A STUDY OF OSTEOCYTE APOPTOSIS IN MECHANICALLY LOADED AND
UNLOADED MURINE TIBIAE

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ABSTRACT

A Study of Osteocyte Apoptosis in Mechanically Loaded and Unloaded Murine Tibiae

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Previous research has provided evidence in support of apoptotic osteocytes playing a role in the bone remodeling process. In this study, we examined the regional and quadrantal variations of apoptotic and viable osteocytes in cyclically loaded and unloaded samples. Left tibiae of C57 Black 6 Taconic mice (C57Bl/6) were cyclically loaded for either 2 weeks or 5 weeks, with the right tibiae being used as controls. After loading, tibiae were resected, processed, and then stained using either a TUNEL stain, to show apoptotic osteocytes, or a 2.0% methyl green solution, to reveal viable cells. Cross-sectional images from each tibia were then captured and analyzed in each region (distal, midshaft and proximal) and quadrant (cranial, lateral, caudal, and medial) by counting the number of osteocytes, both apoptotic and viable, and subsequently calculating the percentages and densities of those osteocytes. Individual analysis of each sample group showed that the 5 week loaded bones, with the most statistically significant p-values, had the most regional variations within the samples, specifically showing decreased apoptotic and viable osteocytes in the lateral quadrants. Comparative analysis revealed a statistically significant higher percentage and density of apoptotic osteocytes in 5 week loaded samples compared to all other samples. This provides further quantitative evidence in support of apoptotic osteocytes playing a role in bone remodeling.

Keywords: osteocyte apoptosis, bone remodeling, cyclic loading

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1. INTRODUCTION

1.1 Bone Biology

1.1.1 Bone Function and Properties

Bone is a connective tissue with unique properties and functions that support the human body. With its mineralized extracellular matrix, collagen packed outer shell and regions of porous bone, bones, in general, are able to be rigid and strong yet maintain some flexibility (Bilezikian, et al. 2008). These properties go hand in hand with its support, protection, movement and metabolic functions. Bones come in various shapes and sizes throughout the human body and provide the framework that holds the human body up, helps it move, and protects the internal organs. For example, bones in the human skull help protect the brain while bones that make up the rib cage protect organs like the heart and lungs. Bones also act as lever arms that allow the human body to move and perform basic tasks. Skeletal muscle is attached to bone via tendons, which is why bones are able to act as a mechanical support system for muscle activity that transfers forces and enables motion. Acting as a source of inorganic ions like calcium and phosphate, bone participates in calcium homeostasis for the human body, releasing minerals into the bloodstream as needed (Marieb 2005). Bone is able to perform its versatile mechanical, protective and metabolic functions so effectively because of its structure.

1.1.2 Bone Structure

There are two main types of bone, cortical (compact) and trabecular (cancellous), each with their own unique structure. Cortical bone, with a porosity of about 5 to 10 percent, is the denser of the two and makes up the outermost layer of bone (Figure 1). The primary structural unit of cortical bone is the osteon. Each osteon is shaped like a

tube and contains concentric layers of packed collagen fibrils called lamellae. Lamellae are also cylindrical in shape and are aligned along the long axis of the bone giving it some strength. At the junctions of the lamellae lie lacunae or spaces in which mature bone cells called osteocytes reside. Canaliculi are small canals that connect lacunae to each other as well as to the central Haversian canal containing blood vessels and nerve fibers. The processes of the osteocytes also jut into these canaliculi connecting them together. Volkmann's canals are a second type of canal that run perpendicular to Haversian canals and connect these Haversian canals to each other as well as to the membrane that lines the outer bone surface or periosteum. The endosteum is the thin, inner membrane that lines the medullary cavity, whose walls are composed of trabecular bone.

Figure 1. Cortical and trabecular bone structure (International Osteoporosis Foundation, 2011)

Trabecular bone, with porosities ranging from 50 to 90 percent, is much lighter than cortical bone and uses this structure to perform its primarily metabolic functions. Trabecular bone is made of trabeculae, beam or strut like structures that are irregularly arranged along lines of stress giving the bone a spongy appearance and providing some strength and resistance. Trabeculae also contain irregularly arranged lamellae and

osteocytes that are connected to each other by processes residing in canaliculi. Due to its porous nature, many of the voids in trabecular bone are filled with marrow where blood cells are produced (Marieb 2005).

1.1.3 Bone Matrix

Bone is primarily made up of bone matrix. Bone matrix is strengthened by calcium deposits and is made up of organic and inorganic components. Approximately 90% of the organic extracellular components are type I collagen and 10% are proteoglycans, numerous proteins and cellular components. The large amount of collagen seen in the organic part of the matrix is what helps give bones their elasticity and tensile strength. The inorganic part of the matrix is made of hydroxyapatite, which accounts for most of the weight in the matrix and thus is responsible for providing much of the hardness and compressive strength seen in bone. When bone is being created, osteoblasts secrete bone matrix around blood vessels. As the matrix hardens, these cells get trapped inside and develop into osteocytes. Even though this formed bone matrix is hard and impervious to nutrients, the canaliculi and cell to cell communication via gap junctions allow the containing cells to receive the nutrients needed.

1.1.4 Bone Cells

Bones contain four different cell types within: osteoblasts, osteocytes, osteoclasts and bone lining cells. Osteoblasts, osteoclasts and bone lining cells can be found on the surfaces of bone while osteocytes are embedded within the bone matrix.

Osteoblasts, much like osteocytes and bone lining cells, develop from osteoprogenitor cells. Osteoblasts are mononuclear, cuboidal, fully differentiated cells whose primary job is to create bone matrix by laying down osteoid. Osteoid is produced

at a rate of 1 micron per day. About ten to twenty percent of these osteoblasts will become embedded in the bone matrix (Noble 2008).

Opposite of osteoblasts are osteoclasts, large, multinucleated bone cells which resorb bone rather than lay down new bone. Osteoclasts resorb bone at a rate of about tens of microns per day by demineralizing the bone and dissolving the present collagen (Martin, et al. 1998). There are two important areas of its plasma membrane, the ruffled border and the clear zone. The ruffled border is the central area in the plasma membrane where bone resorption takes place. The clear zone is the area of the membrane that surrounds the ruffled border and attaches the osteoclast to the bone matrix (Bilezikian, et al. 2008).

Osteocytes, are the most common cell type, of the four, found in bone; there are about 10 times more osteocytes than there are osteoblasts (Aarden, et al. 1994).

Osteocytes, as previously mentioned, are formed when an osteoblast gets embedded in the bone matrix it secretes. When the osteoblast first starts getting embedded in the matrix, that part of the matrix is still relatively weak. As other osteoblasts continue laying down bone, the embedded osteoblast gets further secured in the matrix as the matrix starts to calcify and the osteoblast starts changing shape and reducing in size.

Specifically, the number of organelles is reduced in the mitochondria, golgi apparatus and endoplasmic reticulum (Bell, et al. 2008). At this point, the osteoblast becomes an osteocyte. Osteocytes are stellate shaped and are located within lacunae and communicate with other cells extracellularly via the canaliculi throughout the matrix and intracellularly with other osteocytes via cytoplasmic processes utilizing gap junctions (Aarden, et al. 1994). Osteocytes, in response to loading, can produce a number of molecules that may

be involved in osteogenesis or may undergo apoptosis, or cell death, if a lot of localized remodeling is occurring. It has been thought that osteocytes may also be involved in targeting osteoblasts and osteoclasts for bone remodeling due to sensing applied loads and their related strains (Noble 2003).

A second type of cell that can result from osteoblasts are bone lining cells. These long, elongated cells are inactive and neither lay down bone nor resorb it and are spread out on newly formed surfaces of bone after remodeling has stopped. The cells will detach when osteoclasts start resorbing bone. Bone lining cells are also thought to regulate the influx and outflux of various ions (Bilezikian, et al. 2008).

1.2 Bone Remodeling

1.2.1 Modeling vs. Remodeling

Over the human life span there are two very important processes that occur related to bone. The first, modeling, takes place when humans are young and involves creating bone. This phase is all about bone formation over bone resorption as it is more important to build and shape the mineralized tissue when there is little. For bone modeling, bone can be resorbed in some places and placed down in others, independent of each other (Martin, et al. 1998). The second process, remodeling, involves maintaining and repairing these bones and occurs over the entire human life span by removing and replacing a fraction of the bone every year; microscopic damage caused by fatigue or loading is repaired and prevented by bone remodeling. Unlike bone modeling, bone remodeling doesn't usually affect the shape or size of the bone. In this phase, bone resorption and bone formation are coupled so that all the bone taken away can be equally replaced by new bone at the same site. This is a local event in which local factors recruit

and regulate the necessary cells and is carried out by a groups of osteoblasts and osteoclasts called a basic multicellular units or BMUs (Bilezikian, et al. 2008). A BMU, shown in Figure 2, consists of about ten osteoclasts, hundreds of osteoblasts and a central capillary to supply nutrients. BMUs go through a few stages throughout the remodeling process, shown in Figure 3.

Figure 2. The structure of a Basic Multicellular Unit (BMU): The upper portion shows a longitudinal section through an active BMU while the lower portion displays a cartoon showing how the osteoclasts and osteoblasts work. The blue osteoclasts can be seen resorbing bone, while the green osteoblasts follow laying down unmineralized osteoid, stained blue, that slowly becomes mineralized, which can be seen stained black. (Robling, et al. 2006).

1.2.2 Process

The first stage, activation, happens when osteoclasts are signaled to form by local factors and start resorbing bone. This leads to the resorption stage where osteoclasts, at the front of the BMU, start removing bone moving forward at rate of about 40 micrometers per day; osteoclasts remove bone in a cone like shape for cortical bone and forms flat grooves if removing bone on a bone surface or in trabecular bone. The diameter of the region dug out by osteoclasts reaches about 250 to 300 micrometers, defining the approximate size of the secondary osteon to be formed (Robling, et al. 2006). After resorption comes the reversal stage, a ten day transition period, in which a cement line is formed on top of the resorption space by mononuclear cells and connects

the old bone to the new bone that will be put down on top. Next, is the formation stage and occurs much slower than the resorption stage. Formation takes about 3 months to complete while it took only 3 weeks for the resorption phase. During this time, osteoblasts lay down osteoid, unmineralized matrix, upon which will be mineralized over time. Each BMU leaves space for a Haversian canal in the bone so nutrients can be delivered. The last stage is the resting stage, or quiescence, and involves the osteoblasts either becoming embedded in the matrix to form osteocytes or becoming bone lining cells, thus finishing the completion of a secondary osteon (Martin, et al. 1998).

Figure 3. The cycle of bone remodeling (Hill 1998).

1.2.3 Purpose

The structure and function of bone are recognized as entities that work hand in hand. Bones are not just able to handle the normal loading conditions that occur every day but are able to adjust to any additional stresses that may be placed. This thought process was described by Julius Wolff. Wolff's Law stated that bones will alter their structure in response to a loading condition in order to better accommodate that load or lack thereof (Bilezikian, et al. 2008). This describes what we see in bone remodeling. The

loading of bone, and subsequent damage caused, as well as the disuse of bone has major effects over the structure of the bone and is responsible for remodeling taking place.

Disuse of bone causes more bone to be removed with not as much bone being formed during the remodeling process resulting in an overall loss of bone mass.

Mechanical loading on the other can cause more bone to be formed in response. As bone becomes damaged, osteoclasts are signaled to remove the damaged bone and osteoblasts are signaled to lay down the necessary new bone. A study involving the loading of rat forelimbs showed that increasing the loading frequency increased bone formation (Robling, et al. 2006). So, the loading conditions, including the amount of load, the frequency it is applied and the duration of the load, all play a role in the bone remodeling process. Overloading bone can cause microdamage within the bone, which bone remodeling will try to repair. However, if the bone is overloaded too much, damage may be incurred faster than the bone can repair it. If this continues, larger microcracks can form and propagate causing stress fractures.

1.2.4 Fatigue Microdamage

Throughout our lives, our bones are constantly being subjected to multiple loads every day. Much like any other structure, as these loads are applied over and over again, fatigue microdamage occurs within the bone and is seen visually as microcracks. Frost was the first one to describe microdamage in vivo. Physiologic strains and stresses form microdamage and continued loading increases its presence. As the microdamage increases, the material and structural properties get further weakened until the microcracks grow larger and spread farther leading to the eventual failure of the bone in the form of a fatigue fracture. Luckily, bone contains within it the ability to repair this

fatigue damage in the form of bone remodeling (Robling, et al. 2006). There is evidence that microdamage is associated with recruiting BMUs that will target specific areas where bone remodeling is needed. In a study, by Burr and colleagues, remodeling BMUs in dogs were shown to be likely associated with fatigue induced microcracks (Burr, et al. 1985). Another study showed that bone remodeling could be initiated by inducing microdamage in rats, which don't normally remodel their cortical bone (Bentolila, et al. 1998). These studies indicate a positive correlation between apoptotic osteocytes and the recruitment of osteoclasts involved in BMU remodeling by showing that microdamage was associated with apoptotic osteocytes and resorption cavities (Robling, et al. 2006). For this reason, continued research has been performed regarding osteocyte apoptosis, osteocytes as mechanosensors and osteocytes themselves and how they may be involved in the remodeling process.

1.3 The Osteocyte

1.3.1 Mechanosensors

As previously described, osteocytes are formed when inactive osteoblasts become embedded in the bone matrix. These osteocytes reside within spaces called lacunae that are connected to each other by canals called canaliculi. The processes of the osteocytes jut into these canaliculi, creating pathways throughout the bone matrix. Gap junctions are present at the ends of these osteocyte processes allowing osteocytes to connect with each other. This entire network allows communication between other cells and the transfer of nutrients to occur intracellularly as well as extracellularly (Figure 4). Because of this large network of connectivity and the sheer amount of osteocytes spread throughout the bone matrix, osteocytes are considered the most popular theory in terms of

which bone cell dominates the mechanosensory activity and may be involved in activating the bone remodeling process.

Figure 4. Osteocyte network comprised of osteocyte lacunae (A) that are connected via canaliculi (B) to allow for communication within the network. In the middle, resides a Haversian space (C) (Martin, et al. 1998).

Although many of the inner workings of osteocytes, in regards to their mechanosensory capabilities and ability to support or initiate bone remodeling, remain unknown, we do know that strain plays an important role. Bones are able to take on various loads at different rates and can lay down new bone when the loading felt is increased or resorb bone in the case of disuse. Strains in bone are experienced near osteocyte lacunae. In theory, cells can feel up to seven times the deformation compared to the strain in the tissue itself (Robling, et al. 2006). Because cellular responses within bone control the formation and resorption of bone needed for bone remodeling, a common theory is that the strains experienced by osteocytes are then translated into cellular signals to make this happen, meaning that the osteocytes act as mechanotransducers (Bonewald 2006). Various studies have already provided some evidence to support this theory. In a study of turkey ulna, loading caused an increase in

G6PD, a marker for cell metabolism, in osteocytes relative to local strain magnitudes (Skerry, et al. 1989). However, osteocytes are most likely responding to strain derived fluid flow rather than strain directly caused by mechanical loading (Bilezikian, et al. 2008). Osteocyte lacunae may also act as pumps pushing fluid through the canaliculi because of the strain concentrations located adjacent to them (Robling, et al. 2006). Another study, showed that nitric oxide (NO), a free radical involved in promoting bone formation and inhibiting bone resorption, was generated in osteocytes in response to mechanical strain caused by flow-derived shear stress (Bakker et al. 2001). As mechanical loads are applied to the bone, interstitial fluid can flow extracellularly throughout the bone and osteocytes can use this fluid flow to sense strain information and initiate the necessary cellular response (Figure 5). The means in which osteocytes actually elicit this cellular response is also unclear. One theory suggests that osteocytes control the bone architecture by using the strain experienced in the bone as a feedback mechanism to maintain programmed physiological levels of strain (Lanyon 1993; Hedgecock, et al. 2007). Another way in which osteocytes may send signals for bone resorption or formation is through osteocyte apoptosis (Noble, et al. 1997; Power, et al. 2002; Bentolila, et al. 1998; Hedgecock, et al. 2007).

