss, the majority of studies of systemic BMD and clinical measures of periodontal disease have been cross-sectional in design. Therefore, it is not yet clearly known if generalized low bone density in the jaw directly influences the risks of periodontal disease and tooth loss or the biological mechanisms lead to the associations. Most studies support the existence of a relationship, however, more research is needed to confirm this association [21].

Osteoporosis is a serious health and economic issue and appropriate measures have been taken in order to decrease the occurrences by pharmacological and non-pharmacological intervention. Several antiresorptive therapies like estrogen replacement therapy (ERT), various bisphosphonates such as alendronate, the SERM raloxifene, calcitonin and sodium fluoride,

including calcium and vitamin D have been approved modalities for the treatment and prevention of bone loss [31]. Furthermore, parathyroid hormone is considered anabolic to bone, thereby increasing bone formation [33].

Bone adaptation during skeletal growth and development continuously adjusts skeletal mass and architecture to changing mechanical environments. Literature suggests that the three fundamental rules governing bone adaptation are: 1) it is driven by dynamic, rather than static loading; 2) Only a short duration of mechanical loading is necessary to initiate an adaptive response and 3) Bone cells have the potential to accommodate to any mechanical environment, making them less responsive to routine loading signals [75].

Studies have well established the sensitivity of the long bones (tibia and/or femur) to mechanical signals in enhancing bones quality and quantity [65]. Both the long bone and alveolar bone are very sensitive to altered mechanical environment through static and dynamic loading [54]. Furthermore, studies have demonstrated that mechanical loading, originating from either muscular function or gravity, is an important factor in bone homeostasis and in maintaining structure and mass of the bone throughout lifetime. Unlike the long bones, the alveolar bone is completely dependent on masticatory function, since in the absence of teeth or denture-transmitted mechanical stimulation it undergoes disuse atrophy [39]. It has been predicted that the strain magnitudes required for maintaining bone mass and stimulating bone formation can be much lower than 70 microstrain at very high loading frequency [38, 50, 51]. Studies have shown that the strain on the bone surface induced by low-level mechanical vibrations at 0.3 g is less than 10 microstrain, which are far below than what might cause damage to the bone [52]. Furthermore, in an animal study, the maximum values of bone strain and bite force were recorded at 435 microstrain, which is still lower than what might potentially cause microdamage in the bone [53].

Exercise plays an important role in the management of osteoporosis. However, a factor influencing the effects of exercise on bone integrity is that exercise effects are generally regional. Weight-bearing exercises can increase the density of the lower extremities and the axial skeleton but not the cranial skeleton [76]. This explains the cranial skeleton may not feel

the benefits of whole body vibrations as most of the vibrations get dissipated in the axial skeleton. Therefore, our hypothesis is to assess the transmissibility of the low-level mechanical signals to the alveolar bone and if successful, how effective it is as a non-pharmacological intervention to prevent alveolar bone loss affecting postmenopausal women, osteoporotic patients and in general people prone to tooth loss caused by periodontal diseases.

Low-level vibrations below a magnitude of 0.56 g and high frequency in the range of 20 - 90Hz does not pose a safety threat to patients according to International Safety Organization (ISO) [77]. This has been further confirmed by studies that indicate these signals are anabolic to bone [43, 78]. Hence, it can be assumed that vibrations below a magnitude of 0.3 g will not pose a safety threat when applied to the jaws as the strain during mastication is much higher than that caused by 0.5 g. Furthermore, according to American Dental Association (ADA), a recommended brushing time is between 2 – 4 min daily. Low strains and short bouts of vibration can therefore be assumed to not have an adverse effect on the patients and can also be comparable to ISO-2631 as the frequency of these signals will be in the range of 20 – 90 Hz.

The osteogenic response to low magnitude, high frequency has been extensively studied especially in the trabecular bone. Evidence exists on the positive effects of these low magnitude, high frequency mechanical signals on bone quality and quantity, bone healing and on the inhibition of osteopenia [43, 79, 80]. The role of specific low magnitude high frequency loading parameters including frequency, magnitude, strain, loading duration and period of study have been extensively studied and it is suggested that the loading frequency is possibly the most important factor for the response of bone [81, 82]. Studies have also revealed that osseointegrated dental implants are a well-accepted and predictable treatment method for the restoration of partially or completely edentulous patients [83]. Currently, early and immediate implant loadings, which challenges bone healing and implant osseointegration are accepted protocols for treatments. However, these loading conditions have not been well evidenced for patients with clinical conditions such as diabetes [84]. In another study on mice, the cortical layer of the parietal bone (the skull bone) was drilled. The animals received whole body vibrations and the results showed faster healing rate in the vibrated group compared to the

controls. However, the intact region of the cortical bone found no significant differences between the groups. Therefore, this might imply that only the healing bone responds to vibrations whilst the intact bone might not be sensitive enough to the whole body vibrations [85]. These stimulating effects of low magnitude high frequency loading led us to investigate the effects on the alveolar bone quality and quantity when localized mechanical signals were applied to the jaws of rats.

The effects of loading frequency have been assessed in several animal studies where individual limbs/bones of the animals have been exposed to mechanical loading and whole body vibrations have been exposed to the over body of the animal by means of a vibrating platform. However, in the present study we propose to apply localized vibrations on the jaws of the rats by means of an actuator, which is placed inside the mouth of the animal.

Methods

Design

Actuator

A recent study indicated that the cortical bone of an intact skull is not sensitive to WBV even though the healing bone is sensitive to the vibrations [85]. These results concur with a preliminary experiment we conducted to assess the effects of whole body vibrations on the alveolar bone of a mouse. We found no significant differences in the alveolar bone morphology of the vibrated group and the controls (data not shown). Therefore, we designed this experiment to administer localized vibrations to the jaws to assess how these low magnitude high frequency signals altered the quality and quantity of the alveolar bone.

An actuator was designed by modifying a commercially available, battery operated toothbrush (Oral-B Pulsar, Procter & Gamble, USA). Firstly, the toothbrush was connected to a DC power source in order to eliminate inconsistencies caused by battery discharge and to provide a current source to retain a constant mechanical output. The bristles of this toothbrush

were removed in order to eliminate dissipation of the mechanical signals through the bristles, and to ensure that this is a response to the mechanical signal and not to the cleaning of the teeth. The DC voltage from the power source controlled the magnitude and frequency of the actuator. This actuator was designed such that the neck of the toothbrush, which contained the vibrator, would be placed inside the mouth of the rat (**Fig. 2**).

Bedding

The actuator was designed so that it is placed inside the mouth between the upper and the lower jaws. To implement this set-up, the animals had to be anesthetized and placed in a supine position on the bedding. The bedding was made out of foam and cut to the shape of a 12-week old rat. The foam that supported the head of the rat was carefully cut in order to prevent head movement that could potentially influence varying mechanical signals.

Animal Set-up

Stony Brook University's Institutional Animal Care and Use Committee (IACUC) approved the experimental procedures used in this experiment. The animal model chosen for this experiment was male Sprague Dawley rat at 12-week old. The animals were anesthetized in a chamber with 5% Isoflurane in oxygen with a pressure of 1-2 liters/min. After they were anesthetized, they were removed from the chamber and positioned on the foam bed where they were continued to be given approximately 3% Isoflurane in oxygen with a pressure of 1-2 liters/min using a nosecone for the time they were mechanically stimulated.

While the animals were anesthetized on their foam bedding, the actuator was placed between the jaws in order to provide localized mechanical signals to the alveolar bone. To prevent the dislocation of the actuator during the vibrations, a load bearing weight was suspended across the mouth of the rat (**Fig. 2**).

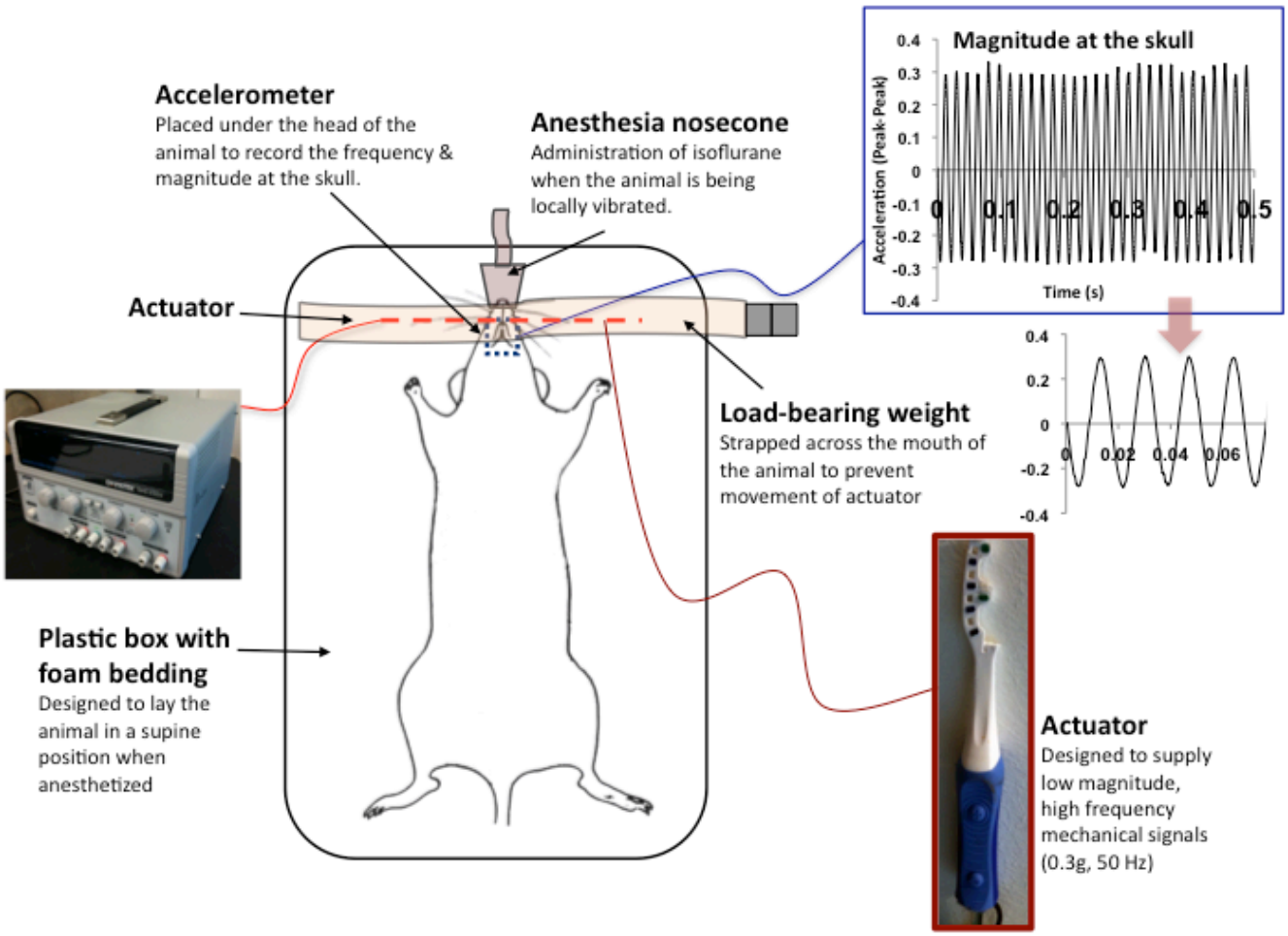


Figure 2: Schematic of the animal set-up during bouts of vibrations. The accelerometer is placed under the head of the animal to generate magnitude and frequency recording. The plot of the magnitude describes the constant strength of the signal at the skull of the rat. This mechanical signal was a sinusoidal wave as shown. The animal is placed in the foam bedding in a supine position. The nosecone is attached to the nose to provide constant flow of isoflurane to anesthetize the rat. The actuator is placed inside the mouth of the rat, between the upper and lower jaws and a load bearing strapped across the mouth to prevent movement caused by the vibrations of the actuator.

Validation Studies

Several studies in the past have shown that applying extremely low magnitude (0.2 - 0.3 g) and high frequency (30 – 90 Hz) improve the quality and the quantity of the trabecular bone in the long bones [65, 78]. Therefore, in this study we determined the feasibility of generating high frequency mechanical signals, which can be locally administered to the alveolar bones.

To verify that the mechanical signal delivered by the actuator was in fact distributed through the teeth to the mandible, an accelerometer was placed under the head of the animal. The accelerometer was embedded in the bedding so that magnitude of the mechanical signals at the skull could be measured. LabView program was used to record the magnitude and the strength of the mechanical signal through the accelerometer.