Figure 5. Mechanotransduction in bone (Klein-Nulend, et al. 1995).

1.4 Osteocyte Apoptosis

Apoptosis is a form of programmed cell death or suicide. Apoptosis is a physiological form of cell death that occurs in both pathological and healthy bone. The process of apoptosis is typically initiated by either proteins released by the mitochondria or by activation of a cell surface death receptor in the tumor necrosis factor (TNF) family (Hock, et al. 2001). Cells that undergo apoptosis, separate from their neighbors, shrink and break into small pieces called apoptotic bodies (Noble 2003). In the nucleus, chromatin starts to clump up around the nuclear membrane. As this nuclear DNA is continually cleaved, the nucleus disappears and these clumps fill up the cytoplasm until eventually breaking the cell into multiple, membrane-bound apoptotic bodies (Bilezikian, et al. 2008). As this process is occurring, fluid is leaving the cell which is what causes the cell to shrink and separate from its surroundings. These formed apoptotic bodies are phagocytosed by specialized cells through a non-inflammatory process (Cardoso, et al. 2009). In bone, the majority of osteocytes are phagocytosed by osteoclasts near resorption sites (Bronckers, et al. 1996). There is another type of cell death, however, and that is necrosis. Necrosis typically affects groups of cells, and unlike apoptosis, causes their nuclei to swell, spill their cellular contents and attract inflammatory cells. Death by necrosis is much faster than apoptosis, is not energy dependent and not as controllable as apoptosis (Noble 2003).

Figure 6. The difference between necrotic and apoptotic cells (Gewies 2003).

1.4.1 Apoptosis and Bone Remodeling

Osteocytes and their connective, canalicular system provide the means to detect any changes occurring throughout the bone and initiate bone remodeling to complete the necessary repairs. One of the ways in which this may be made possible is through apoptotic osteocytes and their role in bone remodeling. Apoptotic osteocytes have been shown to lead to increased resorption of bone material, indicating that the death of osteocytes is important in osteoclast recruitment during bone remodeling (Henriksen, et al. 2009). In one study, Bronckers et al. showed that, in developing bone, the majority of apoptotic osteocytes were located at sites with high levels of bone resorption (Bronckers, et al. 1996). A study using a rabbit tibial lengthening model showed that apoptotic osteocytes were localized in regions with osteoclast activity indicating a close relationship between bone remodeling and apoptosis (Li, et al. 2003).

Many studies have shown there to be a U-shaped relationship between strain and apoptosis seen in osteocytes (Jilka, et al. 2007; Noble, et al. 2003). This means that limbs that experience physiological levels of strain show low levels of apoptotic osteocytes. Conversely, limbs experiencing unloading or levels of strain high enough to induce

microdamage, the two opposite ends of the spectrum, show increased levels of apoptotic osteocytes (Jilka, et al. 2007). Thus, microdamage caused by overloading or fatigue loading in bone also plays an important role in bone remodeling as well as how it connects apoptotic osteocytes to bone remodeling.

A study by Verborgt et al. was performed to attempt to show a relationship between osteocyte apoptosis, bone microdamage and bone resorption. In this study, the ulnae of adult rats were fatigue loaded and sections were stained to be able to determine which cells were undergoing apoptosis. Apoptotic osteocytes were found in regions surrounding microcracks as well as in bone surrounding resorption spaces. However, bone that was distant to the microcracks or resorption areas showed no differences when compared to the controls. These results were consistent from 1 day after loading to 10 days after loading. Additionally, increases in empty lacunae and decreases in normal osteocytes were observed over time. This study shows a strong correlation between microdamage, resorption and apoptotic osteocytes (Verborgt, et al. 2000). A similar study performed by Noble et al., attempted to study the role of apoptotic osteocytes on bone remodeling related to loading as well. This was done by loading rats with varying levels of strain from low to high. The rats had a loading regiment that involved loading them on days 1-5 and days 8-12. The rats that were loaded with high enough levels of strain to cause some damage resulted in an eight-fold increase in apoptotic osteocytes 7 days after the overloading. Next, 14 days after the loading, new Haversian canals were formed. Finally, 28 days after the overloading, the infilling of the resorption spaces was seen (Noble, et al. 2003). Thus, for this study, the formation of Haversian canals never preceded the presence of apoptotic osteocytes, supporting the hypothesis that apoptotic

osteocytes may be the mechanism behind bone remodeling. Rats that were loaded with low levels of strain also showed higher levels of apoptotic osteocytes relative to physiologic levels of strain. This data also seems to support a U-shaped relationship between apoptosis in osteocytes and strain and provides evidence that this U-shaped relationship may be used to influence bone remodeling (Noble, et al. 2003).

A study by Hedgecock et al. correlated the regional variability in remodeling with the regional variability in osteocyte apoptosis in rabbit tibia midshafts.

Histomorphometric analyses of the tibias showed that the remodeling parameters were the lowest in the cranial quadrant compared to the other quadrants. TUNEL staining was also performed in the tibias to see the regional variability of apoptosis within the samples. The densities of apoptotic osteocytes found in the cranial quadrant were seen to be lower than the densities in the medial quadrant. Also, the densities of osteocyte lacunae, empty lacunae and total osteocytes were seen to be higher in the lateral quadrants compared to the caudal quadrants. From this data, a strong statistically significant correlation was found between the remodeling parameters and the apoptotic osteocyte density. Although osteocyte density and lacunar density did not correlate with remodeling parameters like the density of apoptotic osteocytes did, the authors suggested that cell viability could be another factor added when correlating with remodeling parameters (Hedgecock, et al. 2007).

A study by Jessica Chan compared the variations associated with bone remodeling factors and densities of apoptotic osteocytes at different regions and quadrants within unloaded murine tibias. Bone samples were TUNEL stained to detect apoptotic osteocytes and subsequently analyzed. The levels of bone morphogenic protein

antagonists, noggin and gremlin, were measured and compared with the amount of apoptotic osteocytes found for each quadrant and region within the tibia. This study found higher densities of apoptotic osteocytes as well as higher levels of BMP antagonists in the midshaft compared to the other regions. There was also found to be a positive linear correlation between apoptotic osteocytes and gremlin and a negative linear correlation between noggin and percentage of apoptotic osteocytes (Chan 2011).

1.5 Study Goals (And Previous Studies)

This study continues the work done by Jessica Chan, as previously described, by incorporating bones that have been cyclically loaded. The objective of this study is to calculate the density of apoptotic and viable osteocytes, filtered by region, quadrant and a combination of both, in loaded and unloaded murine cortical bone and attempt to point out any statistically significant differences that may be present between the loaded and unloaded data. This study will focus on the tibias taken from the left hind limbs of murine specimens, which were mechanically loaded for a time period of either 2 or 5 weeks. Densities of osteocytes will be compared between three regions (proximal, midshaft and distal) and four anatomic quadrants (cranial, lateral, caudal, and medial) present throughout the limb as well as between loaded and unloaded specimens. It is hypothesized that osteocyte apoptosis will increase in the loaded limbs for both the 2 week and 5 week periods as a result of the additional cyclic stress placed on the bones. In addition, regional variations in apoptotic osteocytes are expected to be enhanced as a result of the increased loading in the limbs for both the 2 week and 5 week periods, with the most variation expected to be seen within the 5 week loaded samples due to the further loading seen by the samples.

2. MATERIALS AND METHODS

Twelve C57 Black 6 Taconic mice (C57Bl/6) were procured at 7 to 9 weeks of age (Taconic, Oxnard, CA), and were housed in microisolation chambers located at the California Polytechnic State University vivarium. A previous study showed that skeletal maturity in male and female C57Bl/6 mice is reached between 3 and 6 months of age by recording any changes in body weight, length of tibiae, bone density, bone modulus and bone composition over a 12 month period (Somerville, et al. 2004). For this reason, the mice used in this study were mechanically loaded once they reached 20 to 25 weeks of age and were subsequently sacrificed at around 6 months of age, which means they would have reached young adulthood and full skeletal maturity. The loading protocol used in this study was approved by the animal care committee.

2.1 Mechanical Loading

Of the twelve mice procured, 4 mice were to be mechanically loaded for a period of 2 weeks and the other 8 mice for a period of 5 weeks. For each of the mice, the left tibia was mechanically loaded for the designated amount of time while the right tibia was used as a control and was not loaded throughout this process; in between loading bouts, mice experienced normal loading due to daily motion in their cages. However, due to user error, three of the mice had to be prematurely sacrificed and were not used as data in this study. Therefore, for this study, 3 mice were mechanically loaded for 2 weeks and 6 mice were mechanically loaded for 5 weeks using a Bose Enduratec 3220, Enduratec signal box and WinTest PCI control system with its relevant WinTest software. In order to use the loading equipment the mice needed to be successfully anesthetized beforehand.

This was done by placing the mice in an isolation chamber with 4 to 5 percent isoflurane being input at 3 liters per minute. This anesthesia equipment can be seen in Figure 7.

Figure 7. Equipment used to provide isoflurane anesthesia to subjects.

Once anesthetized, the mice were placed on the loading fixture and attached to a nose cone channeling 2% isoflurane at 1 liter per minute (Verborgt, et al. 2000). The loading fixture, as seen in Figure 8, is a ramp that supports most of their body weight with a shallow groove indented into the fixture that allows the left hindlimb to be secured in one spot so the loading plunger can apply the compressive force.

Figure 8. Loading fixture used to support and secure the mouse during the loading process.

With the mouse anesthetized and secured in the fixture, the Enduratec and WinTest equipment can then be used to cyclically apply a mechanical load to the left tibia of each mouse. This is achieved by creating a sinusoidal waveform within the WinTest program as can be seen in Figures 9 and 10.

Figure 9. Interface in WinTest program that shows the cyclical loading procedure.

Figure 10. Sinusoidal waveform observed while running the cyclical loading procedure.

When the program was run, the subject's left tibia was ramped up to the preload of 0.2 N of compression by displacing the plunger onto the tibia at 0.05 mm per second. A sinusoidal waveform cycles the force applied between 1.0 Newton of compression and 3.2 Newtons of compression at 4 Hz for 1200 cycles before finally ramping back down to 0.02 Newtons of compression at the end of the mechanical loading. The force values and number of cycles were chosen to produce fatigue loading, well below the amount need to cause any fracture, within the murine bone (Noble, et al. 2003; Verborgt, et al. 2000). A frequency of 4 Hz was chosen to be similar to previous cyclical loading experiments and matches normal murine stride frequency (Noble, et al. 2003; Verborgt, et al. 2000; Mosley, et al. 1997). After the loading process was complete, each mouse was monitored throughout the week to ensure that there were no abnormalities and that neither limb was

being favored over the other. The mice loading protocol in its entirety can be seen in Appendix A.

2.2 Specimen Sacrifice, Fixation and Decalcification

After 2 and 5 weeks of loading, each respective group was sacrificed. Each mouse was successfully euthanized via cervical dislocation. After euthanization, each of the right and left tibias, were resected and placed in sealed microcentrifuge tubes filled with a fixative, Histochoice. Tibiae were then fixated by immersion fixation over the next 3 to 4 days and subsequently transferred into a decalcification solution for another 3 to 4 days prior to sample embedding. The decalcification solution was composed of 150 grams disodium EDTA dehydrate and 15 grams NaOH. Water was added until the pH of the solution reached 7.4 (between 700-800ml), yielding an approximate 15% EDTA solution.

2.3 Sample Processing

Following sacrifice, tissue immersion fixation, and decalcification, the tissue samples were embedded in paraffin wax using the Shandon Excelsior ES system (Thermo Fisher Scientific, Waltham, MA). Next, the embedded tissue blocks were secured on the Leica RM2255 rotary microtome (Leica Microsystems, Bannockburn, IL) and seven micron thick sections were cut from the block. Using tweezers, the sections were then carefully floated in a warm distilled water bath (Boekel Scientific, Feasterville, PA) and subsequently placed onto microscope slides (Thermo Fisher Scientific). Three sections were placed on each microscope slide and were allowed to air dry for 24 hours before the staining process. The full protocols used for the microtome and staining processes are listed in Appendices B and C, respectively. To prepare for staining, slides were incubated in a 40°C oven for twenty to thirty minutes or until the paraffin wax became more

transparent and malleable. For each staining process completed, half of the sections were stained using the DeadEnd Colorimetric TUNEL (Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick-end labeling) System (Promega Corp., Madison, WI) to identify cellular DNA fragmentation that is characteristic of osteocyte apoptosis. The other half of the sections were stained with a created 2.0% methyl green solution that reveals non-apoptotic nuclei within each cross section. The methyl green solution was created by first mixing 2.72 grams of sodium acetate into 200 ml of distilled water, then adjusting the pH of the mixture to 4.2 using glacial acetic acid, and finally dissolving 4.0 g methyl green (Aldrich Chemical Co., St. Louis, MO) into the resulting mixture. Microtomed sections were split between two stains to ensure that both viable and apoptotic osteocytes would be clearly visible throughout the counting process. To ensure likeness between cross-sections, the sections used for the TUNEL staining were directly adjacent to the sections used for the methyl green staining and, thus, were approximately only seven to thirty microns apart relative to original tissue placement.

2.4 TUNEL staining and Osteocyte Apoptosis

The first step in the TUNEL staining process is to deparaffinize sections by immersing the slides in xylene for 5 minutes. The tissue section were then rehydrated by graded ethanol washes: 8 minutes in 100%, 3 minutes in 95%, 3 minutes in 85%, 3 minutes in 70%, and 3 minutes in 50% ethanol. The sections were then allowed to air dry for 20 minutes. After drying, the sections were rinsed for 5 minutes in phosphate buffered saline (PBS), refixed in Histochoice for 15 minutes and rinsed again in PBS for 10 minutes. To help permeabilize the tissue sections, 100 microliters of a 20µg/mL proteinase K solution was used to cover the tissue sections for 10 minutes at room

temperature. After another PBS rinse, Histochoice refixing and second PBS rinse, 100 microliters of equilibration buffer was placed on each slide to cover the sections for 10 minutes. A rTdT reaction mix was created by mixing 98 parts equilibration buffer with 1 part biotinylated nucleotide mix and 1 part rTdT enzyme. After gently blotting away the previously equilibrated areas with tissue paper, 100 microliters of the rTdT reaction mix was added to each slide and then covered with a plastic coverslip to evenly distribute the reagent. The slides were then incubated for 1 hour in a humidifying chamber at 37°C to allow the end-labeling reaction to occur. Following incubation, coverslips were removed and the slides were immersed in a 2x sodium-chloride sodium-citrate (SSC) wash to terminate the reaction. Three subsequent 5 minute rinses in PBS were then performed to remove unincorporated biotinylated nucleotides. A 0.3% hydrogen peroxide in PBS mix was created and slides were allowed to sit in the mixture for 5 minutes to block endogenous peroxidases. Again, the slides were rinsed three times in PBS for five minutes each. The streptavidin HRP solution was diluted with PBS and 100 microliters of the mixture was added to each slide and allowed to sit for 30 minutes. After three PBS rinses, the DAB components (DAB substrate 20x buffer and DAB 20xchromagen) were mixed with deionized water and hydrogen peroxide 20x just before use. Once mixed, 100 microliters of the DAB mixture were added to each slide and allowed to develop until there was a light brown background. Finally, slides were rinsed several times in deionized water, excess liquid was removed and then the slides were mounted with Permount (Thermo Fischer Scientific).

To perform the methyl green stain, sections were deparaffinized by immersing the slides in xylene for 5 minutes. The tissue sections were rehydrated by graded ethanol

washes: 8 minutes in 100%, 3 minutes in 95%, 3 minutes in 85%, 3 minutes in 70%, and 3 minutes in 50% ethanol. The sections were then allowed to air dry for 20 minutes. After drying, the sections were rinsed in deionized water for 5 minutes, allowed to sit in the 2% methyl green solution (Aldrich Chemical Co., St. Louis, MO) for 30 seconds, and then rinsed again in distilled water. Excess liquid was removed and then the slides were mounted with Permount (Thermo Fischer Scientific).