The voltage of the power source, which was connected to the actuator, controlled the strength of the signal. Therefore, the voltage was adjusted until the desired frequency and the magnitude was recorded. In this power source set-up, the magnitude was dependent on the frequency and the frequency was dependent on the voltage supply. In order to ensure the voltage was not too high or low at the start of the experiment, daily a calibration animal was set-up in vivo, just so the actuator was adjusted to the correct voltage for the experimental animals.

Firstly, to determine the repeatability and consistency of this set-up, before the commencement of the 6 weeks animal study, a feasibility study on one male Sprague Dawley rat was performed. This feasibility study was conducted to determine if the localized, low magnitude, high frequency mechanical signal was transmitted to the skull of the rat in the proposed set-up. Due to the nature of the set-up to the power source, it was established that the magnitude and frequency were dependent on the voltage of the power source. Increasing the voltage resulted in an increase in both frequency and magnitude, suggesting that the frequency and the magnitude could not be independently controlled. Therefore, at different voltage values ranging from 0.2 - 0.8 V, combinations of magnitude and frequency were recorded at the skull of the rat. Five measurements at each voltage were recoded to test for the repeatability of the set-up. It was established from our feasibility study, described above, that 50 Hz and 0.3 g (peak) was an ideal combination of parameters for the study as the literature indicated that most studies that used low magnitude high frequency signals used magnitude around 0.3 g.

Furthermore, in order to validate the repeatability and consistency of this set-up and the low magnitude high frequency signal (50 Hz and 0.3g) over a longer period of time, a 1-week validation study was conducted during the 6 weeks study. A Matlab program was used to record

the frequency and the magnitude at 4 time points (1 min, 1.5 min, 2 min & 2.5 min) during the 3 min vibration bout. The data was collected at a sampling rate of 2000 Hz for 3 s at each of the 4 time points for each animal in 1-week. The *in vivo* magnitude data at each of the 4 time points was plotted individually and the peak magnitude was described as the total height of the two opposite peaks divided by 2.

Experimental Design

Twenty 12-week old male Sprague Dawley rats were obtained from Charles River (Wilmington, MA) and were randomly divided into two groups (n=10): (1) Sham control and (2) Vibe group. The Vibe group was subjected to localized vibrations at 50 Hz, 0.3 g for 3mins daily for 5 days/week under isoflurane anesthesia. While the Sham group was not vibrated, the entire mandible set-up and procedure was simulated but without the power turned on to the device.

Results

Validation Studies

The feasibility study indicated that with increasing voltage, both the magnitude and the frequency increased. Suggesting that, due to the analogue nature of the power source, the magnitude and the frequency were dependent on the voltage. For a range of voltages ranging from 0.2 – 0.8 V, the magnitude and frequency is plotted (**Fig. 3**). The magnitude and frequency parameters were chosen as 0.34 ± 0.04 g and 50 ± 0.5 Hz, respectively as most studies in the past have used parameters in a similar range.

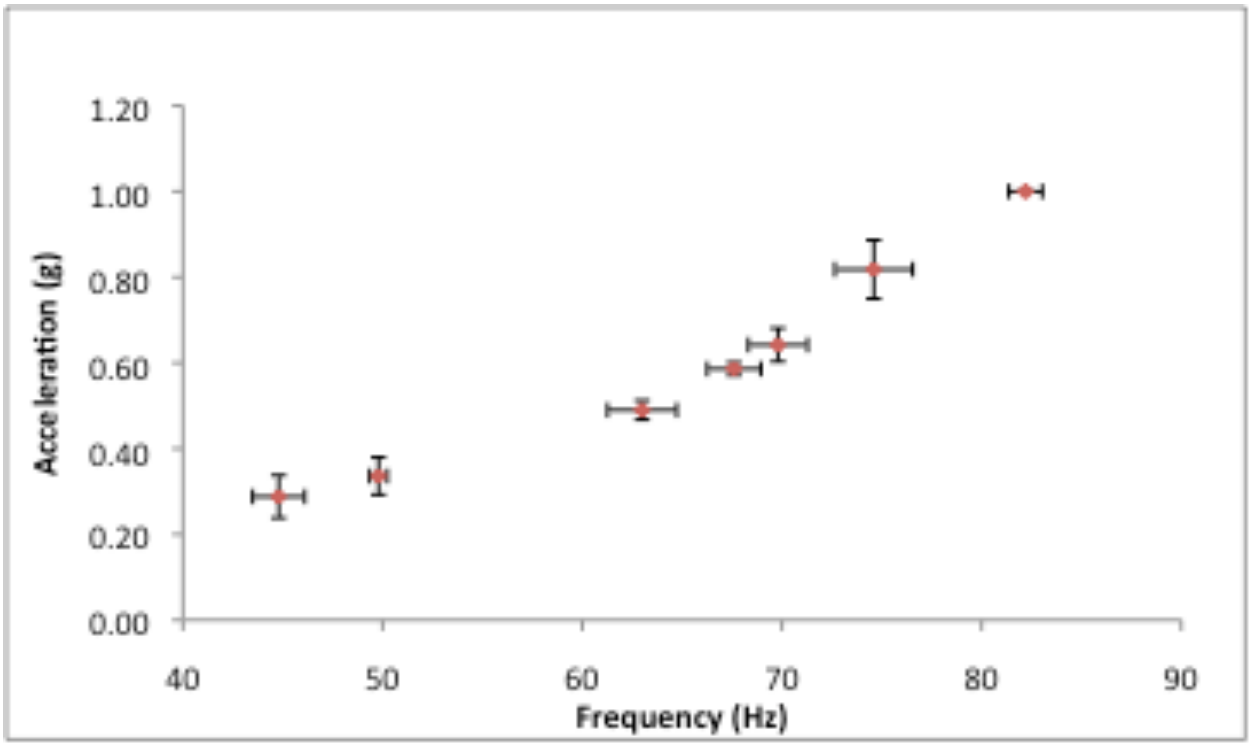
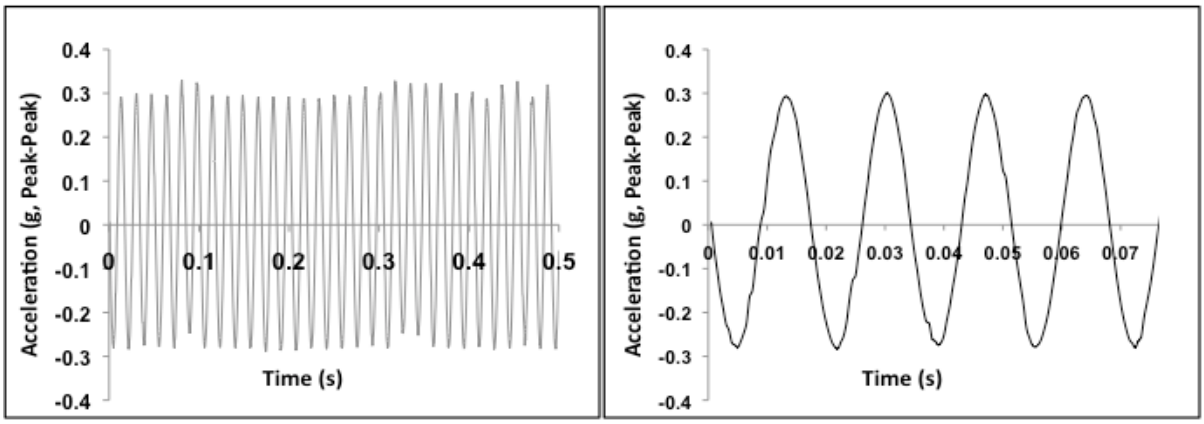


Figure 3: The range of frequency-magnitude obtained by changing the voltage range from 0.2 – 0.8 V. For the purpose of this study, 50 Hz and 0.3 g was used. These acceleration (± SD) and frequency (± SD) measurements were recorded at the skull of a rat. The acceleration might be higher at the teeth as at the actuator is more closely in contact to the teeth than to the skull, resulting in some loss of transmission at the skull.

After the frequency and magnitude were determined as 50 Hz and 0.3 g, respectively, a 1-week validation study was conducted with these parameters to assess the repeatability and consistency of the mechanical signal and the set-up. The sinusoidal nature of the actuator was confirmed by the recording from the accelerometer (**Fig. 4**). Matlab program was used to record the average frequency and magnitude at each of the 4 time points in 1-week (**Table 1**). The recorded signals suggested that the average (±SD) peak magnitude was 0.3 ± 0.01 g (peak) and the average frequency was 58 ± 0.6 Hz for the week these signals were recorded at the skull of the rats.



a) b)

Figure 4: Strength and consistency of the mechanical signal from the actuator, recorded at the skull of the rat by the accelerometer. a) Strength and consistency of the mechanical signal from the actuator over 0.5 s and b) Sinusoidal nature of the mechanical signal.

Discussion

Several studies in the past have suggested a positive correlation between estrogen deficiency and an accelerated destruction of the alveolar bone causing tooth loss particularly in elderly women suffering from periodontal diseases [86, 87]. However, this study is a pilot study, therefore we used male rats to avoid interferences caused from hormonal disruptions. Studies in the past have established that low-level mechanical signals are a non-pharmacological intervention to prevent bone loss caused by estrogen deficiency [57]. Whole body vibrations were used to administer these low level mechanical signals to the long bones. A recent study suggested that whole body vibrations resulted in a faster healing rate of the skull, which was drilled compared to the control group. However, the intact region of the cortical bone found no significant differences between the two groups [85]. These results overlap with our findings from an earlier study, suggesting that whole body vibrations do not transmit mechanical signals to an unaffected and healthy alveolar bone. Therefore, in order to deliver mechanical signals to the alveolar bones, localized vibration of the jaws of the animals was employed.

To test the hypothesis that the actuator provides consistent and stable mechanical signals, an accelerometer was placed under the head of the rat when it was being vibrated.

Because it was hard to place strain gauges inside the mouth of the rats, it was easier to validate the results at the skull. Therefore, we assumed that the frequency and magnitude parameters recorded at the skull are identical to that being transmitted to the alveolar bone through the teeth. The initial feasibility study suggested a range of frequency and magnitude parameters for a range of increasing voltages, suggesting that as the voltage was increased, the frequency and magnitude was also increased. This is a drawback of this pilot study, as the parameter values could not be independently changed as the voltage from the power source was controlling the magnitude and the frequency. However, the frequency and magnitude parameters were chosen at 50 Hz and 0.3 g (peak) as recorded at the skull of the rats (**Fig 3**). These parameters were chosen as most studies on long bones have used parameters in a similar range and have found these low intensity vibrations to be anabolic to trabecular bone.

Furthermore, the power source that was used was an analog power source, which made it harder to adjust to the voltage to generate the exact mechanical signals at 0.3 g and 50 Hz, which was established during the calibration before the study. The nature of the analog power signal caused minor deviations in the voltage to generate 0.3 g and 50 Hz signal at the start of the bout every day. This was also evident from the validation study at week 3, which recorded a signal of 0.3g and 60Hz, suggesting a drift only in the recorded frequency. However, the calibration study was conducted only on 1 day while the validation study was for 1-week, which may have caused the discrepancy. Despite this shortcoming, the 1-week calibration study suggested a very repeatable and consistent frequency and magnitude recordings (**Fig 4b**). Therefore, in order to minimize drifting of the voltage that could potentially alter the mechanical signals drastically, a calibration animal was set-up and vibrated for 3 min daily before vibrating the experimental animals.

This study was designed to provide the basis for a short-term 6-week pilot study with only two groups: sham control (n=10) and vibrated (n=10) groups. If successful, it might be important to redesign this set-up in order to make it less manual, accommodate more groups and be able to provide a range of frequencies and magnitudes, which can be controlled independently.

CHAPTER 3

Low intensity vibrations (LIV) will enhance the bone quantity of the alveolar bone when localized vibrations are applied to the mandible.