2.5 Image Capture, Processing, and Analysis

To quantify osteocyte apoptosis and perform subsequent analyses, slides were observed under full-spectrum white light using a BX41 polarizing light microscope (Olympus Optical Co., Ltd., Center Valley, PA) at 40x magnification. Images were captured using a Retiga EXi color camera (QImaging, Surrey, BC, Canada) and the QCapture Pro imaging program (QImaging). After capturing all of the necessary images, Adobe Photoshop was used to combine all of the images taken from a specific cross-section into a single image so that there was only one image for each cross section. Three cross sections of each stain, TUNEL and methyl green, were taken from each region, distal, midshaft and proximal, within the right and left tibias of all 9 subjects amounting to a grand total of 324 cross sections captured and created. A Ronchi ruler with known lines at a size of 150 lines per mm was used to calibrate the images at 40x magnification. Image analysis was performed using Image J (Wayne Rashband(NIH)) where images were calibrated with the Ronchi ruler, yielding total field dimensions of 0.23 mm by 0.17 mm.

Regarding analysis, the area for each bone cross-section and the quadrants therein contained, were calculated using the freehand selection tool within Image J. Two types of

osteocytes were counted for each stain. For the TUNEL stain, osteocytes in lacunae with their nuclei stained brown (Figure 11) were counted as apoptotic osteocytes. For the methyl green stain, osteocytes in lacunae with their nuclei stained blue (Figure 12) were counted as viable osteocytes.

Figure 11. TUNEL stained image showing stained osteocytes and empty lacunae.

Figure 12. Methyl green stained image showing stained osteocytes and empty lacunae.

The total number of cells in each cross-section were counted in each anatomic quadrant (Cranial, Lateral, Caudal, Medial) and in each region (Proximal, Midshaft, Distal) along the tibia for all subjects (Figure 13). To ensure that only cells in-plane were counted, stained cells were only counted if they had a clearly defined lacunar wall. The amount of apoptotic and viable osteocytes present in each subject was expressed by calculating four different terms: the apoptotic osteocyte density (number of TUNEL stained cells per cross-sectional area), percent apoptotic osteocytes (number of TUNEL stained cells as a percent of the total cells counted in the cross section), viable osteocyte density (number of methyl green stained cells per cross-sectional area) and percent viable osteocytes (number of methyl green stained cells as a percent of the total cells counted in the cross section).

Figure 13. An example of what cross-sectional views across 3 regions of murine tibia look like stacked on top of each other, with samples on the left side(A) being TUNEL stained cross-sections, and samples directly to the right (B) being methyl green stained cross-sections (Cr=cranial, Ca=caudal, M=medial, L=lateral). To the right of the cross-sections is an image of a tibia and fibula divided regionally to show the corresponding regions in which each cross-section sample was taken from.

2.6 Statistical Analysis

For this study, the statistical software Minitab 16 (Minitab) was used to analyze and compare all data collected using general linear models (GLM, ANOVA); pairwise comparisons were made using the Tukey method. Firstly, the four groups of tibias, 2 week loaded left tibias, 2 week control right tibias, 5 week loaded left tibias and 5 week control right tibias, were analyzed individually to see if there were any statistically significant differences to be seen within each group independent of the others. Next, the groups of tibias were compared between each other. Comparisons were made between the 2 week loaded and 2 week control samples, the 5 week loaded and 5 week control samples, the 2 week loaded and 5 week loaded samples, and the 2 week control and 5 week control samples. For all data analyzed, differences in apoptotic osteocyte density, percentage of apoptotic osteocytes, viable osteocyte density and percentage of viable osteocytes were determined. These four variables were analyzed in each quadrant (Cranial, Lateral, Caudal, Medial), region (Proximal, Midshaft, Distal), and combination of both. Data was considered to be statistically significant if its related p-value was less than 0.05.

3. RESULTS

3.1 Individual statistics for left tibias loaded for 2 weeks

Below are the statistics for all of the 2 week loaded left tibias, including a summary, analysis by quadrant, analysis by region and combinations of the latter two. For each statistical analysis, apoptotic osteocyte density, percentage of apoptotic osteocytes, viable osteocyte density and percentage of viable osteocytes were the variables considered. Mean values, standard deviations and relevant p-values are shown, with p-values less than 0.05 indicating statistically significant data.

A statistically significant lower percentage of apoptotic osteocytes can be seen in the lateral quadrants of 2 week loaded bones compared to the cranial and medial quadrants, when averaged across all regions. When specifically looking in the proximal region, the lateral quadrant had significantly lower percentage of apoptotic osteocytes than all other quadrants and a lower apoptotic osteocyte density compared to the medial quadrant. The caudal quadrant also showed a significantly lower percentage of apoptotic osteocytes when compared to the medial quadrant, within the proximal region. Only analyzing samples within the midshaft region showed that the percentage of apoptotic osteocytes within the lateral quadrant was lower than the medial quadrant. Looking within just the distal region showed that the percentage of viable osteocytes was significantly higher in the cranial quadrant compared to the lateral quadrant. No statistically significant differences between regions were seen when averaged across all quadrants. The only regional difference seen for 2 week loaded subjects, when looking only at data from medial quadrants, was that the distal region had a significantly less percentage of apoptotic osteocytes compared to the proximal region.

Table 1. Apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded Mean

SUBJECT	APOPTOTIC OSTEOCYTE DENSITY (#/mm²)	PERCENTAGE APOPTOTIC OSTEOCYTES (%)	VIABLE OSTEOCYTE DENSITY (#/mm²)	PERCENTAGE VIABLE OSTEOCYTES (%)
2WL1	84.274	31.6416	166.157	41.8833
2WL2	129.092	38.7803	268.705	54.2325
2WL3	162.850	41.2053	232.974	48.2845
Overall	125.4(39.4)	37.21(4.97)	222.6(52.1)	48.13(6.18)

Table 2. Quadrantal differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	150.1(38.4)	42.03(6.40)	258.3(63.3)	53.54(7.96)
Lateral(L)	88.8(40.7)	26.62(5.80)	139.82(12.08)	33.41(1.79)
Caudal(Cd)	112.1(46.8)	33.89(7.47)	211.4(52.1)	45.61(6.39)
Medial(M)	146.1(34.6)	43.42(1.92)	244.7(109.0)	51.73(11.52)
Cr-L	.3162	.0461	.2252	.0514
Cr-Cd	.6711	.3726	.8340	.6129
Cr-M	.9993	.9905	.9945	.9912
L-Cd	.8915	.4611	.5984	.2896
L-M	.3662	.0306	.3087	.0770
Cd-M	.7374	.2581	.9303	.7704

Table 3. Mean quadrantal data gathered from the *proximal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded Proximal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	139.26(26.7)	42.49(4.23)	248.18(118.0)	54.36(14.24)
Lateral(L)	73.68(47.6)	22.37(3.88)	113.62(61.2)	30.82(12.34)
Caudal(Cd)	119.79(25.9)	35.97(4.43)	185.28(30.6)	46.44(8.26)
Medial(M)	178.15(40.5)	51.88(6.50)	193.30(62.0)	54.82(17.6)
Cr-L	.2001	.0043	.2029	.2231
Cr-Cd	.9105	.4105	.7387	.8878
Cr-M	.5818	.1630	.8068	1.000
L-Cd	.4526	.0368	.6598	.5264
L-M	.0321	.0003	.5865	.2110
Cd-M	.2757	.0166	.9991	.8708

Table 4. Mean quadrantal data gathered from the *midshaft region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded Midshaft Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	169.52(67.1)	40.59(4.18)	236.57(68.0)	49.54(8.58)
Lateral(L)	71.92(34.1)	25.75(6.21)	109.51(53.8)	28.75(8.46)
Caudal(Cd)	104.01(55.5)	33.25(8.37)	192.70(114.6)	40.36(10.80)
Medial(M)	157.92(62.4)	41.00(2.56)	256.64(168.4)	46.93(14.93)
Cr-L	.2238	.0534	.5296	.1737
Cr-Cd	.5184	.4498	.9602	.7419
Cr-M	.9939	.9997	.9958	.9908
L-Cd	.8946	.4319	.7950	.5924
L-M	.3102	.0472	.4164	.2561
Cd-M	.6580	.4072	.8914	.8817

Table 5. Mean quadrantal data gathered from the *distal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded Distal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	141.53(24.6)	43.01(11.61)	290.43(47.3)	56.72(4.66)
Lateral(L)	120.86(64.2)	31.75(12.99)	196.34(23.2)	40.67(1.78)
Caudal(Cd)	112.62(62.2)	32.43(10.07)	256.25(35.3)	50.03(3.32)
Medial(M)	102.34(18.4)	37.39(6.00)	284.29(112.9)	53.44(8.14)
Cr-L	.9479	.5801	.3484	.0193
Cr-Cd	.8747	.6247	.9139	.4190
Cr-M	.7456	.9105	.9994	.8548
L-Cd	.9963	.9998	.6813	.1853
L-M	.9615	.9099	.3999	.0582
Cd-M	.9929	.9360	.9492	.8402

Table 6. Regional differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	123.53(42.6)	37.06(8.04)	262.57(50.0)	51.66(3.72)
Midshaft(MS)	129.12(48.0)	36.67(2.95)	206.56(90.3)	44.08(9.63)
Proximal(P)	123.56(29.8)	37.90(4.46)	198.71(65.2)	48.65(9.80)
D-MS	.9847	.9959	.6185	.5325
D-P	1.000	.9812	.5431	.8968
MS-P	.9848	.9603	.9898	.7827

Table 7. Mean regional data gathered from the *cranial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded Cranial Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	141.53(24.6)	43.01(11.61)	290.43(47.3)	56.72(4.66)
Midshaft(MS)	169.52(67.1)	40.59(4.18)	236.57(68.0)	49.54(8.58)
Proximal(P)	139.26(26.7)	42.49(4.23)	248.18(118.)	54.36(14.24)
D-MS	.7290	.9192	.7210	.6704
D-P	.9978	.9961	.8142	.9551
MS-P	.6933	.9489	.9841	.8294

Table 8. Mean regional data gathered from the *lateral quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded Lateral Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	120.86(64.2)	31.75(12.99)	196.34(23.2)	40.67(1.78)
Midshaft(MS)	71.92(34.1)	25.75(6.21)	109.51(53.8)	28.75(8.46)
Proximal(P)	73.68(47.6)	22.37(3.88)	113.62(61.2)	30.82(12.34)
D-MS	.4977	.6866	.1548	.2875
D-P	.5203	.4293	.1765	.4048
MS-P	.9990	.8829	.9942	.9544

Table 9. Mean regional data gathered from the *caudal quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded Caudal Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	112.62(62.2)	32.43(10.07)	256.25(35.3)	50.03(3.32)
Midshaft(MS)	104.01(55.5)	33.25(8.37)	192.70(114.6)	40.36(10.8)
Proximal(P)	119.79(25.9)	35.97(4.43)	185.28(30.6)	46.44(8.26)
D-MS	.9763	.9913	.5540	.3697
D-P	.9835	.8535	.4868	.8532
MS-P	.9232	.9098	.9911	.6475

Table 10. Mean regional data gathered from the *medial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded Medial Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	102.34(18.4)	37.39(6.00)	284.29(112.9)	53.44(8.14)
Midshaft(MS)	157.92(62.4)	41.00(2.56)	256.64(168.4)	46.93(14.93)
Proximal(P)	178.15(40.5)	51.88(6.50)	193.30(62.0)	54.82(17.6)
D-MS	.3400	.6984	.9590	.8434
D-P	.1704	.0361	.6540	.9921
MS-P	.8455	.1015	.8078	.7812

3.2 Individual statistics for right tibias used as 2 week controls

Below are the statistics for all of the 2 week unloaded right tibias used as controls, including a summary, analysis by quadrant, analysis by region and combinations of the latter two. For each statistical analysis, apoptotic osteocyte density, percentage of apoptotic osteocytes, viable osteocyte density and percentage of viable osteocytes were the variables considered. Mean values, standard deviations and relevant p-values are shown, with p-values less than 0.05 indicating statistically significant data.

For 2 week unloaded tibias, the percentage of apoptotic and viable osteocytes within the lateral quadrants were significantly lower than the medial quadrants, when averaged across all regions. The caudal quadrants showed a lower percentage of apoptotic osteocytes compared to the medial quadrants when averaged across all regions as well. When analyzing only within the proximal region, the lateral quadrant showed a significantly lower percentage of apoptotic osteocytes compared to all other quadrants. The caudal quadrant showed a lower percentage of apoptotic osteocytes as well when compared to the cranial and medial quadrants. The apoptotic osteocyte density of the lateral and the caudal quadrants were both significantly lower than that of the medial quadrant. When looking at data only within the midshaft region, the percentage of viable osteocytes was significantly lower in the lateral quadrant when compared to the cranial and medial while the caudal quadrant was only lower than the medial. No statistically significant differences were seen between regions when they were averaged across all quadrants; the percentage of apoptotic osteocytes in the proximal region was lower than that in the distal region, when solely looking at lateral quadrants.

Table 11. Apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Control Mean

SUBJECT	APOPTOTIC OSTEOCYTE DENSITY (#/mm²)	PERCENTAGE APOPTOTIC OSTEOCYTES (%)	VIABLE OSTEOCYTE DENSITY (#/mm²)	PERCENTAGE VIABLE OSTEOCYTES (%)
2WR1	86.712	30.2028	234.962	50.6915
2WR2	128.724	35.1610	309.972	58.2828
2WR3	154.713	34.8880	322.758	56.7443
Overall	123.4(34.3)	33.42(2.79)	289.2(47.4)	55.24(4.01)

Table 12. Quadrantal differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Control Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	128.08(34.1)	36.20(2.08)	305.68(62.2)	59.06(6.36)
Lateral(L)	107.65(31.1)	28.30(3.83)	242.22(34.7)	46.37(1.55)
Caudal(Cd)	112.28(33.9)	30.21(2.91)	277.54(52.9)	52.05(5.01)
Medial(M)	167.96(40.6)	38.92(4.06)	344.32(35.6)	61.03(6.19)
Cr-L	.8894	.0745	.4175	.0651
Cr-Cd	.9436	.1992	.8861	.3992
Cr-M	.5376	.7507	.7592	.9637
L-Cd	.9984	.8917	.8030	.5598
L-M	.2305	.0184	.1144	.0337
Cd-M	.2843	.0488	.3782	.2214

Table 13. Mean quadrantal data gathered from the *proximal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Control Proximal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	119.69(7.01)	37.96(2.02)	314.25(20.9)	60.82(0.92)
Lateral(L)	64.08(23.4)	20.74(2.88)	194.92(47.2)	41.14(7.09)
Caudal(Cd)	87.54(4.45)	29.74(1.01)	252.90(52.5)	49.47(5.94)
Medial(M)	168.77(35.7)	39.63(4.38)	312.88(92.7)	58.51(13.4)
Cr-L	.0552	.0004	.1405	.0708
Cr-Cd	.3351	.0314	.6050	.3809
Cr-M	.0928	.8879	1.000	.9846
L-Cd	.5757	.0200	.6440	.6151
L-M	.0016	.0002	.1461	.1156
Cd-M	.0079	.0119	.6208	.5561

Table 14. Mean quadrantal data gathered from the *midshaft region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Control Midshaft Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	133.45(46.1)	36.55(6.04)	291.28(113.2)	58.95(7.50)
Lateral(L)	115.74(41.0)	28.43(7.06)	238.32(27.8)	43.87(1.80)
Caudal(Cd)	115.72(46.9)	29.34(5.27)	270.63(79.6)	49.77(3.04)
Medial(M)	172.28(58.3)	37.96(6.36)	421.17(65.7)	63.17(4.14)
Cr-L	.9683	.4294	.8375	.0170
Cr-Cd	.9681	.5213	.9873	.1489
Cr-M	.7637	.9919	.2496	.6913
L-Cd	1.000	.9978	.9548	.4494
L-M	.5173	.3079	.0792	.0041
Cd-M	.5170	.3831	.1613	.0312