Abstract

Low intensity vibrations have proven to be anabolic to bone when subjected to whole body vibrations. There is growing evidence that these low-level, high frequency mechanical signals are anabolic to bone and thereby a potential non-invasive and non-pharmacological treatment for osteoporosis specifically in post-cranial skeleton. However, the effect of these signals on the quality and quantity of the cranial skeleton, particularly in the alveolar bone is yet to be elucidated. Twelve-week old male Sprague Dawley rats were randomly divided into two groups (n=10): Sham control and Vibe group. The Vibe group was mechanically stimulated (0.3 g, 50 Hz) for 3 min/day for 5 days/week over 6 weeks. While the Sham was not vibrated, the entire mandible set-up and procedure was simulated but without the power turned on to the device. The bone quantity was assessed using micro-computed tomography (μCT) and dynamic histomorphometry. For μCT, three regions of interest (ROI) were chosen under each of the three molars: 1) Whole mandible, 2) Alveolar bone and 3) $180\mu m^3$ cube in the alveolar bone. The results from μCT suggested a significant increase in the bone mineral density in the alveolar bone under the first and second molar in the whole mandible and alveolar bone ($p < 0.01$) and under all three molars in the $180\mu m^3$ cube ($p < 0.05$). The bone mineralization increased by approximately 5% in the Vibe group in the $180\mu m^3$ cube ROI. However, LIV could not influence other bone parameters such as total volume (TV), bone volume (BV) and bone volume fraction (BV/TV). Results from dynamic histomorphometry also showed no significant differences in the indices of bone formation such as total labeled surface (LS/BS), single labeled surface (sL.S/BS), double labeled surface (dL.S/BS), mineral apposition rate (MAR) and bone formation rate (BFR/BS) between the two groups. This data indicates that short bouts (3 min/day) of mechanical signal could not influence the bone formation in the alveolar bone but was able to

enhance the bone quality as seen in the 5% increase in bone mineralization. Therefore, to assess the quality of the alveolar, further studies need to be performed to understand the processes underlying the increase in mineralization. If successful, it can provide as a promising modality to prevent alveolar bone loss caused by estrogen depletion, osteoporosis and periodontal diseases, which have serious clinical implication of early tooth loss.

Introduction

According to NIH and the Surgeon General's Report on Oral Health, 42% of Americans over the age of 65 are toothless due to unnatural causes. Alveolar bone loss has major economic impact on individuals. It has been reported that medical intervention to treat alveolar bone loss costs an estimated $5-6 billion/year for just the surgical treatments related [11]. The most common cause of alveolar bone loss is attributed to chronic periodontitis. It affects approximately 35% of dentate adults in the United States between ages 30 – 90, and approximately 13% have a moderate to severe form of the disease [13]. Like osteoporosis, periodontitis is considered a "silent" disease, not causing symptoms until late in the disease process when tooth loss may occur [14].

In addition to periodontal diseases, estrogen deprivation arising from menopause increases the risk of developing osteoporosis and oral diseases [67]. Approximately 36 million women in the United States are in the postmenopausal phase of life. Estrogen deprivation arising from menopause increases the risk of developing osteoporosis and oral diseases[67]. Approximately 40% of women and 15% of men worldwide can expect to suffer an osteoporotic fracture during their lifetime [68, 69]. Studies also show that postmenopausal women with osteoporosis and periodontitis are likely to exhibit a loss of dentoalveolar bone height and a decreased BMD of the alveolar bone. As a result, these women are at a high risk of experiencing early loss of teeth caused by decreased BMD [70, 73, 74]. Osteoporosis is a serious health and economic issue and appropriate measures have been taken in order to decrease the occurrences by pharmacological treatment such as anti-resorptive and treatments that are anabolic to bone.

Lately, studies have indicated non-pharmacological treatment as a viable treatment to enhance bone quantity [31]. Studies have established that low-level mechanical signals are a non-pharmacological intervention to prevent bone loss caused by estrogen deficiency [57]. These studies have indicated a high sensitivity of bone to low-level mechanical signals (< 0.3 g, 20 - 90 Hz) when subjected to whole body vibrations in both clinical trials [58] and in animal models [43, 57], specifically in long bones to enhance the bone quantity and quality.

Mechanical loading, originating from either muscular function or gravity, is an important factor in bone homeostasis and in maintaining structure and mass of the bone throughout lifetime. Unlike the long bones, the alveolar bone is completely dependent on masticatory function, since in the absence of teeth or denture-transmitted mechanical stimulation it undergoes disuse atrophy [39]. Alveolar bone grows in response to tooth eruption and resorbs when teeth are lost [88]. It has been predicted that the strain magnitudes required for maintaining bone mass and stimulating bone formation can be much lower than 70 microstrain at very high loading frequency [38, 50, 51]. Studies have also revealed the sensitivity of the alveolar bone to altered mechanical environment through both static and dynamic loading [54]. Also, orthodontic tooth movement is achieved by mechanical loading by the process of repeated alveolar bone resorption on the pressure side and alveolar bone formation on the tension side [89].

Osteoporosis is characterized by progressive bone loss, particularly in the weight-supporting areas of the skeleton. The bone that is remaining in osteoporotic patients is considered to be normal and is capable of repair, however, the strength of the skeleton and the bone mass are compromised. Studies have indicated that bones anabolic response to mechanical loading can enhance the bone quantity and quality, thereby combating diseases such as osteoporosis [65]. Studies have indicated a correlation between osteoporosis and alveolar bone loss [70, 73, 74]. Therefore, based on this premise and on the knowledge obtained from Hypothesis 1, site specific, localized low intensity vibrations (LIV) were applied on the jaws of a rat model. In the work presented here, we determined whether these LIV could improve the quantity of the alveolar bone when subjected to mechanical signals for 3min/day, 5days/week

over 6 weeks. The morphological parameters were investigated using micro-computed tomography (μCT) and dynamic histomorphometry.

Methods

Experimental Design

Twenty 12-week old male Sprague Dawley rats were obtained from the Charles River (Wilmington, MA) and were randomly divided into two groups (n=10): (1) Sham control and (2) Vibe group. The Vibe group was subjected to localized vibrations at 50 Hz, 0.3 g for 3mins daily for 5 days/week under isoflurane anesthesia. While the Sham group was not vibrated, the entire set-up and procedure was simulated without the mechanical signals.

Stony Brook University's Institutional Animal Care and Use Committee (IACUC) approved the experimental procedures used in this experiment. The animal model chosen for this experiment was male Sprague Dawley rat at 12-week old. The animals were anesthetized in a chamber with 5% Isoflurane in oxygen with a pressure of 1-2 liters/min. After they were anesthetized, they were removed from the chamber and positioned on the foam bed where they were continued to be given approximately 3% Isoflurane in oxygen with a pressure of 1-2 liters/min using a nosecone for the time they were mechanically stimulated.

While the animals were anesthetized on their foam bedding, the actuator was placed between the jaws in order to provide localized mechanical signals to the alveolar bone. To prevent the dislocation of the actuator during the vibrations, a load bearing weight was suspended across the mouth of the rat (Fig 1).

In order to measure dynamic indices of bone formation, the rats were injected calcein ($10mg.kg^{-1}$), a fluorescent label, subcutaneously. Animals were injected at two time points, 5 & 6 days and 14 & 15 days prior to the end of the study. They labeled on 2 consecutive days to be able to achieve better fluorescence imaging during the analysis.

Following the 6-week experimental procedure, the animals were euthanized by cardiac puncture, cervical dislocation followed by decapitation to extract the mandible from the skull and each mandible was divided into left and right hemi-mandibles. Each hemi-mandible was fixed in 10% neutral buffered formalin (NBF), if there was a need to perform histological analysis such as osteoclasts and osteoblasts activity in the future. Two days after fixing in 10% NBF, the hemi-mandible tissues were harvested in 70% ethanol for micro-computed tomography (µCT), dynamic histomorphometry and FTIR analysis.

Micro-computed Tomography

Changes in the alveolar bone morphology over the 6-week experiment were evaluated using µCT. The left hemi-mandible was scanned (µCT 40, Scanco Medical SUI) at a resolution of 18µm. The bones were scanned in a coronal plane from the first to the third molar (**Fig. 5**). The landmarks chosen for evaluation were defined as the center of the root canal for each of the three molars. Three regions of interest (ROI) were chosen to evaluate the changes in bone morphology: 1) Whole mandible, 2) Alveolar bone and 3) 180µm^3 cube in the alveolar bone.

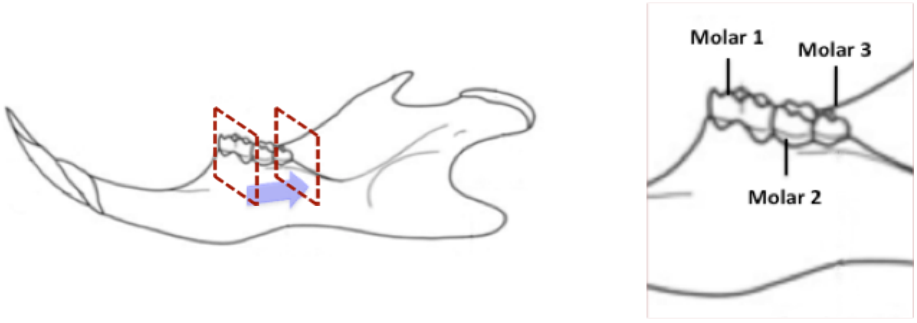
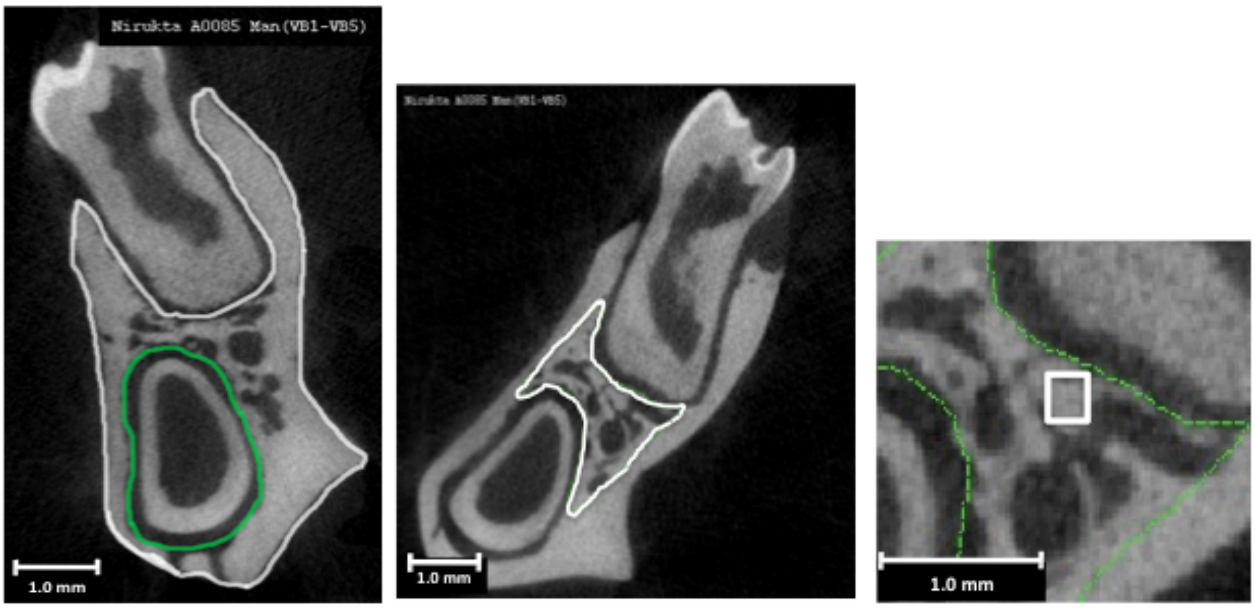


Figure 5: A schematic of the hemi-mandible of the rat depicting the *ex vivo* region scanned using micro-computed tomography as enclosed within the red dotted boxes. Molar 1 - Molar 3 were scanned in the coronal plane at a resolution of 18µm.

The first ROI was chosen as the whole cortical and trabecular region in the mandible (**Fig. 6a**). The landmark of the center of the root canal was used and sections of 378µm (21x18µm) thickness were analyzed from the center of each of the three molars. The threshold parameter was set to 310 for the evaluation of the bone. Total volume (TV), bone volume (BV), bone volume fraction (BV/TV) and bone mineral density (BMD) were evaluated.

The second ROI was chosen as only the alveolar bone ignoring the cortical shell of the mandible (**Fig 6b**). The landmark of the center of the root canal was used and sections of 378µm (21x18µm) thickness were analyzed from the center of each of the three molars. Alveolar bone is identified as the region with porosities and voids (**Fig 6b**). The vertical distance was identified as the distance between the molar and the incisor root while the horizontal distance was identified as the end point of the last porosity and void. The horizontal distance was observed to be consistent at 2mm ± 0.2mm for each mandible. The threshold parameter was set to 310 for the evaluation of the bone. Total volume (TV), bone volume (BV), bone volume fraction (BV/TV) and bone mineral density (BMD) were evaluated.