Table 15. Mean quadrantal data gathered from the *distal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Control Distal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	131.09(54.8)	34.08(3.96)	311.52(82.5)	57.40(11.25)
Lateral(L)	143.13(36.0)	35.72(5.44)	293.43(67.2)	54.09(9.46)
Caudal(Cd)	133.58(57.0)	31.55(5.84)	309.09(48.5)	56.91(6.88)
Medial(M)	162.81(42.1)	39.17(3.84)	298.90(38.3)	61.43(6.88)
Cr-L	.9894	.9745	.9829	.9655
Cr-Cd	.9999	.9166	1.000	.9999
Cr-M	.8505	.5965	.9940	.9414
L-Cd	.9946	.7255	.9887	.9781
L-M	.9570	.8200	.9995	.7421
Cd-M	.8777	.2915	.9968	.9203

Table 16. Regional differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Control Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	138.65(49.4)	34.19(4.82)	305.10(61.4)	57.41(8.62)
Midshaft(MS)	127.87(47.9)	33.16(5.05)	290.88(76.4)	54.39(2.97)
Proximal(P)	103.63(11.37)	32.90(1.36)	271.71(21.5)	53.92(1.24)
D-MS	.9430	.9495	.9518	.7749
D-P	.5674	.9229	.7691	.7153
MS-P	.7518	.9968	.9147	.9937

Table 17. Mean regional data gathered from the *cranial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Control Cranial Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	131.09(54.8)	34.08(3.96)	311.52(82.5)	57.40(11.25)
Midshaft(MS)	133.45(46.1)	36.55(6.04)	291.28(113.2)	58.95(7.50)
Proximal(P)	119.69(7.01)	37.96(2.02)	314.25(20.9)	60.82(0.92)
D-MS	.9973	.7728	.9510	.9683
D-P	.9403	.5490	.9991	.8579
MS-P	.9146	.9169	.9375	.9545

Table 18. Mean regional data gathered from the *lateral quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Control Lateral Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	143.13(36.0)	35.72(5.44)	293.43(67.2)	54.09(9.46)
Midshaft(MS)	115.74(41.0)	28.43(7.06)	238.32(27.8)	43.87(1.80)
Proximal(P)	64.08(23.4)	20.74(2.88)	194.92(47.2)	41.14(7.09)
D-MS	.6152	.2969	.4224	.2440
D-P	.0676	.0338	.1145	.1326
MS-P	.2339	.2667	.5691	.8818

Table 19. Mean regional data gathered from the *caudal quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Control Caudal Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	133.58(57.0)	31.55(5.84)	309.09(48.5)	56.91(6.88)
Midshaft(MS)	115.72(46.9)	29.34(5.27)	270.63(79.6)	49.77(3.04)
Proximal(P)	87.54(4.45)	29.74(1.01)	252.90(52.5)	49.47(5.94)
D-MS	.8683	.8293	.7376	.3233
D-P	.4355	.8806	.5404	.2986
MS-P	.7120	.9938	.9348	.9976

Table 20. Mean regional data gathered from the *medial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Control Medial Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	162.81(42.1)	39.17(3.84)	298.90(38.3)	61.43(6.88)
Midshaft(MS)	172.28(58.3)	37.96(6.36)	421.17(65.7)	63.17(4.14)
Proximal(P)	168.77(35.7)	39.63(4.38)	312.88(92.7)	58.51(13.4)
D-MS	.9663	.9532	.1571	.9698
D-P	.9865	.9929	.9671	.9180
MS-P	.9953	.9127	.2147	.8080

3.3 Individual statistics for left tibias loaded for 5 weeks

Below are the statistics for all of the 5 week loaded left tibias, including a summary, analysis by quadrant, analysis by region and combinations of the latter two. For each statistical analysis, apoptotic osteocyte density, percentage of apoptotic osteocytes, viable osteocyte density and percentage of viable osteocytes were the variables considered. Mean values, standard deviations and relevant p-values are shown, with p-values less than 0.05 indicating statistically significant data.

For 5 week loaded data averaged across all regions, the lateral quadrants showed significantly lower percentages of viable osteocytes when compared to all other quadrants and significantly lower density of viable osteocytes when compared to the cranial quadrants. When looking at samples only taken from proximal regions, the lateral quadrants showed significantly lower percentages and densities of viable osteocytes compared to all other quadrants. These proximal lateral quadrants also showed lower percentages and densities of apoptotic osteocytes compared to the medial quadrants in the same region. Only analyzing samples within the midshaft region showed that there were significantly less percentages of viable osteocytes in the lateral quadrants compared to the medial quadrants. Looking only at samples within the distal region showed that there were significantly lower percentages of viable osteocytes in the lateral quadrants compared to the cranial and medial quadrants.

When averaged across all quadrants, midshaft samples showed higher viable osteocyte densities than the distal and proximal regions. When looking only within the cranial quadrant, distal samples showed significantly less viable osteocyte densities than midshaft samples. Isolating lateral quadrant samples showed significantly higher densities of apoptotic osteocytes, percentages of apoptotic osteocytes and densities of

viable osteocytes in the midshaft compared to in the proximal region. Regional data solely from the caudal quadrant showed significantly higher apoptotic osteocyte densities in the midshaft compared to the proximal regions and significantly higher densities of viable osteocytes in the midshaft compared to both the distal and proximal regions.

Table 21. Apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded Mean

SUBJECT	APOPTOTIC OSTEOCYTE DENSITY (#/mm²)	PERCENTAGE APOPTOTIC OSTEOCYTES (%)	VIABLE OSTEOCYTE DENSITY (#/mm²)	PERCENTAGE VIABLE OSTEOCYTES (%)
5WL1	254.335	58.0080	288.214	61.8257
5WL2	237.385	60.0225	321.218	64.8525
5WL3	276.643	61.7180	338.626	64.3634
5WL4	236.360	62.2560	296.044	63.0723
5WL5	253.074	58.8612	374.637	63.5759
5WL6	168.333	54.4083	339.155	66.5362
Overall	237.7(37.0)	59.21(2.86)	326.3(52.1)	64.04(1.62)

Table 22. Quadrantal differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	236.27(37.8)	59.90(3.83)	348.55(35.4)	67.22(1.50)
Lateral(L)	232.46(56.8)	58.00(3.47)	272.02(63.1)	54.68(5.81)
Caudal(Cd)	239.50(38.3)	58.40(3.39)	330.77(23.34)	63.75(2.72)
Medial(M)	256.88(59.1)	60.31(2.46)	333.68(42.3)	67.36(1.34)
Cr-L	.9991	.7587	.0298	.0000
Cr-Cd	.9995	.8635	.8927	.3089
Cr-M	.8847	.9964	.9333	.9999
L-Cd	.9944	.9967	.1224	.0008
L-M	.8238	.6336	.0986	.0000
Cd-M	.9264	.7555	.9994	.2780

Table 23. Mean quadrantal data gathered from the *proximal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded Proximal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	222.01(28.9)	58.68(4.20)	331.65(49.7)	66.40(2.15)
Lateral(L)	173.98(25.0)	52.43(4.98)	198.41(64.5)	47.12(11.49)
Caudal(Cd)	210.18(18.24)	56.97(4.00)	309.71(40.4)	64.43(5.67)
Medial(M)	277.59(80.9)	63.09(4.59)	307.77(63.3)	66.61(4.91)
Cr-L	.2927	.1043	.0025	.0006
Cr-Cd	.9691	.9089	.9012	.9600
Cr-M	.1845	.3423	.8770	.9999
L-Cd	.5296	.3207	.0116	.0018
L-M	.0042	.0026	.0133	.0005
Cd-M	.0811	.1135	.999	.9472

Table 24. Mean quadrantal data gathered from the *midshaft region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded Midshaft Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	255.87(71.0)	62.48(4.44)	392.50(54.7)	67.41(3.49)
Lateral(L)	277.99(81.8)	64.35(5.50)	348.03(102.4)	58.25(7.78)
Caudal(Cd)	287.88(68.9)	62.29(3.30)	382.38(54.9)	63.60(5.68)
Medial(M)	287.24(114.6)	60.99(5.37)	383.97(112.5)	68.84(5.07)
Cr-L	.9698	.9013	.8037	.0527
Cr-Cd	.9162	.9999	.9968	.6626
Cr-M	.9206	.9477	.9981	.9717
L-Cd	.9971	.8732	.8970	.3901
L-M	.9976	.6172	.8842	.0211
Cd-M	1.000	.9644	1.000	.4080

Table 25. Mean quadrantal data gathered from the *distal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded Distal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	230.93(44.9)	58.54(6.44)	321.50(31.1)	67.85(1.45)
Lateral(L)	245.40(77.7)	57.22(6.52)	269.62(68.5)	58.67(2.89)
Caudal(Cd)	220.42(48.0)	55.96(6.80)	300.20(14.07)	63.22(4.82)
Medial(M)	205.80(43.4)	56.84(7.32)	309.30(47.7)	66.62(3.26)
Cr-L	.9682	.9864	.2240	.0006
Cr-Cd	.9873	.9107	.8454	.1075
Cr-M	.8594	.9719	.9652	.9170
L-Cd	.8614	.9880	.6492	.1162
L-M	.6093	.9997	.4425	.0027
Cd-M	.9673	.9958	.9849	.3175

Table 26. Regional differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	225.88(41.0)	57.08(5.41)	302.42(19.36)	64.32(2.00)
Midshaft(MS)	272.75(68.7)	62.78(3.77)	377.70(52.9)	64.97(3.61)
Proximal(P)	214.44(24.9)	57.78(2.74)	298.83(49.0)	62.82(3.85)
D-MS	.2459	.0727	.0219	.9363
D-P	.9122	.9534	.9886	.7102
MS-P	.1263	.1230	.0165	.5032

Table 27. Mean regional data gathered from the *cranial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded Cranial Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	230.93(44.9)	58.54(6.44)	321.50(31.1)	67.85(1.45)
Midshaft(MS)	255.87(71.0)	62.48(4.44)	392.50(54.7)	67.41(3.49)
Proximal(P)	222.01(28.9)	58.68(4.20)	331.65(49.7)	66.40(2.15)
D-MS	.6836	.4022	.0448	.9490
D-P	.9514	.9989	.9239	.5878
MS-P	.5036	.4259	.0905	.7718

Table 28. Mean regional data gathered from the *lateral quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded Lateral Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	245.40(77.7)	57.22(6.52)	269.62(68.5)	58.67(2.89)
Midshaft(MS)	277.99(81.8)	64.35(5.50)	348.03(102.4)	58.25(7.78)
Proximal(P)	173.98(25.0)	52.43(4.98)	198.41(64.5)	47.12(11.49)
D-MS	.6811	.1106	.2405	.9956
D-P	.1862	.3396	.3028	.0665
MS-P	.0411	.0067	.0147	.0783

Table 29. Mean regional data gathered from the *caudal quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded Caudal Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	220.42(48.0)	55.96(6.80)	300.20(14.07)	63.22(4.82)
Midshaft(MS)	287.88(68.9)	62.29(3.30)	382.38(54.9)	63.60(5.68)
Proximal(P)	210.18(18.24)	56.97(4.00)	309.71(40.4)	64.43(5.67)
D-MS	.0781	.1004	.0078	.9919
D-P	.9322	.9341	.9120	.9213
MS-P	.0400	.1829	.0177	.9620

Table 30. Mean regional data gathered from the *medial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for left tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded Medial Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	205.80(43.4)	56.84(7.32)	309.30(47.7)	66.62(3.26)
Midshaft(MS)	287.24(114.6)	60.99(5.37)	383.97(112.5)	68.84(5.07)
Proximal(P)	277.59(80.9)	63.09(4.59)	307.77(63.3)	66.61(4.91)
D-MS	.2508	.4578	.2651	.6743
D-P	.3338	.1895	.9994	1.000
MS-P	.9788	.8119	.2522	.6719

3.4 Individual statistics for right tibias used as 5 week controls

Below are the statistics for all of the 5 week unloaded right tibias used as controls, including a summary, analysis by quadrant, analysis by region and combinations of the latter two. For each statistical analysis, apoptotic osteocyte density, percentage of apoptotic osteocytes, viable osteocyte density and percentage of viable osteocytes were the variables considered. Mean values, standard deviations and relevant p-values are shown, with p-values less than 0.05 indicating statistically significant data.

No statistically significant data differences were seen between quadrants, when averaged across all regions, or between regions, when averaged across all quadrants. The only statistically significant difference seen showed higher viable osteocyte densities in midshafts compared to samples in the proximal region, when analyzing within the medial quadrant only.

Table 31. Apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Control Mean

SUBJECT	APOPTOTIC OSTEOCYTE DENSITY (#/mm²)	PERCENTAGE APOPTOTIC OSTEOCYTES (%)	VIABLE OSTEOCYTE DENSITY (#/mm²)	PERCENTAGE VIABLE OSTEOCYTES (%)
5WR1	132.827	41.1027	351.098	60.0203
5WR2	148.814	35.8902	399.276	57.1673
5WR3	125.390	32.8866	392.888	60.4484
5WR4	186.986	46.9419	300.056	68.5789
5WR5	169.658	43.1078	271.562	72.1905
5WR6	126.423	33.8280	389.805	64.6432
Overall	148.3(25.3)	38.96(5.62)	350.8(53.8)	63.84(5.72)

Table 32. Quadrantal differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Control Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	151.11(17.04)	41.24(4.74)	358.88(83.2)	66.72(3.11)
Lateral(L)	154.51(36.2)	36.82(6.58)	319.53(27.5)	57.41(10.05)
Caudal(Cd)	141.49(28.4)	37.35(6.70)	368.86(59.6)	64.33(7.15)
Medial(M)	154.90(45.1)	38.35(6.07)	336.28(58.0)	64.63(6.75)
Cr-L	.9980	.5964	.6770	.1466
Cr-Cd	.9581	.6867	.9916	.9384
Cr-M	.9972	.8415	.9151	.9575
L-Cd	.9046	.9987	.5054	.3673
L-M	1.000	.9715	.9626	.3316
Cd-M	.8971	.9916	.7872	.9999

Table 33. Mean quadrantal data gathered from the *proximal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Control Proximal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	150.31(18.42)	42.06(4.85)	312.83(88.0)	64.48(3.61)
Lateral(L)	135.35(48.8)	35.54(8.14)	265.19(37.4)	54.35(11.81)
Caudal(Cd)	132.08(25.1)	38.56(6.21)	350.45(97.9)	66.50(9.72)
Medial(M)	165.20(61.2)	38.51(9.86)	268.59(73.3)	59.62(9.94)
Cr-L	.9261	.4532	.7152	.2644
Cr-Cd	.8758	.8497	.8352	.9812
Cr-M	.9272	.8450	.7583	.8022
L-Cd	.9991	.8972	.2587	.1402
L-M	.6179	.9011	.9998	.7607
Cd-M	.5368	1.000	.2908	.5841

Table 34. Mean quadrantal data gathered from the *midshaft region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Control Midshaft Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	164.57(27.5)	40.87(7.98)	408.90(123.1)	67.21(4.17)
Lateral(L)	191.78(69.1)	39.62(8.77)	365.40(99.9)	58.69(11.38)
Caudal(Cd)	155.41(25.9)	38.86(7.33)	396.47(83.9)	64.60(6.96)
Medial(M)	155.11(10.82)	37.32(6.66)	397.45(95.8)	65.21(5.78)
Cr-L	.6421	.9920	.8794	.2397
Cr-Cd	.9779	.9687	.9965	.9317
Cr-M	.9758	.8557	.9973	.9675
L-Cd	.4088	.9982	.9510	.5416
L-M	.4018	.9546	.9466	.4596
Cd-M	1.000	.9855	1.000	.9990

Table 35. Mean quadrantal data gathered from the *distal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Control Distal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Cranial(Cr)	138.46(35.3)	40.80(10.09)	354.90(89.4)	68.47(6.75)
Lateral(L)	136.41(26.3)	35.30(6.60)	328.00(64.6)	59.19(9.15)
Caudal(Cd)	136.99(60.2)	34.62(10.86)	359.66(81.8)	61.89(9.12)
Medial(M)	144.39(79.4)	39.21(11.13)	342.81(65.2)	69.06(8.48)
Cr-L	.9999	.7686	.9267	.2565
Cr-Cd	1.000	.7008	.9995	.5414
Cr-M	.9975	.9920	.9925	.9994
L-Cd	1.000	.9994	.8873	.9443
L-M	.9941	.9005	.9864	.2113
Cd-M	.9953	.8503	.9802	.4710

Table 36. Regional differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Control Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	139.09(45.8)	37.63(8.96)	350.68(71.9)	64.41(7.51)
Midshaft(MS)	163.32(22.7)	39.67(7.46)	394.35(83.1)	64.55(5.68)
Proximal(P)	142.64(22.93)	39.58(5.51)	307.31(69.6)	62.56(6.33)
D-MS	.4179	.8836	.5838	.9993
D-P	.9803	.8937	.5881	.8774
MS-P	.5242	.9997	.1447	.8601

Table 37. Mean regional data gathered from the *cranial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Control Cranial Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	138.46(35.3)	40.80(10.09)	354.90(89.4)	68.47(6.75)
Midshaft(MS)	164.57(27.5)	40.87(7.98)	408.90(123.1)	67.21(4.17)
Proximal(P)	150.31(18.42)	42.06(4.85)	312.83(88.0)	64.48(3.61)
D-MS	.2685	.9999	.6354	.9015
D-P	.7469	.9594	.7568	.3785
MS-P	.6585	.9637	.2602	.6249

Table 38. Mean regional data gathered from the *lateral quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Control Lateral Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	136.41(26.3)	35.30(6.60)	328.00(64.6)	59.19(9.15)
Midshaft(MS)	191.78(69.1)	39.62(8.77)	365.40(99.9)	58.69(11.38)
Proximal(P)	135.35(48.8)	35.54(8.14)	265.19(37.4)	54.35(11.81)
D-MS	.1799	.6194	.6487	.9965
D-P	.9993	.9985	.3140	.7243
MS-P	.1697	.6507	.0709	.7706

Table 39. Mean regional data gathered from the *caudal quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Control Caudal Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	136.99(60.2)	34.62(10.86)	359.66(81.8)	61.89(9.12)
Midshaft(MS)	155.41(25.9)	38.86(7.33)	396.47(83.9)	64.60(6.96)
Proximal(P)	132.08(25.1)	38.56(6.21)	350.45(97.9)	66.50(9.72)
D-MS	.7163	.6623	.7539	.8523
D-P	.9760	.7002	.9821	.6357
MS-P	.5898	.9978	.6460	.9240

Table 40. Mean regional data gathered from the *medial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes for right tibias used as controls in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Control Medial Region

Region	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
Distal(D)	144.39(79.4)	39.21(11.13)	342.81(65.2)	69.06(8.48)
Midshaft(MS)	155.11(10.82)	37.32(6.66)	397.45(95.8)	65.21(5.78)
Proximal(P)	165.20(61.2)	38.51(9.86)	268.59(73.3)	59.62(9.94)
D-MS	.9457	.9361	.4739	.7043
D-P	.8122	.9910	.2667	.1510
MS-P	.9518	.9741	.0328	.4856

3.5 Comparison Between 2 Week Loaded Left Tibias and Unloaded Right Tibia Controls

Below are the statistics for all of the 2 week loaded left tibias compared against all of the 2 week unloaded right tibias used as controls, including a summary, analysis by quadrant, analysis by region and combinations of the latter two. For each statistical analysis, apoptotic osteocyte density, percentage of apoptotic osteocytes, viable osteocyte density and percentage of viable osteocytes were the variables considered. Mean values, standard deviations and relevant p-values are shown, with p-values less than 0.05 indicating statistically significant data.

When comparing the 2 week loaded data against the unloaded data, only one statistically significant difference was found; the percentage of apoptotic osteocytes in the medial quadrant of the proximal region for the 2 week loaded bones was significantly higher than in the 2 week unloaded bones.

Table 41. Quadrantal differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes between loaded left tibias and control right tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded/2 Week Control Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	% Viable Osteocytes
2WL_Cranial(Cr_{2WL})	150.1(38.4)	42.03(6.40)	258.3(63.3)	53.54(7.96)
2WL_Lateral(L_{2WL})	88.8(40.7)	26.62(5.80)	139.82(12.08)	33.41(1.79)
2WL_Caudal(Cd_{2WL})	112.1(46.8)	33.89(7.47)	211.4(52.1)	45.61(6.39)
2WL_Medial(M_{2WL})	146.1(34.6)	43.42(1.92)	244.7(109.0)	51.73(11.52)
2WR_Cranial(Cr_{2WR})	128.08(34.1)	36.20(2.08)	305.68(62.2)	59.06(6.36)
2WR_Lateral(L_{2WR})	107.65(31.1)	28.30(3.83)	242.22(34.7)	46.37(1.55)
2WR_Caudal(Cd_{2WR})	112.28(33.9)	30.21(2.91)	277.54(52.9)	52.05(5.01)
2WR_Medial(M_{2WR})	167.96(40.6)	38.92(4.06)	344.32(35.6)	61.03(6.19)
Cr_{2WL}-Cr_{2WR}	.9953	.7897	.9709	.9631
L_{2WL}-L_{2WR}	.9982	.9998	.4406	.2993
Cd_{2WL}-Cd_{2WR}	1.000	.9748	.8570	.9207
M_{2WL}-M_{2WR}	.9955	.9298	.4732	.6691

Table 42. Mean quadrantal data gathered from the *proximal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded/2 Week Control Proximal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Cranial(Cr_{2WL})	139.26(26.7)	42.49(4.23)	248.18(118.0)	54.36(14.24)
2WL_Lateral(L_{2WL})	73.68(47.6)	22.37(3.88)	113.62(61.2)	30.82(12.34)
2WL_Caudal(Cd_{2WL})	119.79(25.9)	35.97(4.43)	185.28(30.6)	46.44(8.26)
2WL_Medial(M_{2WL})	178.15(40.5)	51.88(6.50)	193.30(62.0)	54.82(17.6)
2WR_Cranial(Cr_{2WR})	119.69(7.01)	37.96(2.02)	314.25(20.9)	60.82(0.92)
2WR_Lateral(L_{2WR})	64.08(23.4)	20.74(2.88)	194.92(47.2)	41.14(7.09)
2WR_Caudal(Cd_{2WR})	87.54(4.45)	29.74(1.01)	252.90(52.5)	49.47(5.94)
2WR_Medial(M_{2WR})	168.77(35.7)	39.63(4.38)	312.88(92.7)	58.51(13.4)
Cr_{2WL}-Cr_{2WR}	.9906	.8489	.9211	.9955
L_{2WL}-L_{2WR}	.9999	.9995	.8097	.9400
Cd_{2WL}-Cd_{2WR}	.8788	.5610	.9121	1.000
M_{2WL}-M_{2WR}	.9999	.0285	.4167	.9999

Table 43. Mean quadrantal data gathered from the *midshaft region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded/2 Week Control Midshaft Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Cranial(Cr_{2WL})	169.52(67.1)	40.59(4.18)	236.57(68.0)	49.54(8.58)
2WL_Lateral(L_{2WL})	71.92(34.1)	25.75(6.21)	109.51(53.8)	28.75(8.46)
2WL_Caudal(Cd_{2WL})	104.01(55.5)	33.25(8.37)	192.70(114.6)	40.36(10.80)
2WL_Medial(M_{2WL})	157.92(62.4)	41.00(2.56)	256.64(168.4)	46.93(14.93)
2WR_Cranial(Cr_{2WR})	133.45(46.1)	36.55(6.04)	291.28(113.2)	58.95(7.50)
2WR_Lateral(L_{2WR})	115.74(41.0)	28.43(7.06)	238.32(27.8)	43.87(1.80)
2WR_Caudal(Cd_{2WR})	115.72(46.9)	29.34(5.27)	270.63(79.6)	49.77(3.04)
2WR_Medial(M_{2WR})	172.28(58.3)	37.96(6.36)	421.17(65.7)	63.17(4.14)
Cr_{2WL}-Cr_{2WR}	.9875	.9888	.9958	.9603
L_{2WL}-L_{2WR}	.9638	.9991	.7168	.4041
Cd_{2WL}-Cd_{2WR}	1.000	.9906	.9682	.8598
M_{2WL}-M_{2WR}	1.000	.9980	.4507	.3244

Table 44. Mean quadrantal data gathered from the *distal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded/2 Week Control Distal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Cranial(Cr_{2WL})	141.53(24.6)	43.01(11.61)	290.43(47.3)	56.72(4.66)
2WL_Lateral(L_{2WL})	120.86(64.2)	31.75(12.99)	196.34(23.2)	40.67(1.78)
2WL_Caudal(Cd_{2WL})	112.62(62.2)	32.43(10.07)	256.25(35.3)	50.03(3.32)
2WL_Medial(M_{2WL})	102.34(18.4)	37.39(6.00)	284.29(112.9)	53.44(8.14)
2WR_Cranial(Cr_{2WR})	131.09(54.8)	34.08(3.96)	311.52(82.5)	57.40(11.25)
2WR_Lateral(L_{2WR})	143.13(36.0)	35.72(5.44)	293.43(67.2)	54.09(9.46)
2WR_Caudal(Cd_{2WR})	133.58(57.0)	31.55(5.84)	309.09(48.5)	56.91(6.88)
2WR_Medial(M_{2WR})	162.81(42.1)	39.17(3.84)	298.90(38.3)	61.43(6.88)
Cr_{2WL}-Cr_{2WR}	1.000	.8716	.9999	1.000
L_{2WL}-L_{2WR}	.9988	.9984	.5799	.3569
Cd_{2WL}-Cd_{2WR}	.9992	1.000	.9635	.9285
M_{2WL}-M_{2WR}	.7710	1.000	1.000	.8615

Table 45. Regional differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes between loaded left tibias and control right tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded/2 Week Control Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Distal(D_{2WL})	123.53(42.6)	37.06(8.04)	262.57(50.0)	51.66(3.72)
2WL_Midshaft(MS_{2WL})	129.12(48.0)	36.67(2.95)	206.56(90.3)	44.08(9.63)
2WL_Proximal(P_{2WL})	123.56(29.8)	37.90(4.46)	198.71(65.2)	48.65(9.80)
2WR_Distal(D_{2WR})	138.65(49.4)	34.19(4.82)	305.10(61.4)	57.41(8.62)
2WR_Midshaft(MS_{2WR})	127.87(47.9)	33.16(5.05)	290.88(76.4)	54.39(2.97)
2WR_Proximal(P_{2WR})	103.63(11.37)	32.90(1.36)	271.71(21.5)	53.92(1.24)
D_{2WL}-D_{2WR}	.9969	.9761	.9607	.9038
MS_{2WL}-MS_{2WR}	1.000	.9450	.6130	.4870
P_{2WL}-P_{2WR}	.9889	.8047	.7344	.9301

Table 46. Mean regional data gathered from the *cranial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded/2 Week Control Cranial Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Distal(D_{2WL})	141.53(24.6)	43.01(11.61)	290.43(47.3)	56.72(4.66)
2WL_Midshaft(MS_{2WL})	169.52(67.1)	40.59(4.18)	236.57(68.0)	49.54(8.58)
2WL_Proximal(P_{2WL})	139.26(26.7)	42.49(4.23)	248.18(118.)	54.36(14.24)
2WR_Distal(D_{2WR})	131.09(54.8)	34.08(3.96)	311.52(82.5)	57.40(11.25)
2WR_Midshaft(MS_{2WR})	133.45(46.1)	36.55(6.04)	291.28(113.2)	58.95(7.50)
2WR_Proximal(P_{2WR})	119.69(7.01)	37.96(2.02)	314.25(20.9)	60.82(0.92)
D_{2WL}-D_{2WR}	.9996	.5112	.9995	1.000
MS_{2WL}-MS_{2WR}	.8983	.9612	.9597	.7873
P_{2WL}-P_{2WR}	.9920	.9385	.9157	.9438

Table 47. Mean regional data gathered from the *lateral quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded/2 Week Control Lateral Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Distal(D_{2WL})	120.86(64.2)	31.75(12.99)	196.34(23.2)	40.67(1.78)
2WL_Midshaft(MS_{2WL})	71.92(34.1)	25.75(6.21)	109.51(53.8)	28.75(8.46)
2WL_Proximal(P_{2WL})	73.68(47.6)	22.37(3.88)	113.62(61.2)	30.82(12.34)
2WR_Distal(D_{2WR})	143.13(36.0)	35.72(5.44)	293.43(67.2)	54.09(9.46)
2WR_Midshaft(MS_{2WR})	115.74(41.0)	28.43(7.06)	238.32(27.8)	43.87(1.80)
2WR_Proximal(P_{2WR})	64.08(23.4)	20.74(2.88)	194.92(47.2)	41.14(7.09)
D_{2WL}-D_{2WR}	.9859	.9813	.2292	.3523
MS_{2WL}-MS_{2WR}	.8050	.9969	.0665	.2447
P_{2WL}-P_{2WR}	.9997	.9997	.3898	.6082

Table 48. Mean regional data gathered from the *caudal quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded/2 Week Control Caudal Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Distal(D_{2WL})	112.62(62.2)	32.43(10.07)	256.25(35.3)	50.03(3.32)
2WL_Midshaft(MS_{2WL})	104.01(55.5)	33.25(8.37)	192.70(114.6)	40.36(10.8)
2WL_Proximal(P_{2WL})	119.79(25.9)	35.97(4.43)	185.28(30.6)	46.44(8.26)
2WR_Distal(D_{2WR})	133.58(57.0)	31.55(5.84)	309.09(48.5)	56.91(6.88)
2WR_Midshaft(MS_{2WR})	115.72(46.9)	29.34(5.27)	270.63(79.6)	49.77(3.04)
2WR_Proximal(P_{2WR})	87.54(4.45)	29.74(1.01)	252.90(52.5)	49.47(5.94)
D_{2WL}-D_{2WR}	.9926	1.000	.9192	.8206
MS_{2WL}-MS_{2WR}	.9995	.9731	.7104	.5759
P_{2WL}-P_{2WR}	.9526	.8407	.8096	.9934

Table 49. Mean regional data gathered from the *medial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 2 weeks. Standard deviations in parentheses.

2 Week Loaded/2 Week Control Medial Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Distal(D_{2WL})	102.34(18.4)	37.39(6.00)	284.29(112.9)	53.44(8.14)
2WL_Midshaft(MS_{2WL})	157.92(62.4)	41.00(2.56)	256.64(168.4)	46.93(14.93)
2WL_Proximal(P_{2WL})	178.15(40.5)	51.88(6.50)	193.30(62.0)	54.82(17.6)
2WR_Distal(D_{2WR})	162.81(42.1)	39.17(3.84)	298.90(38.3)	61.43(6.88)
2WR_Midshaft(MS_{2WR})	172.28(58.3)	37.96(6.36)	421.17(65.7)	63.17(4.14)
2WR_Proximal(P_{2WR})	168.77(35.7)	39.63(4.38)	312.88(92.7)	58.51(13.4)
D_{2WL}-D_{2WR}	.5935	.9978	1.000	.9570
MS_{2WL}-MS_{2WR}	.9985	.9753	.3827	.5691
P_{2WL}-P_{2WR}	.9998	.1045	.6862	.9987

3.6 Comparison Between 5 Week Loaded Left Tibias and Unloaded Right Tibia Controls

Below are the statistics for all of the 5 week loaded left tibias compared against all of the 5 week unloaded right tibias used as controls, including a summary, analysis by quadrant, analysis by region and combinations of the latter two. For each statistical analysis, apoptotic osteocyte density, percentage of apoptotic osteocytes, viable osteocyte density and percentage of viable osteocytes were the variables considered. Mean values, standard deviations and relevant p-values are shown, with p-values less than 0.05 indicating statistically significant data.