The third ROI was chosen as 180µm^3 cube within the alveolar bone region devoid of porosities (**Fig 6c**). The landmark of the center of the root canal was used and sections of 180µm (10x18µm) thickness were analyzed from the center of each of the three molars. The threshold parameter was set to 310 for the evaluation of the bone. For this region only the bone mineral density (BMD) was evaluated.



a) b) c)

Figure 6: Micro-computed tomography images of the rat mandible showing the molar and the incisor and the three regions of interest. A) Whole mandible: the entire bone excluding the incisor and the molar, b) Alveolar bone: the region enclosed in the white outline and c) 180µm^3 cube: as enclosed in the white box, devoid of porosities.

Histomorphometry

After the µCT scanning of the left hemi-mandible of twenty rats, the same hemi-mandibles were embedded in a polymethylmethacrylate resin (PMMA) using a standard protocol. Following the bone embedding protocol, these bone blocks were sectioned longitudinally in the coronal plane as shown in Fig. 4. Only the region under the first molar was sectioned and analyzed. The ROI was similar to the region scanned by µCT, the center of the root canal under the first molar (**Fig. 7**). Sections of 4µm were taken from the center of root canal with a microtome using a

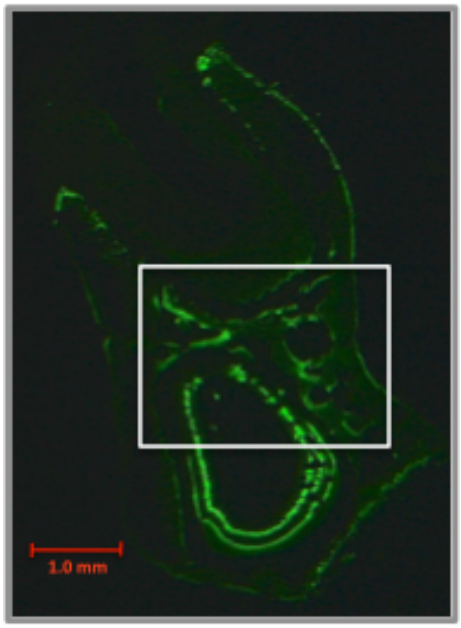


Figure 7: Region of interest (ROI) to determine dynamic histomorphometry parameters is enclosed in the white box.

tungsten carbide blade (RM 2165 microtome, Leica, Bensheim, Germany). Histomorphometry software, Osteomeasure (OsteoMetrics Inc., Atlanta, GA) was then used to trace the fluorescent labels from the bone using a fluorescence microscope. The time span between the two labels was 9 days and using this reference, the bone formation parameters were evaluated. The ratio of total labeled (LS), single labeled (sL.S) and double (dL.S) surfaces to bone surface(BS) were evaluated. The mineral apposition rate (MAR) is defined as the ratio between the distance between the two labels and the time between the two labels (9 days). Finally, the standard measure of bone formation rate (BFR/BS, $\mu m.day^{-1}$) was calculated by multiplying MAR with the ratio of mineralizing surface (MS) and bone area (BA).

Statistical Analysis

The data were expressed as mean ± SD. Differences amongst the groups were tested using an unpaired two-tailed t-test where p-value lower than 0.05 was considered as significant. Percentage changes in the groups were calculated by subtracting the mean of the sham group from the mean of the vibe group and dividing by the mean of the sham group.

Results

There were no significant differences in the mean body mass between the two groups at the end of the 6-week experiment (**Fig. 8**). It is unknown if anesthetizing the animals had an effect on the body mass as both the groups were anesthetized. However, both the groups gained similar amounts of body mass at the end of the 6-week experiment (**Table 1**).

Index	Sham (n=10)	Vibe (n=10)	p-value
Initial body mass (g)	389 ± 11	385 ± 13	0.432
Final body mass (g)	512 ± 33	510 ± 40	0.895
Percent increase in body mass	35.7%	36.7%	0.895

Table 1: Mean (± SD) of the initial body mass at the start of the experiment at week 0, final body mass at the end of the experiment at week 6 and the percentage increases in body mass between Sham and Vibe groups. The initial and final body masses between the two groups did not vary at the end of the 6-week study.

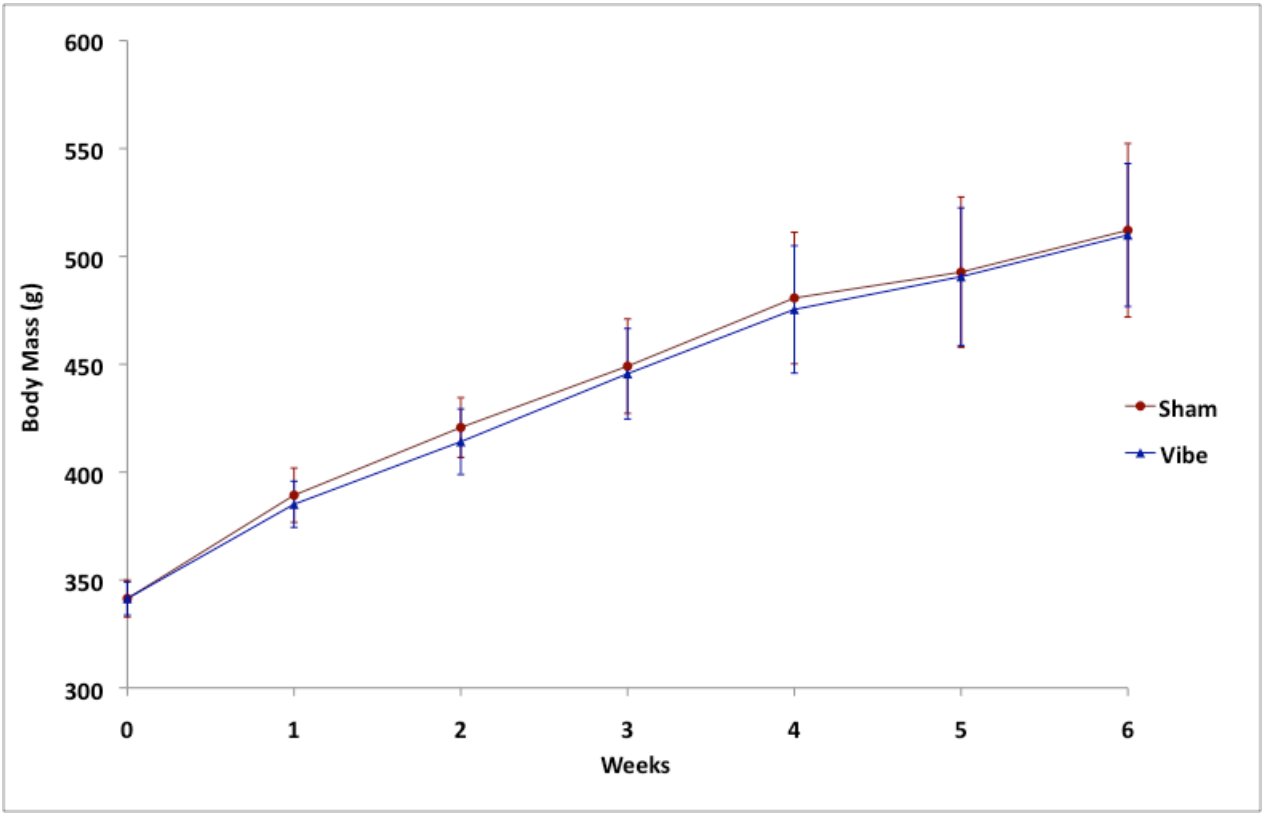


Figure 8: Graph showing the growth of the animals over 6 weeks between the Sham and the Vibe groups. The plot shows a constant growth pattern and no significant differences between the weights of the two groups.

Micro-computed Tomography

The ROI that was first evaluated was the whole mandible (**Fig. 6a**). Different parameters of the bone morphology such as total volume (TV), bone volume (BV), bone volume fraction (BV/TV) and bone mineral density (BMD) were evaluated (**Table 2**). There were no significant differences ($p > 0.05$) in the TV, BV, BV/TV between the two groups under all three molars. However, the vibe group showed significant increases ($p < 0.05$) in BMD under the first and the second molar by approximately 3%. Furthermore, the TV of the Vibe group under all three molars was decreased by approximately 3.3% also causing a decrease in BV but it was not significantly altered ($p > 0.05$) (**Fig. 9**).

Bone Parameters		Sham	Vibe	Percent Change	p-value
Total Volume (mm^3)	***Molar 1***	3.67 ± 0.33	3.57 ± 0.27	-2.9	0.444
	Molar 2	4.04 ± 0.27	3.96 ± 0.25	-2.2	0.460
	Molar 3	3.95 ± 0.30	3.75 ± 0.31	-4.9	0.174
Bone Volume (mm^3)	***Molar 1***	3.07 ± 0.25	3.04 ± 0.23	-1.1	0.751
	Molar 2	3.17 ± 0.19	3.15 ± 0.20	-0.8	0.765
	Molar 3	3.15 ± 0.20	3.09 ± 0.23	-2.1	0.499
Bone Volume Fraction (%)	***Molar 1***	84 ± 2	85 ± 2	+1.7	0.130
	Molar 2	79 ± 3	79 ± 3	+1.3	0.445
	Molar 3	80 ± 4	80 ± 4	+2.8	0.141
Bone Mineral Density (mg HA/cm^3)	***Molar 1***	818.94 ± 8.33	**843.06 ± 15.45**	**+2.9**	**0.0007**
	Molar 2	811.19 ± 12.56	**836.49 ± 21.91**	**+3.1**	**0.007**
	Molar 3	827.35 ± 8.96	843.73 ± 22.65	+2.0	0.055

Table 2: Indices of micro-computed tomography bone parameters at the whole mandible region of interest expressed as Mean ± SD. Bone mineral density under Molar 1 and Molar 2 are significantly higher in the Vibe group compared to the Sham by approximately 3.0% ($p < 0.01$), with relative increase in molar 3 bordering on significance.

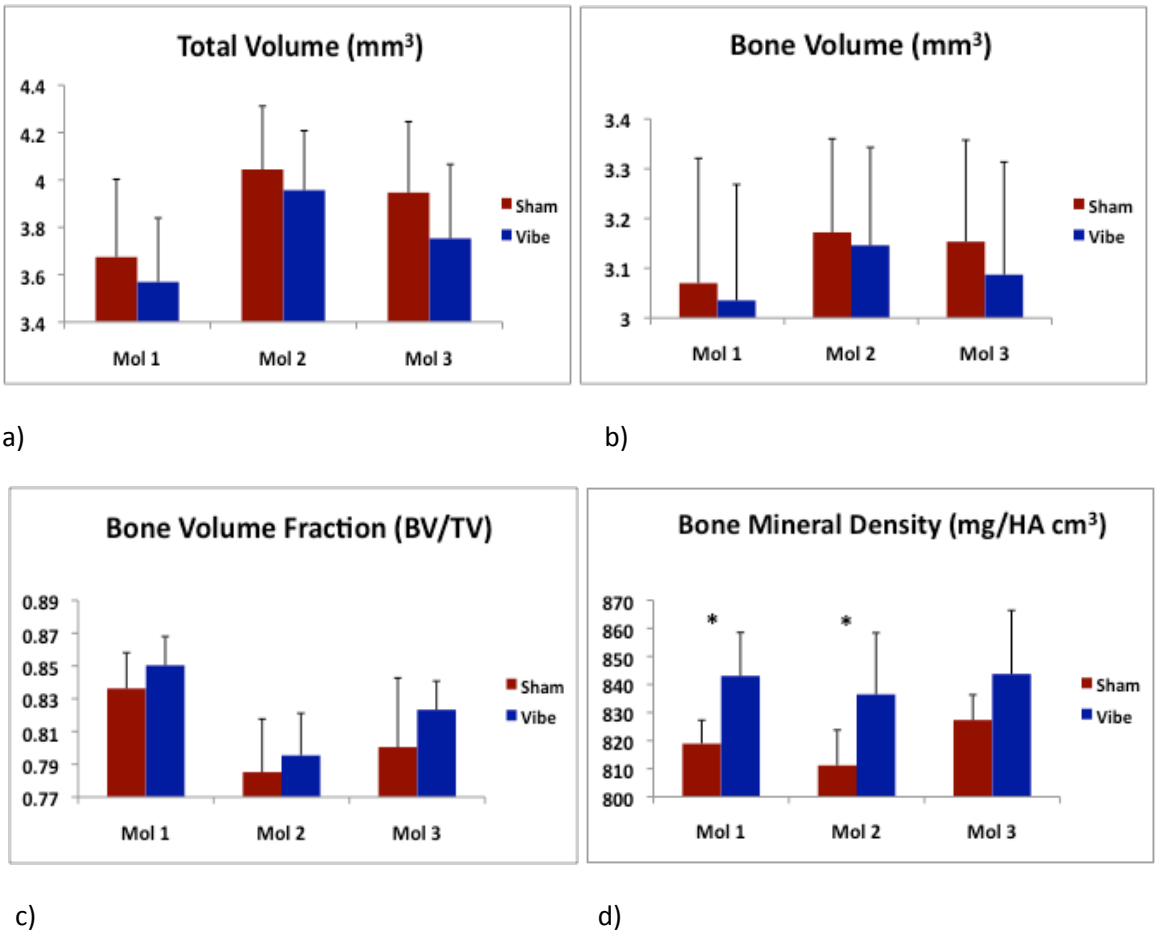
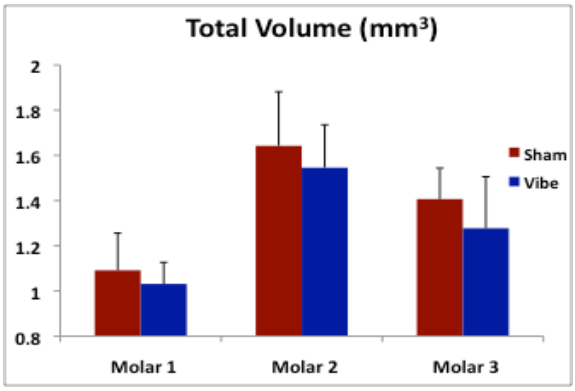


Figure 9: Bone morphological parameters obtained for the whole mandible region of interest from micro-computed tomography under Molar 1, Molar 2 and Molar 3. Localized LIV on the mandible influenced the bone mineral density (BMD) parameter under Molar 1 and Molar 2 by increasing the BMD in the Vibe group by approximately 3.0% ($p < 0.01$).

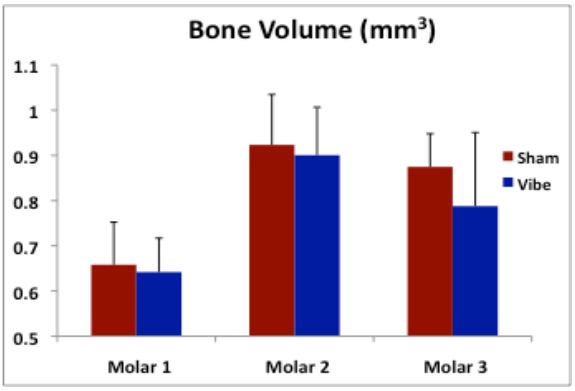
After having identified an increase in BMD under the first and second molar, the second ROI was chosen to focus only the alveolar bone, excluding the cortical shell (**Fig 6b**). There were no significant differences in the TV, BV or BV/TV between the two groups under any of the three molars (**Table 3**). However, the Vibe group showed approximately 3.0% increases ($p < 0.05$) in BMD under the first and the second molar, but a marginal decrease of 0.3% under the third molar (**Fig. 10**). Furthermore, the TV of the Vibe group under all three molars was decreased by approximately 6.9% also causing a decrease in BV but it was not significantly altered. The decrease in TV and BV was consistent with the decrease in the TV and BV in the first ROI.

Bone Parameters		Sham	Vibe	Percent Change	p-value
Total Volume (mm^3)	***Molar 1***	1.09 ± 0.16	1.03 ± 0.10	-5.5	0.164
	Molar 2	1.64 ± 0.24	1.55 ± 0.19	-5.9	0.165
	Molar 3	1.41 ± 0.14	1.28±0.23	-9.2	0.071
Bone Volume (mm^3)	***Molar 1***	0.66 ± 0.09	0.64±0.08	-2.5	0.337
	Molar 2	0.92 ± 0.11	0.90±0.11	-2.5	0.323
	Molar 3	0.87 ± 0.07	0.79±0.16	-9.9	0.071
Bone Volume Fraction (%)	***Molar 1***	60.6 ± 5.2	62.2 ± 3.9	+2.7	0.221
	Molar 2	56.5 ± 4.4	58.5 ± 5.2	+3.6	0.183
	Molar 3	62.4 ± 4.1	61.5 ± 5.7	-1.3	0.356
Bone Mineral Density (mg HA/cm^3)	***Molar 1***	748.83 ± 11.66	**770.09 ± 17.20**	**+2.8**	**0.005**
	Molar 2	755.17 ± 12.24	**777.97 ± 27.26**	**+3.0**	**0.032**
	Molar 3	798.16 ± 16.56	795.54 ± 36.33	-0.3	0.839

Table 3: Indices of micro-computed tomography bone parameters at the alveolar bone region of interest expressed as Mean ± SD. Bone mineral density under Molar 1 and Molar 2 are significantly higher in the Vibe group compared to the Sham by approximately 2.9% ($p < 0.05$).



a)



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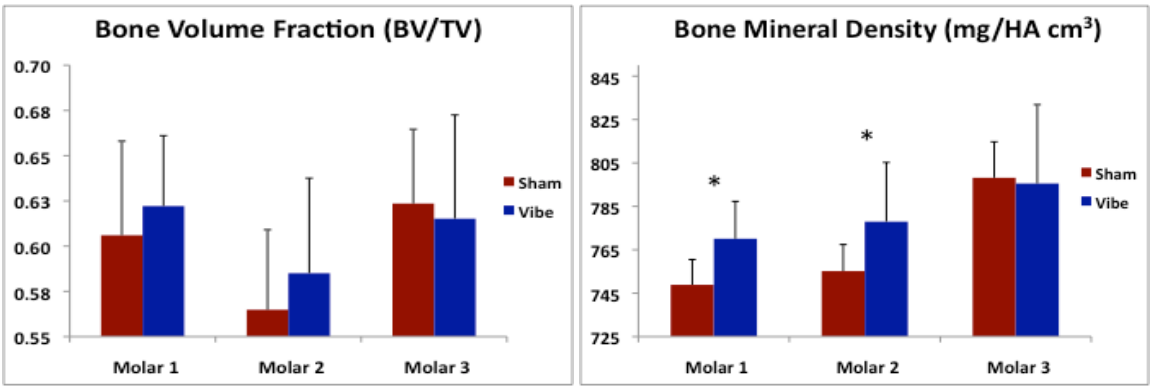


c) d)

Figure 10: Bone morphological parameters obtained for the alveolar bone region of interest from micro-computed tomography under Molar 1, Molar 2 and Molar 3. Localized LIV on the mandible influenced the bone mineral density (BMD) parameter under Molar 1 and Molar 2 by increasing the BMD in the Vibe group by approximately 2.9% ($p < 0.05$).

Another set of results was obtained to assess if the decrease in TV of the alveolar bone (**Fig 10a**) was attributed to the increase in the cortical shell with a sample size of 3. Data suggested a similar trend as above where a decrease in the cortical shell was observed (**Fig 11**). This suggested that LIV did not only slow down the growth in alveolar bone, but also in the cortical shell.

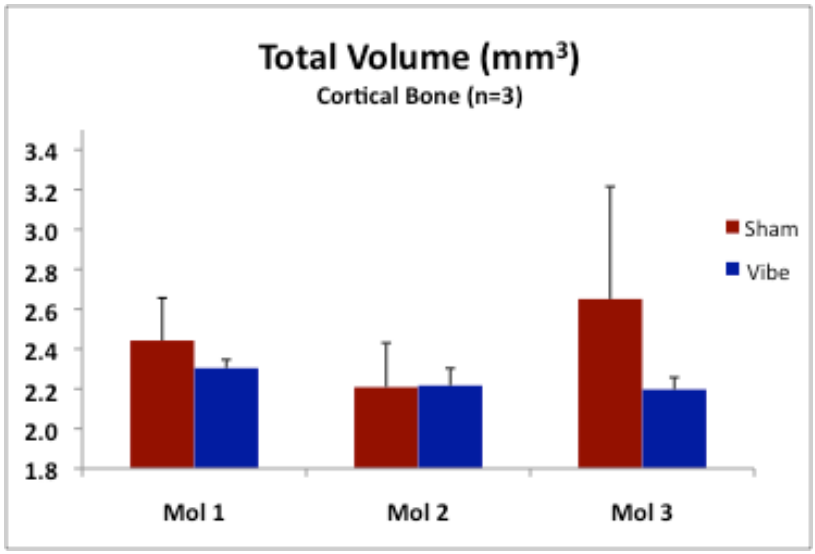


Figure 11: Total volume of the cortical shell obtained for n=3 to assess changes in the cortical bone under the influence of LIV. Data was obtained by subtracting the whole mandible TV with the alveolar bone TV. Data suggested a decrease in the TV in the cortical shell, indicating that LIV showed the growth of the mandible homogenously throughout the bone (both cortical and alveolar bone).

The first and second ROI as shown above have clearly shown an increase in the BMD in the vibe groups. In order to assess the quality of just the bone devoid of porosities within the alveolar bone, the third ROI was chosen as an 180µm^3 cube. Only the BMD parameter was evaluated as the BV, TV and BV/TV were the same for both groups. Mechanical signals caused a significant increase ($p < 0.05$) in the BMD under all three molars. The Vibe group had a 5% higher BMD as compared to the sham group (**Table 4, Fig. 12**).

		Sham	Vibe	Percent Change	p-value
Bone Mineral Density (mg HA/cm^3)	***Molar 1***	761.81 ± 37.76	**801.68 ± 38.93**	**5.2**	**0.032**
	Molar 2	824.82 ± 33.69	**868.24 ± 42.25**	**5.3**	**0.021**
	Molar 3	804.26 ± 38.47	**840.95 ± 35.56**	**4.6**	**0.039**

Table 4: Bone mineral density (BMD) index of micro-computed tomography bone parameters at the 180µm^3 region of interest expressed as Mean ± SD. This region of interest was chosen without any voids and porosities to assess the BMD of a small region of bone. BMD under all three molars are significantly higher in the Vibe group compared to the Sham by approximately 5.0% ($p < 0.05$).

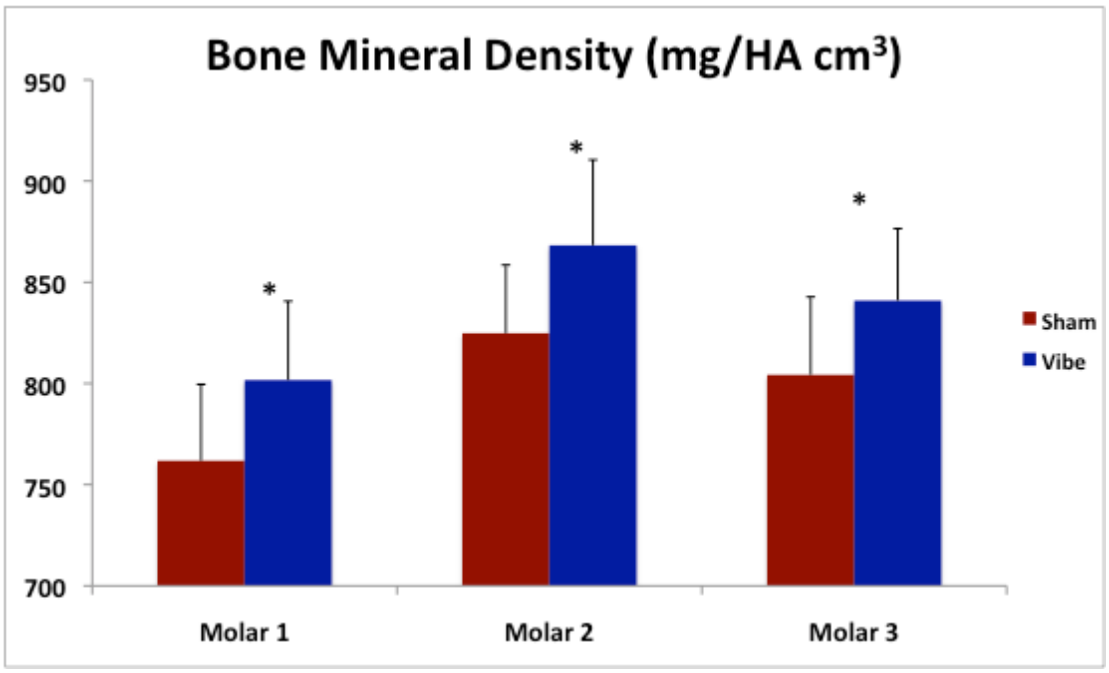


Figure 12: Bone mineral density (BMD) parameter obtained from micro-computed tomography at the 180µm^3 under all three molars. BMD under all three molars were higher in the Vibe group compared to the Sham by approximately 5.0% ($p < 0.05$).