When comparing the 5 week loaded data against the unloaded data, the percentage of apoptotic osteocytes, in each region, quadrant and combination of the two, were higher in 5 week loaded bones when compared against the 5 week unloaded bones from the same region and quadrant. No statistically significant differences in viable osteocyte density or percentage of viable osteocytes were seen between the 5 week loaded samples and the 5 week unloaded control samples. The density of apoptotic osteocytes in each quadrant, averaged across all regions, were significantly higher in the 5 week loaded bones than their respective counterparts in the 5 week unloaded bones. The medial quadrants in proximal and midshaft samples, the caudal quadrants in midshaft samples and the lateral quadrants in distal samples showed significantly higher densities of apoptotic osteocytes in 5 week loaded bones compared to 5 week unload bones. The density of apoptotic osteocytes in the distal and midshaft regions, averaged across all quadrants, were higher in 5 week loaded bones when compared against the 5 week unloaded bones from the same region. The distal regions of cranial, lateral and caudal quadrants showed statistically significantly higher apoptotic osteocyte densities in 5 week

loaded samples compared to 5 week unloaded samples. The 5 week loaded midshaft regions in the cranial, caudal and medial quadrants of bone showed statistically significantly higher apoptotic osteocyte densities compared to 5 week unloaded samples.

Table 50. Quadrantal differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes between loaded left tibias and control right tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded/5 Week Control Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
5WL_Cranial(Cr_{5WL})	236.27(37.8)	59.90(3.83)	348.55(35.4)	67.22(1.50)
5WL_Lateral(L_{5WL})	232.46(56.8)	58.00(3.47)	272.02(63.1)	54.68(5.81)
5WL_Caudal(Cd_{5WL})	239.50(38.3)	58.40(3.39)	330.77(23.34)	63.75(2.72)
5WL_Medial(M_{5WL})	256.88(59.1)	60.31(2.46)	333.68(42.3)	67.36(1.34)
5WR_Cranial(Cr_{5WR})	151.11(17.04)	41.24(4.74)	358.88(83.2)	66.72(3.11)
5WR_Lateral(L_{5WR})	154.51(36.2)	36.82(6.58)	319.53(27.5)	57.41(10.05)
5WR_Caudal(Cd_{5WR})	141.49(28.4)	37.35(6.70)	368.86(59.6)	64.33(7.15)
5WR_Medial(M_{5WR})	154.90(45.1)	38.35(6.07)	336.28(58.0)	64.63(6.75)
Cr_{5WL}-Cr_{5WR}	0.0221	0.0000	1.0000	1.0000
L_{5WL}-L_{5WR}	0.0471	0.0000	0.7685	0.9896
Cd_{5WL}-Cd_{5WR}	0.0051	0.0000	0.9102	1.0000
M_{5WL}-M_{5WR}	0.0032	0.0000	1.0000	0.9895

Table 51. Mean quadrantal data gathered from the *proximal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded/5 Week Control Proximal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
5WL_Cranial(Cr_{5WL})	222.01(28.9)	58.68(4.20)	331.65(49.7)	66.40(2.15)
5WL_Lateral(L_{5WL})	173.98(25.0)	52.43(4.98)	198.41(64.5)	47.12(11.49)
5WL_Caudal(Cd_{5WL})	210.18(18.24)	56.97(4.00)	309.71(40.4)	64.43(5.67)
5WL_Medial(M_{5WL})	277.59(80.9)	63.09(4.59)	307.77(63.3)	66.61(4.91)
5WR_Cranial(Cr_{5WR})	150.31(18.42)	42.06(4.85)	312.83(88.0)	64.48(3.61)
5WR_Lateral(L_{5WR})	135.35(48.8)	35.54(8.14)	265.19(37.4)	54.35(11.81)
5WR_Caudal(Cd_{5WR})	132.08(25.1)	38.56(6.21)	350.45(97.9)	66.50(9.72)
5WR_Medial(M_{5WR})	165.20(61.2)	38.51(9.86)	268.59(73.3)	59.62(9.94)
Cr_{5WL}-Cr_{5WR}	.1170	.0009	.9997	.9999
L_{5WL}-L_{5WR}	.7908	.0007	.6777	.7899
Cd_{5WL}-Cd_{5WR}	.0662	.0002	.9640	.9998
M_{5WL}-M_{5WR}	.0017	0.000	.9708	.8157

Table 52. Mean quadrantal data gathered from the *midshaft region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded/5 Week Control Midshaft Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
5WL_Cranial(Cr_{5WL})	255.87(71.0)	62.48(4.44)	392.50(54.7)	67.41(3.49)
5WL_Lateral(L_{5WL})	277.99(81.8)	64.35(5.50)	348.03(102.4)	58.25(7.78)
5WL_Caudal(Cd_{5WL})	287.88(68.9)	62.29(3.30)	382.38(54.9)	63.60(5.68)
5WL_Medial(M_{5WL})	287.24(114.6)	60.99(5.37)	383.97(112.5)	68.84(5.07)
5WR_Cranial(Cr_{5WR})	164.57(27.5)	40.87(7.98)	408.90(123.1)	67.21(4.17)
5WR_Lateral(L_{5WR})	191.78(69.1)	39.62(8.77)	365.40(99.9)	58.69(11.38)
5WR_Caudal(Cd_{5WR})	155.41(25.9)	38.86(7.33)	396.47(83.9)	64.60(6.96)
5WR_Medial(M_{5WR})	155.11(10.82)	37.32(6.66)	397.45(95.8)	65.21(5.78)
Cr_{5WL}-Cr_{5WR}	.2884	0.0000	1.0000	1.0000
L_{5WL}-L_{5WR}	.3575	0.0000	1.0000	1.0000
Cd_{5WL}-Cd_{5WR}	.0283	0.0000	1.0000	1.0000
M_{5WL}-M_{5WR}	.0290	0.0000	1.0000	.9804

Table 53. Mean quadrantal data gathered from the *distal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded/5 Week Control Distal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
5WL_Cranial(Cr _{5WL})	230.93(44.9)	58.54(6.44)	321.50(31.1)	67.85(1.45)
5WL_Lateral(L _{5WL})	245.40(77.7)	57.22(6.52)	269.62(68.5)	58.67(2.89)
5WL_Caudal(Cd _{5WL})	220.42(48.0)	55.96(6.80)	300.20(14.07)	63.22(4.82)
5WL_Medial(M _{5WL})	205.80(43.4)	56.84(7.32)	309.30(47.7)	66.62(3.26)
5WR_Cranial(Cr _{5WR})	138.46(35.3)	40.80(10.09)	354.90(89.4)	68.47(6.75)
5WR_Lateral(L _{5WR})	136.41(26.3)	35.30(6.60)	328.00(64.6)	59.19(9.15)
5WR_Caudal(Cd _{5WR})	136.99(60.2)	34.62(10.86)	359.66(81.8)	61.89(9.12)
5WR_Medial(M _{5WR})	144.39(79.4)	39.21(11.13)	342.81(65.2)	69.06(8.48)
Cr _{5WL} -Cr _{5WR}	.0957	.0162	.9816	1.0000
L _{5WL} -L _{5WR}	.0272	.0014	.7369	1.0000
Cd _{5WL} -Cd _{5WR}	.1745	.0020	.7191	1.0000
M _{5WL} -M _{5WR}	.5345	.0171	.9813	.9976

Table 54. Regional differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes between loaded left tibias and control right tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded/5 Week Control Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
5WL_Distal(D _{5WL})	225.88(41.0)	57.08(5.41)	302.42(19.36)	64.32(2.00)
5WL_Midshaft(MS _{5WL})	272.75(68.7)	62.78(3.77)	377.70(52.9)	64.97(3.61)
5WL_Proximal(P _{5WL})	214.44(24.9)	57.78(2.74)	298.83(49.0)	62.82(3.85)
5WR_Distal(D _{5WR})	139.09(45.8)	37.63(8.96)	350.68(71.9)	64.41(7.51)
5WR_Midshaft(MS _{5WR})	163.32(22.7)	39.67(7.46)	394.35(83.1)	64.55(5.68)
5WR_Proximal(P _{5WR})	142.64(22.93)	39.58(5.51)	307.31(69.6)	62.56(6.33)
D _{5WL} -D _{5WR}	.0115	0.0001	.7468	1.0000
MS _{5WL} -MS _{5WR}	.0009	0.0000	.9968	1.0000
P _{5WL} -P _{5WR}	.0523	0.0002	.9999	1.0000

Table 55. Mean regional data gathered from the *cranial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded/5 Week Control Cranial Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	% Viable Osteocytes
5WL_Distal(D _{5WL})	230.93(44.9)	58.54(6.44)	321.50(31.1)	67.85(1.45)
5WL_Midshaft(MS _{5WL})	255.87(71.0)	62.48(4.44)	392.50(54.7)	67.41(3.49)
5WL_Proximal(P _{5WL})	222.01(28.9)	58.68(4.20)	331.65(49.7)	66.40(2.15)
5WR_Distal(D _{5WR})	138.46(35.3)	40.80(10.09)	354.90(89.4)	68.47(6.75)
5WR_Midshaft(MS _{5WR})	164.57(27.5)	40.87(7.98)	408.90(123.1)	67.21(4.17)
5WR_Proximal(P _{5WR})	150.31(18.42)	42.06(4.85)	312.83(88.0)	64.48(3.61)
D _{5WL} -D _{5WR}	.0065	.0009	.9761	.9998
MS _{5WL} -MS _{5WR}	.0073	.0001	.9991	1.0000
P _{5WL} -P _{5WR}	.0542	.0021	.9983	.9581

Table 56. Mean regional data gathered from the *lateral quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded/5 Week Control Lateral Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	% Viable Osteocytes
5WL_Distal(D _{5WL})	245.40(77.7)	57.22(6.52)	269.62(68.5)	58.67(2.89)
5WL_Midshaft(MS _{5WL})	277.99(81.8)	64.35(5.50)	348.03(102.4)	58.25(7.78)
5WL_Proximal(P _{5WL})	173.98(25.0)	52.43(4.98)	198.41(64.5)	47.12(11.49)
5WR_Distal(D _{5WR})	136.41(26.3)	35.30(6.60)	328.00(64.6)	59.19(9.15)
5WR_Midshaft(MS _{5WR})	191.78(69.1)	39.62(8.77)	365.40(99.9)	58.69(11.38)
5WR_Proximal(P _{5WR})	135.35(48.8)	35.54(8.14)	265.19(37.4)	54.35(11.81)
D _{5WL} -D _{5WR}	.0367	.0001	.7686	1.0000
MS _{5WL} -MS _{5WR}	.1525	.0000	.9986	1.0000
P _{5WL} -P _{5WR}	.8669	.0024	.6565	.7815

Table 57. Mean regional data gathered from the *caudal quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded/5 Week Control Caudal Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
5WL_Distal(D_{5WL})	220.42(48.0)	55.96(6.80)	300.20(14.07)	63.22(4.82)
5WL_Midshaft(MS_{5WL})	287.88(68.9)	62.29(3.30)	382.38(54.9)	63.60(5.68)
5WL_Proximal(P_{5WL})	210.18(18.24)	56.97(4.00)	309.71(40.4)	64.43(5.67)
5WR_Distal(D_{5WR})	136.99(60.2)	34.62(10.86)	359.66(81.8)	61.89(9.12)
5WR_Midshaft(MS_{5WR})	155.41(25.9)	38.86(7.33)	396.47(83.9)	64.60(6.96)
5WR_Proximal(P_{5WR})	132.08(25.1)	38.56(6.21)	350.45(97.9)	66.50(9.72)
D_{5WL}-D_{5WR}	.0354	.0001	.6649	.9995
MS_{5WL}-MS_{5WR}	.0003	.0000	.9992	.9999
P_{5WL}-P_{5WR}	.0565	.0008	.9041	.9959

Table 58. Mean regional data gathered from the *medial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between loaded left tibias and control right tibias in all subjects that were loaded for 5 weeks. Standard deviations in parentheses.

5 Week Loaded/5 Week Control Medial Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
5WL_Distal(D_{5WL})	205.80(43.4)	56.84(7.32)	309.30(47.7)	66.62(3.26)
5WL_Midshaft(MS_{5WL})	287.24(114.6)	60.99(5.37)	383.97(112.5)	68.84(5.07)
5WL_Proximal(P_{5WL})	277.59(80.9)	63.09(4.59)	307.77(63.3)	66.61(4.91)
5WR_Distal(D_{5WR})	144.39(79.4)	39.21(11.13)	342.81(65.2)	69.06(8.48)
5WR_Midshaft(MS_{5WR})	155.11(10.82)	37.32(6.66)	397.45(95.8)	65.21(5.78)
5WR_Proximal(P_{5WR})	165.20(61.2)	38.51(9.86)	268.59(73.3)	59.62(9.94)
D_{5WL}-D_{5WR}	.6896	.0062	.9764	.9873
MS_{5WL}-MS_{5WR}	.0393	.0002	.9997	.9309
P_{5WL}-P_{5WR}	.1100	.0001	.9541	.4670

3.7 Comparison Between 2 Week Loaded Left Tibias and 5 Week Loaded Left Tibias

Below are the statistics for all of the 2 week loaded left tibias compared against all of the 5 week loaded left tibias, including a summary, analysis by quadrant, analysis by region and combinations of the latter two. For each statistical analysis, apoptotic osteocyte density, percentage of apoptotic osteocytes, viable osteocyte density and percentage of viable osteocytes were the variables considered. Mean values, standard deviations and relevant p-values are shown, with p-values less than 0.05 indicating statistically significant data.

The lateral quadrants, isolated to each region individually and averaged across all regions; the caudal quadrants, isolated to the midshaft region and averaged across all regions; and the medial quadrants, averaged across all regions, showed significantly higher densities of apoptotic osteocytes in 5 week loaded bone compared to 2 week loaded samples. The midshaft regions, when isolated to lateral quadrants, caudal quadrants and an averaged across all quadrants, showed significantly higher densities of apoptotic osteocytes in 5 week loaded bones compared to 2 week loaded bones as well. The 5 week loaded quadrants, isolated to the proximal region, midshaft region and average across all regions, and 5 week loaded regions, isolated to the cranial quadrant, lateral quadrant, caudal quadrant and average across all quadrants, showed significantly higher percentages of apoptotic osteocytes compared to the respective quadrants or regions of the 2 week loaded samples. The lateral, caudal and medial quadrants, isolated to the distal region, and the distal and midshaft regions, both isolated to medial quadrant, also showed a significantly higher percentage of apoptotic osteocytes in the 5 week loaded samples compared to the 2 week load samples. Lateral quadrants, when averaged

across all regions and when isolated to the midshaft region, showed significantly higher densities of viable osteocytes in 5 week loaded bones compared to 2 week loaded bones. Midshaft regions, when isolated to cranial quadrants, lateral quadrants, caudal quadrants and averaged across all quadrants, and proximal regions, isolated to the caudal quadrant, showed significantly higher densities of viable osteocytes in 5 week loaded bones compared to 2 week loaded bones as well. The 5 week loaded quadrants, isolated to the distal region, midshaft region and averaged across all regions, and the 5 week loaded regions, averaged across all quadrants, showed a significantly higher percentage of viable osteocytes compared to the respective quadrants or regions in the 2 week loaded samples. The midshafts, when isolated by each individual quadrant, and the proximal regions, isolated to the caudal quadrant, also showed a significantly less percentage of viable osteocytes in the 2 week loaded samples compared to the 5 week samples.

Table 59. Quadrantal differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes between left tibias loaded for 2 weeks and left tibias loaded for 5 weeks. Standard deviations in parentheses.