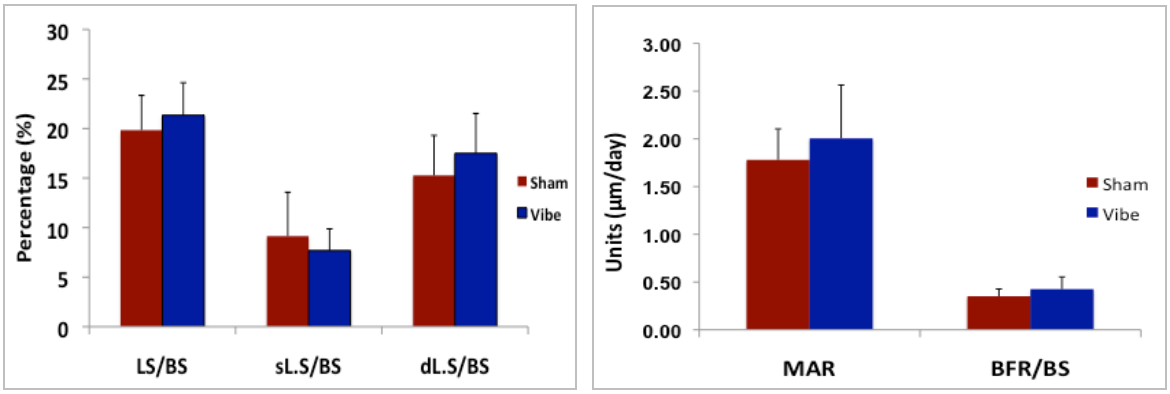
Histomorphometry

The indices of bone formation such as LS/BS, sL.S/BS, dL.S/BS, MAR and BFR/BS were not significantly different between the Sham and the Vibe groups under Molar 1 (**Table 5, Fig. 13**). All these parameters, excluding sL.S/BS, indicated a 7.5 - 21.2% increase when subjected to localized mechanical signals, however, they were not significantly increased by the LIV. The overall labeled surface (LS/BS) showed a 7.5% increase in the Vibe group compared to the Sham. Although the single labeled surfaces (sL.S/BS) showed a decrease in the Vibe group by 16%, the double-labeled surfaces (dLS/BS) showed an increase by 14.5% in the Vibe group. The localized vibrations at the mandible failed to influence significant changes in the bone formation parameters, however, the Vibe group had a 12.6% higher mineralization compared to the Sham group, which may partially explain the increased bone mineral density observed in the µCT data, consistent with a study which found a correlation between reduced mineralization density and

reduced bone stiffness [90]. However, the full extent of the MAR having an influence on the mineralization is not known, as the osteoid parameters were not studied.

Bone Formation Indices	Sham (n=5)	Vibe (n=6)	Percent Change	p-value
LS/BS (%)	19.84 ± 3.50	21.34 ± 3.29	+7.5	0.488
sL.S/BS (%)	9.14 ± 4.43	7.68 ± 2.19	-15.9	0.530
dL.S/BS (%)	15.27 ± 4.03	17.50 ± 4.03	+14.5	0.387
MAR (µm.day^{-1})	1.78 ± 0.32	2.01 ± 0.56	+12.6	0.428
BFR/BS (µm.day^{-1})	0.35 ± 0.08	0.43 ± 0.13	+21.2	0.265

Table 5: Bone formation indices obtained by dynamic histomorphometry under Molar 1 expressed as Mean ± SD, indicating no significant differences between the Sham and the Vibe groups.



a) b)

Figure 13: Plots showing the bone formation indices obtained from dynamic histomorphometry under Molar 1. No significant differences were found in any of the parameters between the Sham and the Vibe groups even though the MAR showed a 12.6% increase in the Vibe group.

Discussion

In order to test the hypothesis that short term localized, low intensity vibrations can increase the bone quantity in the alveolar bone, adult Sprague Dawley rats were subjected to localized vibrations for 6 weeks. The Vibe group was subjected to low intensity mechanical signals (0.3 g, 50 Hz) for 3 min/day, 5 days/wk for 6 weeks. μCT data suggested that low intensity vibrations did not significantly alter the bone morphological parameters such as TV, BV and BV/TV. Furthermore, dynamic histomorphometric parameters such as MAR, BFR, LS/BS, sL.S/BS and dL.S/BS were not significantly different between the two groups. However, the bone mineral density (BMD) parameter in μCT suggested a significant increase in the Vibe group compared to the Sham. These results were consistent under all three ROI's. In addition, the MAR saw a 12.6% increase in the Vibe group compared to the Sham, although the increase was not significant. This set of results indicate to us that LIV caused a change in the bone mineralization property of the bone, even though the bone quantity has not been modified by these short bouts of mechanical signals.

The bone mineralization density in Vibe group was increased in the different ROI that were chosen. This is consistent with the *in vitro* [91, 92] and *in vivo* [93, 94] studies, which have shown an increase in the mineralization matrix when subjected to mechanical loading. Bone mineral density is an important measurement predictor of osteoporotic fracture. Typically in clinical trails, conclusions of significant change cannot be drawn until an observed increase is of at least 5.5% or an observed decrease of at least 7.5% [95]. The first two ROI in μCT: whole mandible and the alveolar bone saw a 3% increase in the BMD under the first and second molar. The third ROI saw a 5% increase in BMD under all three molars.

Compared to the Sham group, the Vibe group showed a decrease in the whole mandible TV by 2 – 5 % and a decrease in the alveolar bone TV by 6-9%, consequently resulting in an increase in BMD in the vibe group. The decrease in the TV of the alveolar bone was not caused by the increase in the cortical shell, as the cortical shell also showed a decrease in the TV in the Vibe group. The exact cause of this has not been elucidated but the data could be interpreted as suggesting that the vibrations slowed the growth of the mandible. Due to the lack of a baseline

group, it cannot be determined by how much, if at all, these LIV slowed growth of the mandible. Furthermore, there were no significant differences between the weights of the animals in the groups, suggesting that growth was not impacted by the vibrations. This is expected as both groups of animals were anesthetized and were set-up under similar conditions. However, had we had data from an age match group, we would be able to assess if the anesthesia could cause changes in the body mass of the animals.

Studies in the past have found LIV to be anabolic to long bones, especially the trabecular bone [43, 65, 78]. However, in this study LIV did not seem to influence the bone formation rate by increasing the activity of the osteoblasts. In another study, low magnitude high frequency mechanical signals had a positive effect in healing bony lesion in the skull of a mouse, by significantly increasing the healing capacity compared to the control group [85]. These positive results coincides with several other observations that the fracture healing process is sensitive to small periods of daily strain and that controlled cyclic loading may provide a mechanical stimulus to the osteoblasts activity [85, 96]. Another study suggested a genetic basis for the sensitivity of the trabecular bone to exogenous mechanical stimuli [59].

In our current study, there are other factors can be attributed to the lack of significant results in the bone quantity parameters. Firstly, due to the nature of this pilot study, healthy male adult rats were chosen to eliminate the hormonal interferences. It is possible that in a healthy bone LIV that we administered was incapable of enhancing the activity of the osteoblasts, as a healthy bone will undergo a steady-state balance of resorption and formation activity. Therefore, as indicated in previous studies, a diseased bone might respond to these mechanical signals very differently compared to a healthy bone [85].

Despite the inconclusive results about quantity of the alveolar bone when subjected to LIV, data from μCT suggested that 6 weeks was sufficient to induce up to 5% increase in the bone mineralization indicating that the alveolar bone is receptive to mechanical loading as reported in literature, and resulted in an improvement in bone quality [54]. This suggests a means of controlling bone quality parameters independent of osteoclast and osteoblast activity. It is commonly perceived that osteocytes are most likely sensitive to mechanotransduction in

bone and more easily translate mechanical strains into biochemical signals [97]. Which suggests that osteocytes regulates the mineralization of bone osteoid and thereby plays a crucial role in mineral metabolism [97]. The results of the BMD concur with this theory as these mechanical signals triggered the activity of the osteocyte, even though osteoblasts and osteoclasts seemed to have been unaffected. These results are evidence that the alveolar bone is responsive to the LIV; perhaps a longer bout of these signals and a long-term study could yield more conclusive results. It is also possible that a different mechanism might be promoting mineralization, which is independent of cells. However, in order to understand the cellular and chemical causes of an increase in BMD, further analysis using Fourier transform infrared spectroscopy will need to be performed.

CHAPTER 4

Low intensity vibrations increase bone mineralization caused by changes in the chemical composition in the alveolar bone at a cellular level.

Abstract

The biomechanical quality of bone is correlated with bone mass and is a good predictor of fracture risk and is believed that the mechanical properties of bone are due to its macroscopic anatomical dimension to the micro and the nanometer range. These parameters include shape and geometry, cortical and trabecular architecture, mineralization density and the material properties of the organic matrix. Our previous hypothesis suggested that localized LIV has the potential to increase the bone mineralization when subjected to mechanical signals for as little as 3 min/day even though it could not enhance bone quantity. Twelve-week old male Sprague Dawley rats were randomly divided into two groups (n=10): Sham control and Vibe group. The Vibe group was mechanically stimulated (0.3 g, 50 Hz) for 3 min/day for 5 days/week over 6 weeks. While the Sham was not vibrated, the entire mandible set-up and procedure was simulated but without the power turned on to the device. Fourier transform infrared (FTIR) spectroscopy was used to understand the chemical composition of the mineralized bone and to assess if the 5% changes in mineralization using μCT can be attributed to changes in chemical composition. LIV did not significantly influence any of the four chemical parameters such as the mineralization, carbonate incorporation, collagen cross-linking and crystallinity. However, the lack of significant results in the chemical composition can be attributed to several factors such as the use of a healthy animal model, bout period and possibly length of the study. It can be concluded that even though the bone chemical properties were not altered greatly with LIV, the morphological patterns indicate an enhanced bone mineralization indicating the sensitivity of the alveolar bone to low intensity vibrations. This study has provided the basis for further research on enhancing the quantity and quality of alveolar bone. If successful, it can provide as a

promising modality to prevent alveolar bone loss caused by estrogen depletion, osteoporosis and periodontal diseases, which have serious clinical implication of early tooth loss.

Introduction

Alveolar bone loss has major economic impact on individuals and costs an estimated $5-6 billion/year for just the surgical treatments related [11]. The most common cause of alveolar bone loss is attributed to chronic periodontitis. It affects approximately 35% of dentate adults in the United States between ages 30 – 90, and approximately 13% have a moderate to severe form of the disease [13]. Like osteoporosis, periodontitis is considered a “silent” disease, not causing symptoms until late in the disease process when tooth loss may occur [14]. In addition to periodontal diseases, estrogen deprivation arising from menopause increases the risk of developing osteoporosis and oral diseases [67]. Studies also show that postmenopausal women with osteoporosis and periodontitis are likely to exhibit a loss of dentoalveolar bone height and a decreased BMD of the alveolar bone. As a result, these women are at a high risk of experiencing early loss of teeth caused by decreased bone mineral density [70, 73, 74].

It is well established that skeletal unloading such as that occurs in spinal cord injury, prolonged bed rest, limb immobilization and microgravity results in generalized skeletal loss and loss of mineralization of the skeleton, particularly in bones that bear weight normal conditions. This is caused due to unrestrained bone resorption caused by osteoclast activity and decreased osteoblast mediated bone formation [35, 36]. The two main determinants of bone mineral density are the magnitudes of peak bone mass achieved and amount of bone lost. Therefore, peak bone mass is a major determinant of bone mass later in life and increased amounts of peak bone mass may decrease the risk of osteoporotic fractures [36].

For the prevention and treatment of osteoporosis, physical exercise is highly recommended [40, 41]. In humans, the practical goal of an exercise intervention is not merely to increase bone mass, but also to reduce the incidence of fractures. The animal studies in the past have clearly suggested that bone responds preferentially to certain forms of mechanical loading.

It has been long been known that factors such as high-magnitude loads that induce relatively large bone strains are more osteogenic than low-magnitude loads [42]. At higher frequencies, much lower strains are necessary to maintain bone mass. It has been predicted that the strain magnitudes required for maintaining bone mass can be much lower than 70 microstrain at very high loading frequency [38, 50, 51].

Both the long bone and alveolar bone are very sensitive to altered mechanical environment through static and dynamic loading [54]. Studies have investigated that whole body vibrations at low-magnitude and high frequency are a non-invasive and non-pharmacological methods to prevent post-ovariectomy bone loss in an animal models [55] and also in postmenopausal women [56]. In animal models such as rodents and larger mammals, these signals promote bone formation [59], enhance bone morphology [57] and also increase bone strength [60] with as little as 10 min per day.

Bone adaptation during skeletal growth and development continuously adjusts skeletal mass and architecture to changing mechanical environments. Literature suggests that the three fundamental rules governing bone adaptation are: 1) it is driven by dynamic, rather than static loading; 2) Only a short duration of mechanical loading is necessary to initiate an adaptive response and 3) Bone cells have the potential to accommodate to any mechanical environment, making them less responsive to routine loading signals [75].

Convincing evidence exists in the literature about the treatment modalities to enhance the bone mineralization by pharmacological interventions such as bisphosphonates, which inhibits bone resorption and increase bone formation and thereby increasing the mineralization in the bone. In large clinical trials, it was shown increasing mineralization with the aid of bisphosphonates reduced fracture rate in postmenopausal women [98-100]. The biomechanical quality of bone is correlated with bone mass, which is a good predictor of fracture risk. However, bone strength is also influenced by factors independent of mass. The outstanding mechanical properties of bone are due to it the organization of parameters such as shape and geometry, cortical and trabecular architecture, mineralization density and material properties of the

organic matrix. Therefore, it can be safely assumed that bone remodeling, which may alter some of these factors can most definitely affect the bone quality [101].

From Hypothesis 2, our results suggested that LIV did not enhance bone quality by increasing bone formation. However, we found that these signals increase the bone mineralization across the Vibe group. It can be postulated that the increase in bone quality and not in bone quantity is possibly due to osteocytes being more sensitive to mechanical signals than the osteoclasts and osteoblasts for as little as 3 min each day. Therefore, the chemical and structural changes caused by LIV that resulted in an increase in bone mineralization are yet to be elucidated. In the work presented here, we determined at a microscopic level the cause for increase in mineralization in the Vibe group. Fourier transform infrared spectroscopy (FTIR) was used to study differences in chemical composition between the two groups.

Methods

Experimental Design

Twenty 12-week old male Sprague Dawley rats were obtained from the Charles River (Wilmington, MA) and were randomly divided into two groups (n=10): (1) Sham control and (2) Vibe group. The Vibe group was subjected to localized vibrations at 50 Hz, 0.3g for 3mins daily for 5 days/week under isoflurane anesthesia. While the Sham group was not vibrated, the entire set-up and procedure was simulated without the mechanical signals.

Stony Brook University's Institutional Animal Care and Use Committee (IACUC) approved the experimental procedures used in this experiment. The animal model chosen for this experiment was male Sprague Dawley rat at 12-week old. The animals were anesthetized in a chamber with 5% Isoflurane in oxygen with a pressure of 1-2 liters/min. After they were anesthetized, they were removed from the chamber and positioned on the foam bed where they were continued to be given approximately 3% Isoflurane in oxygen with a pressure of 1-2 liters/min using a nosecone for the time they were mechanically stimulated.

While the animals were anesthetized on their foam bedding, the actuator was placed between the jaws in order to provide localized mechanical signals to the alveolar bone. To prevent the dislocation of the actuator during the vibrations, a load bearing weight was suspended across the mouth of the rat (Fig 1).

In order to measure dynamic indices of bone formation, the rats were injected calcein ($10mg.kg^{-1}$), a fluorescent label, subcutaneously. Animals were injected at two time points, 5 & 6 days and 14 & 15 days prior to the end of the study. They labeled on 2 consecutive days to be able to achieve better fluorescence imaging during the analysis.

Following the 6-week experimental procedure, the mandible of the rat was extracted from the skull and was divided into left and right hemi-mandibles. Each hemi-mandible was fixed in 10% neutral buffered formalin (NBF), if there was a need to perform histological analysis such as osteoclasts and osteoblasts activity in the future. Two days after fixing in 10% NBF, the hemi-mandible tissues were harvested in 70% ethanol for micro-computed tomography (μCT) and FTIR analysis.

Fourier Transform Infrared Spectroscopy (FTIR)

Sample Preparation

To establish the quality of the mineralized bone between the groups, high-resolution analysis of the chemical composition on the alveolar bone was performed using Fourier transform infrared spectroscopy (FTIR). Twenty rat left hemi-mandibles were embedded in PMMA after μCT scanning. Before these bone blocks were used for FTIR analysis, they were sectioned at the ROI for histomorphometry. For reflectance FTIR, the surface of the embedded bone in PMMA were ground and polished. The root canal of the first molar was identified as the ROI for FTIR analysis, similar to that of the μCT and histomorphometry ROI. The PMMA blocks were first ground and polished (Buehler, Lake Bluff, IL) with 600, 800 and 1200 grit carbide paper and with diamond suspended cloth with particle size 3, 1, 0.25 and 0.05μm. It was made sure,

that the grinding and polishing did not remove the bone beyond the first molar, as the alveolar bone under the first molar was the selected ROI.

Data Collection

In order to establish the quality of the alveolar in the sham and the vibrated groups, FTIR analysis were performed to assess the mineral content and composition. The internal light source of a Nicolet Continuum IR microscope (Thermo Electron Corp) at beamline U10B of the National Synchrotron Light Source at Brookhaven National Laboratory was used to collect the FTIR data. The light source was coupled to a Mercury Cadmium Telluride (MCT) detector. The spectra were collected from reflectance mode FTIR over the frequency range of 4000-800 cm^{-1}, 128 scans/pixel at a pixel resolution of 10 µm using a 15x objective for the IR light and by applying 4x4 binning. Furthermore, a 64 x 64 FPA (Focal Plane Array) for data collection was used.

Before the bone blocks were scanned, a reflection spectrum from an Au slide was collected as a background. After the bone blocks were scanned, the reflectance spectrum was converted to an absorbance spectrum using Kramers-Kronig transformation. Bolometer and GeCu detectors were used to obtain data in the spectral ranges from 70 cm^{-1} to 400 cm^{-1} and from 200 cm^{-1} to 1000 cm^{-1} respectively.

The ROI was selected as the alveolar bone under the first molar, similar to that of the µCT ROI. The entire region encompassing the alveolar bone and the cortical bone was scanned, however, only the alveolar bone was analyzed. Transform package was used to use thresholding technique to analyze the chemical parameters of the alveolar bone. The same threshold range was used for the Sham and the Vibe groups, by observing the integrated peaks for each sample and making a sensible estimate of the range. In order to exclude every single PMMA pixel from the data, the PMMA thresholding was also employed. Furthermore, due to the PMMA infiltration into the periphery of the bone, approximately a pixel from the edges were excluded in order to analyze just the bone without the interference of PMMA.

Chemical Parameters

Bruker OPUS 6.5 software was used to determine the bones chemical parameters such as bone mineralization, collagen cross-linking, carbonate incorporation and crystallinity as outlined in **Table 6** [102].

Chemical Parameter	Reflection Integration Range (cm^{-1})
Phosphate	900 – 1200
Protein	1600 – 1700
Carbonate	1414-1424
Mineralization	Phosphate/Protein
Carbonate Incorporation	Carbonate/Phosphate
Collagen cross-linking	(1659 - 1661) / (1689 - 1691)
Crystallinity	(1024 - 1026) / (1034 - 1036)

Table 6: Chemical parameters with their associated spectral range used for integrating the absorbance spectra obtained via reflection FTIR.

Statistical Analysis

The data were expressed as mean ± SD. Differences amongst the groups were tested using an unpaired two-tailed t-test where p lower than 0.05 was considered as significant. Percentage changes in the groups were calculated by subtracting the mean of the sham group from the mean of the vibe group and dividing by the mean of the sham group.

Results

Fourier Transform Infrared Spectroscopy (FTIR)

None of the four chemicals parameters exhibited significant differences between the Sham and Vibe group (**Table 7, Fig 14**). However, consistent with our µCT data, the mineralization was approximately 1.5% higher in the vibe group, but not significantly higher. FTIR also suggested a homogenous distribution of the chemical parameters across the entire mandible as seen in one animal from each group. The cortical or the alveolar bone did not have a distinguishably different spatial distribution of the chemical parameters (**Fig. 15**).

FTIR Parameters	Sham (n=10)	Vibe (n=10)	Percent Change	p-value
Mineralization	5.315 ± 0.306	5.391 ± 0.273	+ 1.43	0.566
Carbonate Incorporation	0.018 ± 0.001	0.017 ± 0.001	- 3.13	0.292
Collagen cross-linking	2.152 ± 0.151	2.100 ± 0.161	- 2.47	0.456
Crystallinity	0.775 ± 0.028	0.770 ± 0.033	- 0.32	0.860

Table 7: Chemical parameters obtained using FTIR under Molar 1 is expressed as Mean ± SD. None of the chemical parameters showed significant differences between the Sham and the Vibe groups.

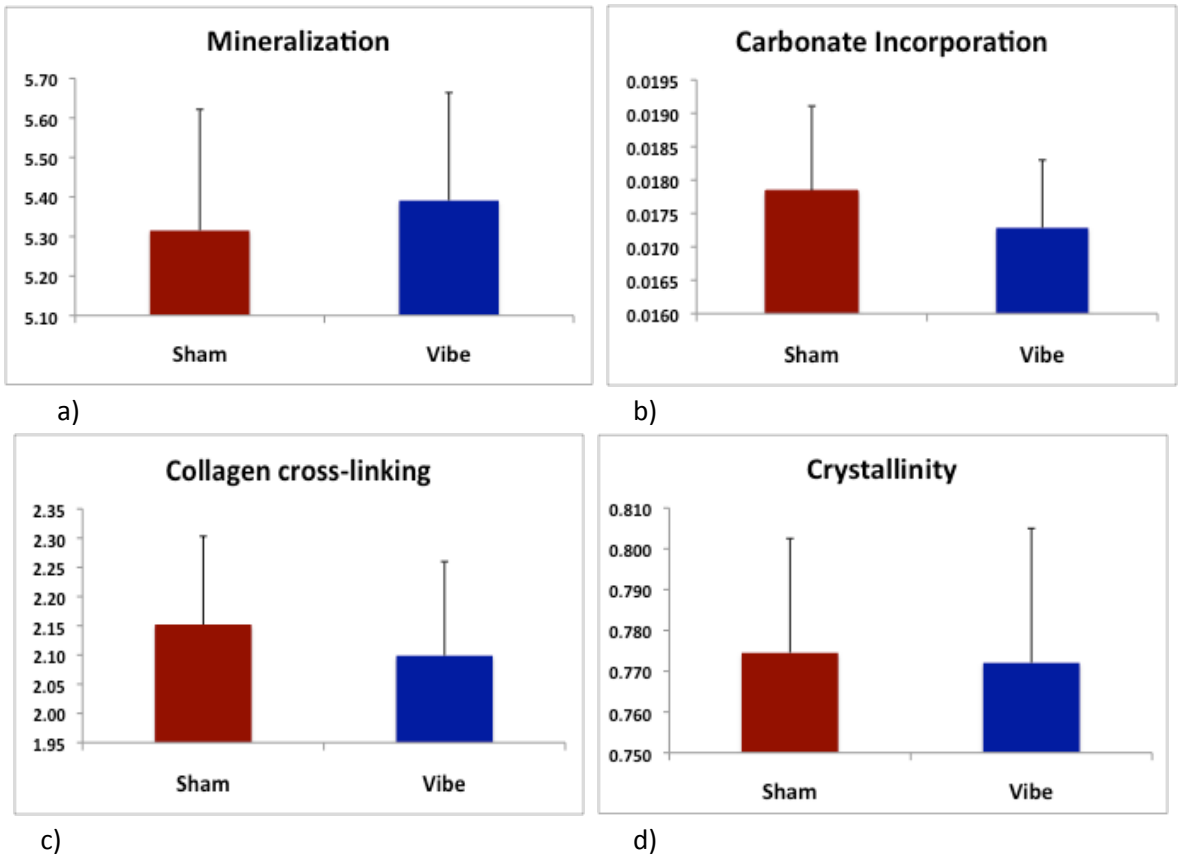


Figure 14: Plots showing the chemical parameters using FTIR imaging under Molar 1. None of the chemical parameters showed significant differences between the two groups. The x-axis has no units as each of the chemical parameters is the ratio of the chemical composition as shown in Table 7.