2 Week Loaded/5 Week Loaded Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Cranial(Cr_{2WL})	150.1(38.4)	42.03(6.40)	258.3(63.3)	53.54(7.96)
2WL_Lateral(L_{2WL})	88.8(40.7)	26.62(5.80)	139.82(12.08)	33.41(1.79)
2WL_Caudal(Cd_{2WL})	112.1(46.8)	33.89(7.47)	211.4(52.1)	45.61(6.39)
2WL_Medial(M_{2WL})	146.1(34.6)	43.42(1.92)	244.7(109.0)	51.73(11.52)
5WL_Cranial(Cr_{5WL})	236.27(37.8)	59.90(3.83)	348.55(35.4)	67.22(1.50)
5WL_Lateral(L_{5WL})	232.46(56.8)	58.00(3.47)	272.02(63.1)	54.68(5.81)
5WL_Caudal(Cd_{5WL})	239.50(38.3)	58.40(3.39)	330.77(23.34)	63.75(2.72)
5WL_Medial(M_{5WL})	256.88(59.1)	60.31(2.46)	333.68(42.3)	67.36(1.34)
Cr_{2WL}-Cr_{5WL}	.1950	.0001	.2539	.0126
L_{2WL}-L_{5WL}	.0036	.0000	.0230	.0001
Cd_{2WL}-Cd_{5WL}	.0125	.0000	.0518	.0005
M_{2WL}-M_{5WL}	.0416	.0001	.2685	.0031

Table 60. Mean quadrantal data gathered from the *proximal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between left tibias loaded for 2 weeks and left tibias loaded for 5 weeks. Standard deviations in parentheses.

2 Week Loaded/5 Week Loaded Proximal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Cranial(Cr_{2WL})	139.26(26.7)	42.49(4.23)	248.18(118.0)	54.36(14.24)
2WL_Lateral(L_{2WL})	73.68(47.6)	22.37(3.88)	113.62(61.2)	30.82(12.34)
2WL_Caudal(Cd_{2WL})	119.79(25.9)	35.97(4.43)	185.28(30.6)	46.44(8.26)
2WL_Medial(M_{2WL})	178.15(40.5)	51.88(6.50)	193.30(62.0)	54.82(17.6)
5WL_Cranial(Cr_{5WL})	222.01(28.9)	58.68(4.20)	331.65(49.7)	66.40(2.15)
5WL_Lateral(L_{5WL})	173.98(25.0)	52.43(4.98)	198.41(64.5)	47.12(11.49)
5WL_Caudal(Cd_{5WL})	210.18(18.24)	56.97(4.00)	309.71(40.4)	64.43(5.67)
5WL_Medial(M_{5WL})	277.59(80.9)	63.09(4.59)	307.77(63.3)	66.61(4.91)
Cr_{2WL}-Cr_{5WL}	.1616	.0007	.5521	.6081
L_{2WL}-L_{5WL}	.0486	.0000	.5331	.2472
Cd_{2WL}-Cd_{5WL}	.0982	.0000	.1211	.1550
M_{2WL}-M_{5WL}	.0517	.0321	.1882	.6323

Table 61. Mean quadrantal data gathered from the *midshaft region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between left tibias loaded for 2 weeks and left tibias loaded for 5 weeks. Standard deviations in parentheses.

2 Week Loaded/5 Week Loaded Midshaft Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	% Viable Osteocytes
2WL_Cranial(Cr_{2WL})	169.52(67.1)	40.59(4.18)	236.57(68.0)	49.54(8.58)
2WL_Lateral(L_{2WL})	71.92(34.1)	25.75(6.21)	109.51(53.8)	28.75(8.46)
2WL_Caudal(Cd_{2WL})	104.01(55.5)	33.25(8.37)	192.70(114.6)	40.36(10.80)
2WL_Medial(M_{2WL})	157.92(62.4)	41.00(2.56)	256.64(168.4)	46.93(14.93)
5WL_Cranial(Cr_{5WL})	255.87(71.0)	62.48(4.44)	392.50(54.7)	67.41(3.49)
5WL_Lateral(L_{5WL})	277.99(81.8)	64.35(5.50)	348.03(102.4)	58.25(7.78)
5WL_Caudal(Cd_{5WL})	287.88(68.9)	62.29(3.30)	382.38(54.9)	63.60(5.68)
5WL_Medial(M_{5WL})	287.24(114.6)	60.99(5.37)	383.97(112.5)	68.84(5.07)
Cr_{2WL}-Cr_{5WL}	.7732	.0000	.2969	.0449
L_{2WL}-L_{5WL}	.0181	.0000	.0224	.0002
Cd_{2WL}-Cd_{5WL}	.0462	.0000	.1165	.0039
M_{2WL}-M_{5WL}	.3161	.0001	.5431	.0073

Table 62. Mean quadrantal data gathered from the *distal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between left tibias loaded for 2 weeks and left tibias loaded for 5 weeks. Standard deviations in parentheses.

2 Week Loaded/5 Week Loaded Distal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Cranial(Cr _{2WL})	141.53(24.6)	43.01(11.61)	290.43(47.3)	56.72(4.66)
2WL_Lateral(L _{2WL})	120.86(64.2)	31.75(12.99)	196.34(23.2)	40.67(1.78)
2WL_Caudal(Cd _{2WL})	112.62(62.2)	32.43(10.07)	256.25(35.3)	50.03(3.32)
2WL_Medial(M _{2WL})	102.34(18.4)	37.39(6.00)	284.29(112.9)	53.44(8.14)
5WL_Cranial(Cr _{5WL})	230.93(44.9)	58.54(6.44)	321.50(31.1)	67.85(1.45)
5WL_Lateral(L _{5WL})	245.40(77.7)	57.22(6.52)	269.62(68.5)	58.67(2.89)
5WL_Caudal(Cd _{5WL})	220.42(48.0)	55.96(6.80)	300.20(14.07)	63.22(4.82)
5WL_Medial(M _{5WL})	205.80(43.4)	56.84(7.32)	309.30(47.7)	66.62(3.26)
Cr _{2WL} -Cr _{5WL}	.2893	.1526	.9879	.0079
L _{2WL} -L _{5WL}	.0451	.0025	.4919	.0000
Cd _{2WL} -Cd _{5WL}	.1178	.0060	.9230	.0012
M _{2WL} -M _{5WL}	.1481	.0347	.9967	.0012

Table 63. Regional differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes between left tibias loaded for 2 weeks and left tibias loaded for 5 weeks. Standard deviations in parentheses.

2 Week Loaded/5 Week Loaded Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Distal(D _{2WL})	123.53(42.6)	37.06(8.04)	262.57(50.0)	51.66(3.72)
2WL_Midshaft(MS _{2WL})	129.12(48.0)	36.67(2.95)	206.56(90.3)	44.08(9.63)
2WL_Proximal(P _{2WL})	123.56(29.8)	37.90(4.46)	198.71(65.2)	48.65(9.80)
5WL_Distal(D _{5WL})	225.88(41.0)	57.08(5.41)	302.42(19.36)	64.32(2.00)
5WL_Midshaft(MS _{5WL})	272.75(68.7)	62.78(3.77)	377.70(52.9)	64.97(3.61)
5WL_Proximal(P _{5WL})	214.44(24.9)	57.78(2.74)	298.83(49.0)	62.82(3.85)
D _{2WL} -D _{5WL}	.0508	.0001	.8858	.0251
MS _{2WL} -MS _{5WL}	.0031	.0000	.0018	.0002
P _{2WL} -P _{5WL}	.1023	.0001	.1168	.0101

Table 64. Mean regional data gathered from the *cranial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between left tibias loaded for 2 weeks and left tibias loaded for 5 weeks. Standard deviations in parentheses.

2 Week Loaded/5 Week Loaded Cranial Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Distal(D_{2WL})	141.53(24.6)	43.01(11.61)	290.43(47.3)	56.72(4.66)
2WL_Midshaft(MS_{2WL})	169.52(67.1)	40.59(4.18)	236.57(68.0)	49.54(8.58)
2WL_Proximal(P_{2WL})	139.26(26.7)	42.49(4.23)	248.18(118.)	54.36(14.24)
5WL_Distal(D_{5WL})	230.93(44.9)	58.54(6.44)	321.50(31.1)	67.85(1.45)
5WL_Midshaft(MS_{5WL})	255.87(71.0)	62.48(4.44)	392.50(54.7)	67.41(3.49)
5WL_Proximal(P_{5WL})	222.01(28.9)	58.68(4.20)	331.65(49.7)	66.40(2.15)
D_{2WL}-D_{5WL}	.1512	.0142	.9742	.1078
MS_{2WL}-MS_{5WL}	.1766	.0004	.0139	.0030
P_{2WL}-P_{5WL}	.2110	.0100	.3792	.0694

Table 65. Mean regional data gathered from the *lateral quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between left tibias loaded for 2 weeks and left tibias loaded for 5 weeks. Standard deviations in parentheses.

2 Week Loaded/5 Week Loaded Lateral Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Distal(D_{2WL})	120.86(64.2)	31.75(12.99)	196.34(23.2)	40.67(1.78)
2WL_Midshaft(MS_{2WL})	71.92(34.1)	25.75(6.21)	109.51(53.8)	28.75(8.46)
2WL_Proximal(P_{2WL})	73.68(47.6)	22.37(3.88)	113.62(61.2)	30.82(12.34)
5WL_Distal(D_{5WL})	245.40(77.7)	57.22(6.52)	269.62(68.5)	58.67(2.89)
5WL_Midshaft(MS_{5WL})	277.99(81.8)	64.35(5.50)	348.03(102.4)	58.25(7.78)
5WL_Proximal(P_{5WL})	173.98(25.0)	52.43(4.98)	198.41(64.5)	47.12(11.49)
D_{2WL}-D_{5WL}	.0928	.0003	.7122	.0583
MS_{2WL}-MS_{5WL}	.0016	.0000	.0017	.0007
P_{2WL}-P_{5WL}	.2487	.0000	.5776	.1033

Table 66. Mean regional data gathered from the *caudal quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between left tibias loaded for 2 weeks and left tibias loaded for 5 weeks. Standard deviations in parentheses.

2 Week Loaded/5 Week Loaded Caudal Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Distal(D _{2WL})	112.62(62.2)	32.43(10.07)	256.25(35.3)	50.03(3.32)
2WL_Midshaft(MS _{2WL})	104.01(55.5)	33.25(8.37)	192.70(114.6)	40.36(10.8)
2WL_Proximal(P _{2WL})	119.79(25.9)	35.97(4.43)	185.28(30.6)	46.44(8.26)
5WL_Distal(D _{5WL})	220.42(48.0)	55.96(6.80)	300.20(14.07)	63.22(4.82)
5WL_Midshaft(MS _{5WL})	287.88(68.9)	62.29(3.30)	382.38(54.9)	63.60(5.68)
5WL_Proximal(P _{5WL})	210.18(18.24)	56.97(4.00)	309.71(40.4)	64.43(5.67)
D _{2WL} -D _{5WL}	.0576	.0002	.8239	.0693
MS _{2WL} -MS _{5WL}	.0005	.0000	.0004	.0005
P _{2WL} -P _{5WL}	.1501	.0008	.0255	.0067

Table 67. Mean regional data gathered from the *medial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between left tibias loaded for 2 weeks and left tibias loaded for 5 weeks. Standard deviations in parentheses.

2 Week Loaded/5 Week Loaded Medial Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WL_Distal(D _{2WL})	102.34(18.4)	37.39(6.00)	284.29(112.9)	53.44(8.14)
2WL_Midshaft(MS _{2WL})	157.92(62.4)	41.00(2.56)	256.64(168.4)	46.93(14.93)
2WL_Proximal(P _{2WL})	178.15(40.5)	51.88(6.50)	193.30(62.0)	54.82(17.6)
5WL_Distal(D _{5WL})	205.80(43.4)	56.84(7.32)	309.30(47.7)	66.62(3.26)
5WL_Midshaft(MS _{5WL})	287.24(114.6)	60.99(5.37)	383.97(112.5)	68.84(5.07)
5WL_Proximal(P _{5WL})	277.59(80.9)	63.09(4.59)	307.77(63.3)	66.61(4.91)
D _{2WL} -D _{5WL}	.4075	.0012	.9989	.2769
MS _{2WL} -MS _{5WL}	.1929	.0009	.4179	.0158
P _{2WL} -P _{5WL}	.4495	.1018	.5305	.3900

3.8 Comparison Between 2 Week and 5 Week Unloaded Right Tibia Controls

Below are the statistics for all of the 2 week unloaded right tibias compared against all of the 5 week unloaded right tibias used as controls, including a summary, analysis by quadrant, analysis by region and combinations of the latter two. For each statistical analysis, apoptotic osteocyte density, percentage of apoptotic osteocytes, viable osteocyte density and percentage of viable osteocytes were the variables considered. Mean values, standard deviations and relevant p-values are shown, with p-values less than 0.05 indicating statistically significant data. No statistically significant differences were seen between the 2 week unloaded controls and the 5 week unloaded controls.

Table 68. Quadrantal differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes between right tibias unloaded for 2 weeks and right tibias unloaded for 5 weeks both used as controls. Standard deviations in parentheses.

2 Week Control/5 Week Control Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WR_Cranial(Cr _{2WR})	128.08(34.1)	36.20(2.08)	305.68(62.2)	59.06(6.36)
2WR_Lateral(L _{2WR})	107.65(31.1)	28.30(3.83)	242.22(34.7)	46.37(1.55)
2WR_Caudal(Cd _{2WR})	112.28(33.9)	30.21(2.91)	277.54(52.9)	52.05(5.01)
2WR_Medial(M _{2WR})	167.96(40.6)	38.92(4.06)	344.32(35.6)	61.03(6.19)
5WR_Cranial(Cr _{5WR})	151.11(17.04)	41.24(4.74)	358.88(83.2)	66.72(3.11)
5WR_Lateral(L _{5WR})	154.51(36.2)	36.82(6.58)	319.53(27.5)	57.41(10.05)
5WR_Caudal(Cd _{5WR})	141.49(28.4)	37.35(6.70)	368.86(59.6)	64.33(7.15)
5WR_Medial(M _{5WR})	154.90(45.1)	38.35(6.07)	336.28(58.0)	64.63(6.75)
Cr _{2WR} -Cr _{5WR}	.9761	.8859	.8845	.7336
L _{2WR} -L _{5WR}	.5253	.3711	.5528	.3097
Cd _{2WR} -Cd _{5WR}	.9187	.5882	.3482	.1981
M _{2WR} -M _{5WR}	.9992	1.0000	1.0000	.9939

Table 69. Mean quadrantal data gathered from the *proximal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between right tibias unloaded for 2 weeks and right tibias unloaded for 5 weeks both used as controls. Standard deviations in parentheses.

2 Week Control/5 Week Control Proximal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WR_Cranial(Cr_{2WR})	119.69(7.01)	37.96(2.02)	314.25(20.9)	60.82(0.92)
2WR_Lateral(L_{2WR})	64.08(23.4)	20.74(2.88)	194.92(47.2)	41.14(7.09)
2WR_Caudal(Cd_{2WR})	87.54(4.45)	29.74(1.01)	252.90(52.5)	49.47(5.94)
2WR_Medial(M_{2WR})	168.77(35.7)	39.63(4.38)	312.88(92.7)	58.51(13.4)
5WR_Cranial(Cr_{5WR})	150.31(18.42)	42.06(4.85)	312.83(88.0)	64.48(3.61)
5WR_Lateral(L_{5WR})	135.35(48.8)	35.54(8.14)	265.19(37.4)	54.35(11.81)
5WR_Caudal(Cd_{5WR})	132.08(25.1)	38.56(6.21)	350.45(97.9)	66.50(9.72)
5WR_Medial(M_{5WR})	165.20(61.2)	38.51(9.86)	268.59(73.3)	59.62(9.94)
Cr_{2WR}-Cr_{5WR}	.9377	.9849	1.0000	.9989
L_{2WR}-L_{5WR}	.1674	.0578	.8654	.4522
Cd_{2WR}-Cd_{5WR}	.6987	.5552	.5660	.1703
M_{2WR}-M_{5WR}	1.0000	1.0000	.9874	1.0000

Table 70. Mean quadrantal data gathered from the *midshaft region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between right tibias unloaded for 2 weeks and right tibias unloaded for 5 weeks both used as controls. Standard deviations in parentheses.