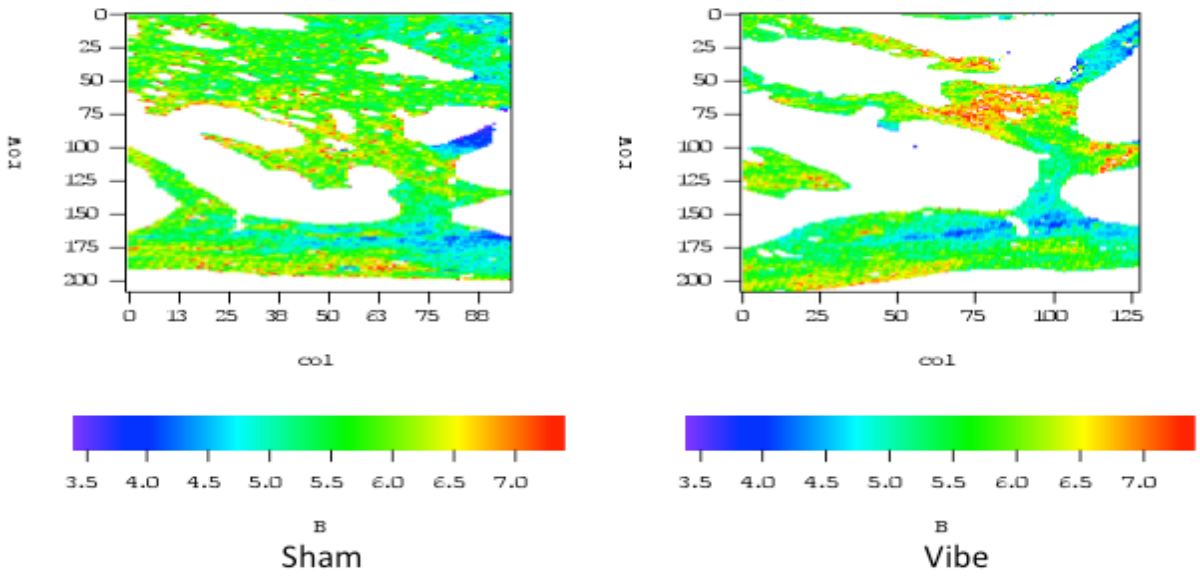


Figure 15: FTIR spectroscopy spatial maps of the alveolar bone showing the distribution of mineralized bone in a) Sham and b) Vibe groups. The red color of the maps depicts the highly mineralized bone while the blue depicts the least mineralized bone. By visual inspection, the spatial maps of a single animal from each group suggest, the Sham and the Vibe group does not show remarkably different spatial mineralization distributions. This is confirmed by the data as seen in Table 7 where there is no significant differences in the mineralization parameter ($p > 0.05$). The bone mineralization in FTIR is a different measure compared to that of μCT (BMD).

Discussion

To test our hypothesis that low intensity vibrations can enhance the quality of alveolar bone, we analyzed the chemical composition in the alveolar bone at a cellular level using FTIR analysis. From our previous experiment, μCT revealed up to 5% increases in the bone mineralization in the alveolar bone as caused by these mechanical signals. Therefore, in order determine the factors that caused an increase in the bone mineralization, the chemical composition of the alveolar bone such as mineralization, carbonate incorporation, collagen cross-linking and crystallinity were studied. Results suggested, no significant differences in the chemical properties between the groups.

Studies have indicated that the biomechanical quality of bone is correlated with bone mass and is a good predictor of fracture risk. It is believed that the outstanding mechanical properties of bone are due to its macroscopic anatomical dimension to the micro and the nanometer range. These parameters include shape and geometry, cortical and trabecular architecture, mineralization density and the material properties of the organic matrix. Therefore, it is clear that bone remodeling, which may alter some of these factors can affect the bone quality [103].

In order to analyze the bone mineral and matrix properties, which are some of the main determinants of bone mineralization in health and disease, we used Fourier transform infrared spectroscopy (FTIR). The spatial arrangement of bone tissue is retained in FTIR imaging; quantitative data can be obtained on bone mineral (hydroxyapatite) size and composition, and on the matrix structure and composition [104]. The data obtained from μCT revealed up to a 5%

increase in mineralization in the Vibe group. Therefore, it was of interest to us to understand the process underlying this mineralization and to further understand if other parameters of the bone were enhanced with LIV that would enhance the quality of the alveolar bone.

Bone is a unique composite composed of 50% wt minerals, 25% wt matrix and 25% wt water. Matrix is a template for bone mineral deposition and is composed of type I collagen. The mineralization of the bone provides the bone hardness to support its functions at different locomotion levels. The mineral component of bone is formed from calcium phosphate, known as the hydroxyapatite crystals. The incorporation and/or substitution of foreign ions, other than calcium and phosphate, tend to modify the basic hydroxyapatite. Several studies have shown that carbonate ions are located inside the bone hydroxyapatite. Studies have revealed, both trauma or disease such as defects and fractures lead to an increase in bone substitutes and thereby change the composition and property of the bone. There is also a nonlinear relationship between crystallinity and age, as younger rats have more crystallized bone than older rats [105].

Studies have explained the causes for changes in mineralization such as lack of gravity caused by disuse [106] and vitamin D deficiency caused a lower mineral density due to decreased mineral formation [107]. However, not all bone defects have led to a decrease in bone mineralization. In a study conducted on the incisor of an osteoporotic rat, a shift in mineralization profile led to higher mineralization caused due to a complete shutdown of bone resorption. This led to an increase in crystal size due to a continual accretion of new mineral on pre-existing crystals [108]. Therefore, to understand the cause behind an increase in mineralization, the chemical parameters of the bone were studies.

Furthermore, studies have also indicated a homogenous distribution or a “reproducible anatomical variation” in a normal bone compared to an osteoporotic bone [109]. However, the mineral composition of the osteoporotic bone in the literature is spare and conflicting [110]. Such studies have reported that the osteoporotic bone mineral consists of crystals that are larger and more well defined that those in normal bones [111, 112], smaller and less defined than normal bones [113] or that there are no differences between them [114]. However, a study conducted by Paschalis et al confirmed that mineral in osteoporotic bones exhibit higher

crystallinity or maturity compared to the normal bone [109]. The cause of this has been attributed to the inhibition of osteoclast activity, which shut the resorption activity. However, in this study, we used perfectly intact bones of adult rats, which might be able to explain the lack of differences in the crystallinity parameter between the two groups. Therefore, in order to assess if LIV slows the bone loss, we might need to perturb the "system", in our case use an OVX animal model.

The FTIR results from our study were inconclusive and it is still not clear what the underlying process behind the increase in mineralization is attributed to. From the results, an increase in mineralization (1.4%) has not caused an increase in the mineral crystallinity (-0.32%). In saying that, the increase in mineralization is very marginal to have caused an increase in the crystallinity. Also, the μCT mineralization parameter is very different from the mineralization parameter in FTIR, the former is the morphological parameter and the latter is a chemical composition parameter. Also, the FTIR bone mineralization is a measure only on a particular surface, while the μCT gives a measure of mineralization in a 3D volumetric space. Therefore, we can assume that LIV did not change the chemical properties of the bone. This is possibly true as the 5% increase in mineralization in μCT is not sufficiently large to see those changes in the chemical composition at a surface of a bone. But these increases in mineralization can definitely suggest that the alveolar bone is responsive to these mechanicals signals. However, the lack of significant results in the chemical composition can be attributed to several factors such as the use of a healthy animal model, bout period and possibly length of the study. It is also possible that an increase in the bone mineralization could have come from a combination of these chemical compositional changes, although there were not significant individually, they might have had an influence on the bone mineralization. Therefore, a longer-term study will need to be performed using a diseased animal model, such as an estrogen deficient or an osteoporotic model (OVX), to assess the effect of LIV on the chemical composition parameters. It can be concluded that even though the bone chemical properties were not altered greatly with LIV, the morphological patterns indicate an enhanced bone mineralization indicating the sensitivity of the alveolar bone to low intensity vibrations.

CHAPTER 5

CONCLUSIONS

Alveolar bone is a clinical condition affecting approximately 42% Americans and has an economic impact of $5-6 billion/year just for the cosmetic and functional surgical treatments related to bone restoration and tooth salvation. Alveolar bone loss is caused due to factors such as estrogen deficiency, osteoporosis and the most commonly occurring periodontal diseases. The clinical implication of alveolar bone loss is pre-mature tooth loss. Several studies have found a correlation between oral bone loss, osteoporosis and periodontitis. Since osteoporosis is characterized by deterioration of the bone structure and a reduction in bone mass such that the bone is susceptible to fracture with very little impact.

The treatment of periodontitis is to mainly control the infection and the medications include antimicrobial mouth rinse, antibiotic gels, antibiotic microspheres, enzyme suppressant and oral antibiotics. However, for chronic cases of periodontitis surgery such as flap surgery and bone and tissue grafts is required to promote bone formation and growth. Treatment for osteoporosis in the long bones include antiresorptive therapies such as estrogen replacement therapy (ERT), various bisphosphonates such as alendronate, the SERM raloxifene, calcitonin and sodium fluoride. However, treatment with parathyroid hormone (PTH) is considered an ideal therapy for osteoporosis as it is considered anabolic to bone. Several studies have also shown a positive correlation between decreased risk of osteoporosis and increase in exercise. This theory is established from the sensitivity of the bone to mechanical signals as additional loading on the skeletal system has proven to enhance the quality and quantity of bone. It has been well established that mechanical loading originating from either muscular function and/or gravity is an important factor in bone homeostasis.

Studies have shown that long bones (post-cranial) and alveolar bone (cranial) are both sensitive to mechanical environment through static and dynamic loading. It also has been suggested that whole body vibrations (WBV) at low-magnitude and high frequency are a non-

invasive and non-pharmacological method to prevent bone loss, particularly in the long bones. These low- magnitudes are associated with extremely small strains, much lower than the physiologically acceptably levels that might potentially cause damage. Similarly, in orthodontic tooth movement, the applied force on the tooth supporting structures causes compressive and tensile forces on the alveolar bone. However, static loads do not seem to play an important role in skeletal osteogenesis as it is primarily driven by dynamic loading, where the most important characteristics are strains rates, frequency and the duration of the dynamic loading.

The premise of our study was to investigate if low-level mechanical signals, which are administered locally, can influence the quantity and quality of the alveolar bone. A study in the past revealed that a healthy alveolar bone is not as sensitive to whole body vibrations as compared to an affected alveolar bone. Therefore, for the purpose of our study, we employed low-level mechanical signals (0.3 g, 50 Hz) on the mandible (lower jaw) of a rat. If these mechanical signals are beneficial to the mandibular morphology and/or alveolar bone, it may serve as a foundation to explore in people to slow tooth loss.

The results obtained from our study, suggested that the alveolar bone is sensitive to these low intensity vibrations as indicated by the increase in bone mineral density obtained from μCT, despite not being able to enhance the quantity of the bone. The chemical parameters of the mineralized bone were not significantly different between the groups as seen in the data obtained from FTIR spectroscopy. This led us to believe that the changes in mineralization as seen in μCT is not attributed to the changes in the chemical composition. It can be speculated that these changes were caused due to the sensitivity of the osteocytes to mechanical signals. We believe that the lack of significant data in the chemical composition is possibly due to the healthy animal model employed in this pilot study. Furthermore, our study suggested that as little as 3 min/day of LIV on the mandible is sufficient to induce changes in the bone properties, however, believe that it may not be sufficient to see significant changes over a period of 6 weeks. Also, a limiting factor in our study was the design of the actuator. We did not have control of the magnitude and frequency independently. Therefore, we used the best possible

combination of the mechanical signal (0.3 g, 50 Hz) as signals of similar magnitude and frequency have proven to be anabolic to long bones.

Due to the nature of this pilot study, we employed healthy adult male rats to eliminate hormonal interferences. Therefore, we believe, that applying these LIV on an osteoporotic animal model, using different combinations of mechanical loading can provide critical information on the potential treatment modality to prevent oral bone loss caused by diseases such as osteoporosis and periodontitis. However, the results obtained from our pilot study has established basis to further the research on alveolar bone loss and provided a critical piece of information on the sensitivity of the alveolar bone to low intensity vibrations. Ultimately, from a broader perspective, our application of LIV on the teeth seeks to explore the potential benefits of brushing teeth with an electric toothbrush, to not only clean teeth, but also to maintain healthy teeth caused by the low magnitude, high frequency signals, in order to improve the quantity and quality of the alveolar bone. Our current study has reinforced that brushing teeth is not only to keep teeth clean but also to potentially improve the bone quality as seen in the bone mineralization parameter in as little as 6 weeks.

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