2 Week Control/5 Week Control Midshaft Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WR_Cranial(Cr_{2WR})	133.45(46.1)	36.55(6.04)	291.28(113.2)	58.95(7.50)
2WR_Lateral(L_{2WR})	115.74(41.0)	28.43(7.06)	238.32(27.8)	43.87(1.80)
2WR_Caudal(Cd_{2WR})	115.72(46.9)	29.34(5.27)	270.63(79.6)	49.77(3.04)
2WR_Medial(M_{2WR})	172.28(58.3)	37.96(6.36)	421.17(65.7)	63.17(4.14)
5WR_Cranial(Cr_{5WR})	164.57(27.5)	40.87(7.98)	408.90(123.1)	67.21(4.17)
5WR_Lateral(L_{5WR})	191.78(69.1)	39.62(8.77)	365.40(99.9)	58.69(11.38)
5WR_Caudal(Cd_{5WR})	155.41(25.9)	38.86(7.33)	396.47(83.9)	64.60(6.96)
5WR_Medial(M_{5WR})	155.11(10.82)	37.32(6.66)	397.45(95.8)	65.21(5.78)
Cr_{2WR}-Cr_{5WR}	.9642	.9894	.6615	.6854
L_{2WR}-L_{5WR}	.2220	.4034	.5735	.0798
Cd_{2WR}-Cd_{5WR}	.8821	.6009	.5851	.0796
M_{2WR}-M_{5WR}	.9990	1.0000	1.0000	.9999

Table 71. Mean quadrantal data gathered from the *distal region* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between right tibias unloaded for 2 weeks and right tibias unloaded for 5 weeks both used as controls. Standard deviations in parentheses.

2 Week Control/5 Week Control Distal Quadrant

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WR_Cranial(Cr_{2WR})	131.09(54.8)	34.08(3.96)	311.52(82.5)	57.40(11.25)
2WR_Lateral(L_{2WR})	143.13(36.0)	35.72(5.44)	293.43(67.2)	54.09(9.46)
2WR_Caudal(Cd_{2WR})	133.58(57.0)	31.55(5.84)	309.09(48.5)	56.91(6.88)
2WR_Medial(M_{2WR})	162.81(42.1)	39.17(3.84)	298.90(38.3)	61.43(6.88)
5WR_Cranial(Cr_{5WR})	138.46(35.3)	40.80(10.09)	354.90(89.4)	68.47(6.75)
5WR_Lateral(L_{5WR})	136.41(26.3)	35.30(6.60)	328.00(64.6)	59.19(9.15)
5WR_Caudal(Cd_{5WR})	136.99(60.2)	34.62(10.86)	359.66(81.8)	61.89(9.12)
5WR_Medial(M_{5WR})	144.39(79.4)	39.21(11.13)	342.81(65.2)	69.06(8.48)
Cr_{2WR}-Cr_{5WR}	1.0000	.9534	.9882	.6046
L_{2WR}-L_{5WR}	1.0000	1.0000	.9970	.9886
Cd_{2WR}-Cd_{5WR}	1.0000	.9996	.9720	.9902
M_{2WR}-M_{5WR}	.9996	1.0000	.9873	.9047

Table 72. Regional differences of apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes between right tibias unloaded for 2 weeks and right tibias unloaded for 5 weeks both used as controls. Standard deviations in parentheses.

2 Week Control/5 Week Control Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WR_Distal(D_{2WR})	138.65(49.4)	34.19(4.82)	305.10(61.4)	57.41(8.62)
2WR_Midshaft(MS_{2WR})	127.87(47.9)	33.16(5.05)	290.88(76.4)	54.39(2.97)
2WR_Proximal(P_{2WR})	103.63(11.37)	32.90(1.36)	271.71(21.5)	53.92(1.24)
5WR_Distal(D_{5WR})	139.09(45.8)	37.63(8.96)	350.68(71.9)	64.41(7.51)
5WR_Midshaft(MS_{5WR})	163.32(22.7)	39.67(7.46)	394.35(83.1)	64.55(5.68)
5WR_Proximal(P_{5WR})	142.64(22.93)	39.58(5.51)	307.31(69.6)	62.56(6.33)
D_{2WR}-D_{5WR}	1.0000	.9760	.9389	.6117
MS_{2WR}-MS_{5WR}	.7029	.7366	.3385	.2339
P_{2WR}-P_{5WR}	.6161	.7174	.9782	.3943

Table 73. Mean regional data gathered from the *cranial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between right tibias unloaded for 2 weeks and right tibias unloaded for 5 weeks both used as controls. Standard deviations in parentheses.

2 Week Control/5 Week Control Cranial Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WR_Distal(D _{2WR})	131.09(54.8)	34.08(3.96)	311.52(82.5)	57.40(11.25)
2WR_Midshaft(MS _{2WR})	133.45(46.1)	36.55(6.04)	291.28(113.2)	58.95(7.50)
2WR_Proximal(P _{2WR})	119.69(7.01)	37.96(2.02)	314.25(20.9)	60.82(0.92)
5WR_Distal(D _{5WR})	138.46(35.3)	40.80(10.09)	354.90(89.4)	68.47(6.75)
5WR_Midshaft(MS _{5WR})	164.57(27.5)	40.87(7.98)	408.90(123.1)	67.21(4.17)
5WR_Proximal(P _{5WR})	150.31(18.42)	42.06(4.85)	312.83(88.0)	64.48(3.61)
D _{2WR} -D _{5WR}	.9995	.7607	.9867	.1349
MS _{2WR} -MS _{5WR}	.7505	.9518	.5296	.3976
P _{2WR} -P _{5WR}	.7624	.9613	1.0000	.9500

Table 74. Mean regional data gathered from the *lateral quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between right tibias unloaded for 2 weeks and right tibias unloaded for 5 weeks both used as controls. Standard deviations in parentheses.

2 Week Control/5 Week Control Lateral Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WR_Distal(D _{2WR})	143.13(36.0)	35.72(5.44)	293.43(67.2)	54.09(9.46)
2WR_Midshaft(MS _{2WR})	115.74(41.0)	28.43(7.06)	238.32(27.8)	43.87(1.80)
2WR_Proximal(P _{2WR})	64.08(23.4)	20.74(2.88)	194.92(47.2)	41.14(7.09)
5WR_Distal(D _{5WR})	136.41(26.3)	35.30(6.60)	328.00(64.6)	59.19(9.15)
5WR_Midshaft(MS _{5WR})	191.78(69.1)	39.62(8.77)	365.40(99.9)	58.69(11.38)
5WR_Proximal(P _{5WR})	135.35(48.8)	35.54(8.14)	265.19(37.4)	54.35(11.81)
D _{2WR} -D _{5WR}	.9999	1.0000	.9751	.9758
MS _{2WR} -MS _{5WR}	.2412	.2886	.1164	.3144
P _{2WR} -P _{5WR}	.3025	.0830	.6711	.4351

Table 75. Mean regional data gathered from the *caudal quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between right tibias unloaded for 2 weeks and right tibias unloaded for 5 weeks both used as controls. Standard deviations in parentheses.

2 Week Control/5 Week Control Caudal Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WR_Distal(D_{2WR})	133.58(57.0)	31.55(5.84)	309.09(48.5)	56.91(6.88)
2WR_Midshaft(MS_{2WR})	115.72(46.9)	29.34(5.27)	270.63(79.6)	49.77(3.04)
2WR_Proximal(P_{2WR})	87.54(4.45)	29.74(1.01)	252.90(52.5)	49.47(5.94)
5WR_Distal(D_{5WR})	136.99(60.2)	34.62(10.86)	359.66(81.8)	61.89(9.12)
5WR_Midshaft(MS_{5WR})	155.41(25.9)	38.86(7.33)	396.47(83.9)	64.60(6.96)
5WR_Proximal(P_{5WR})	132.08(25.1)	38.56(6.21)	350.45(97.9)	66.50(9.72)
D_{2WR}-D_{5WR}	1.0000	.9913	.9478	.9449
MS_{2WR}-MS_{5WR}	.7471	.4870	.2861	.1285
P_{2WR}-P_{5WR}	.6499	.5668	.5509	.0594

Table 76. Mean regional data gathered from the *medial quadrant* for apoptotic osteocyte density, viable osteocyte density, percentage of apoptotic osteocytes, and percentage of viable osteocytes compared between right tibias unloaded for 2 weeks and right tibias unloaded for 5 weeks both used as controls. Standard deviations in parentheses.

2 Week Control/5 Week Control Medial Region

Quadrant	Apoptotic Osteocyte Density	%Apoptotic Osteocytes	Viable Osteocyte Density	%Viable Osteocytes
2WR_Distal(D_{2WR})	162.81(42.1)	39.17(3.84)	298.90(38.3)	61.43(6.88)
2WR_Midshaft(MS_{2WR})	172.28(58.3)	37.96(6.36)	421.17(65.7)	63.17(4.14)
2WR_Proximal(P_{2WR})	168.77(35.7)	39.63(4.38)	312.88(92.7)	58.51(13.4)
5WR_Distal(D_{5WR})	144.39(79.4)	39.21(11.13)	342.81(65.2)	69.06(8.48)
5WR_Midshaft(MS_{5WR})	155.11(10.82)	37.32(6.66)	397.45(95.8)	65.21(5.78)
5WR_Proximal(P_{5WR})	165.20(61.2)	38.51(9.86)	268.59(73.3)	59.62(9.94)
D_{2WR}-D_{5WR}	.9966	1.0000	.9621	.7958
MS_{2WR}-MS_{5WR}	.9976	1.0000	.9976	.9993
P_{2WR}-P_{5WR}	1.0000	1.0000	.9607	1.0000

4. DISCUSSION

The goal of this study was to try to find a correlation between osteocyte apoptosis and the amount of cyclical loading applied, or lack thereof, in order to show that apoptotic osteocytes may be an important signaling factor in the bone remodeling process. For this study, mice were procured and used due to their easy availability, affordability, and size, making them relatively easy to handle. Three mice were cyclically loaded for two weeks and six mice were cyclically loaded for 5 weeks, where the right hindlimb of each mouse was used as a control and the left hindlimb was cyclically loaded. Hindlimbs were used as opposed to forelimbs due to their larger cross sectional area and ability to be cyclically loaded with ease in the loading fixture utilized in this study. After the tibias were resected, processed, and sectioned with a microtome, each section was stained using a TUNEL assay, to detect apoptotic osteocytes, and counterstained with methyl green to detect viable osteocytes. The density of apoptotic osteocytes was measured by region (proximal, midshaft, and distal) and quadrant (cranial, caudal, lateral, and medial) in loaded and unloaded murine cortical bone. The percent of apoptotic osteocytes, density of viable osteocytes and percent of viable osteocytes were also similarly quantified. Statistical analyses were performed on all loaded and unloaded samples independently of each other as well as compared against each other in order to find any statistically significant data. Samples were analyzed by comparing quadrants, averaged across all regions and then isolated to each individual region as well, and then by comparing regions, averaged across all quadrants as well as isolated to each individual quadrant. In this study, it was hypothesized that higher amounts of apoptotic osteocytes would be seen in bones that were cyclically loaded when compared against unloaded bones, with the highest amount seen in the 5 week loaded bones. It was also hypothesized

that more pronounced regional variation would be seen in the 5 week loaded samples compared to the 2 week loaded samples as a result of the additional loading that they experienced.

Looking, individually, at each type of bone analyzed in this study, 2 week loaded, 2 week unloaded, 5 week loaded and 5 week unloaded, a number of trends and statistically significant information could be seen. As a general trend, 2 week loaded bones had densities and percentages of both viable and apoptotic osteocytes that were much lower in the lateral quadrant and slightly lower in the caudal quadrant, when both were compared to the other two quadrants. This trend was generally strongest in the proximal region for apoptotic osteocytes and strongest in the distal region for viable osteocytes. Similarly, for 2 week unloaded bones, viable and apoptotic osteocytes were much lower in the lateral quadrant and slightly lower in the caudal quadrant, when compared to the other two quadrants, with this trend being the strongest in the proximal region for apoptotic osteocytes and strongest in the midshaft for viable osteocytes. Generally, 5 week loaded bone showed lower viable osteocytes in the lateral quadrant, compared to the other quadrants, with this trend being strongest in the proximal region. Another general trend for 5 week loaded bone was that higher apoptotic and viable osteocytes were seen in the midshafts compared to the other regions, when averaged across all quadrants. No trends and only one statistically significant difference was seen within the 5 week unloaded bone data; when looking only within the medial quadrant, a higher density of viable osteocytes were seen in the midshaft compared to the proximal region. Overall, 5 week loaded samples showed the most variation within themselves with 24 significant p-values found in the analysis.

Comparing the results from this study to the data presented in a similar previous study, by Jessica Chan, yields some similarities (Chan 2011). No cyclical loading, however, was performed in the latter study and, thus, only data from individual control samples in this study can be compared to the data presented in her study. In both studies, there weren't a lot of statistically significant findings, when looking solely at differences within unloaded bone samples. However, when analyzing samples by quadrant, the current study showed some general trends, with some statistically significant p-values, of lower apoptotic and viable osteocyte values in the lateral and caudal quadrants when compared to the other quadrants. Jessica's research showed a similar trend for the apoptotic and viable osteocyte densities, although no statistically significant p-values were found to support this trend in her study. Only a few statistically significant differences were found in Jessica's study; midshafts were shown to have higher viable and apoptotic osteocytes when compared to the other regions. In the current study, although not seen in 2 week samples, this trend was similarly observed within the 5 week loaded and unloaded samples, with statistically significant p-values only seen in the 5 week loaded samples. Differences observed between the samples used in this study and the samples from Jessica's study may be due to the fact that a lower quantity of mice that were younger in age, and possibly skeletal maturity, were used in the study performed by her. Overall, given that the samples analyzed in Jessica's study only yielded some statistically significant p-values when looking at the midshafts, and nowhere else, the most prevalent trend between samples used in both studies is the general lack of statistically significant findings seen for unloaded bone samples. This should then

provide some evidence that the control samples in this study and the samples used in Jessica's study are relatively similar (Chan 2011).

In order to shed more light on the role of apoptotic osteocytes in the remodeling process, statistical analyses comparing each type of sample, loaded and unloaded, to one another is necessary. The first comparison analyzed in this study was 2 week loaded samples against 2 week unloaded control samples. When comparing 2 week loaded bones against 2 week unloaded control samples, only one statistically significant difference was found; the percentage of apoptotic osteocytes in 2 week loaded samples was shown to be higher than the 2 week unloaded bones when analyzed within the medial quadrant of the proximal region. Overall, the 2 week loaded samples showed little statistically significant differences to the unloaded samples. However, 2 week loaded bones showed a trend of slightly more apoptotic osteocytes and less viable osteocytes than 2 week control samples, even though there weren't any statistically significant p-values to support this trend. This could be because 2 weeks is only enough time to reach the start of the remodeling process. A study by Noble et al. showed that load induced DNA damage is followed by cellular apoptosis process within 2 weeks of initial damage stimulus (Noble, et al. 2003). In the study, at 14 days after loading, less cells with DNA fragmentation and less viable cells were observed as well as the first signs of Haversian remodeling (Noble, et al. 2003). This observation from Noble's study could explain the general trend of slightly more apoptotic osteocytes, less viable osteocytes and overall lack of statistically significant differences seen in this study's samples loaded for 2 weeks, when compared to the controls. 5 week unloaded and 2 week unloaded samples were also compared against one another. As expected, the 2 week unloaded and 5 week

unloaded samples showed no statistically significant differences when compared against each other. Given that the samples cyclically loaded for the most amount of time were expected to show the most differences when compared against unloaded samples, 5 week loaded samples were compared against both 5 week unloaded and 2 week loaded samples.

As previously mentioned before, no significant differences were found between 2 week loaded samples and 2 week unloaded samples nor between 2 week unloaded samples and 5 week unloaded samples. It makes sense, then, that comparing 5 week loaded samples against both 5 week unloaded samples and 2 week loaded samples yielded many similarities. Except for two statistically insignificant p-values when compared with 2 week loaded samples, all locations within 5 week loaded samples showed significantly higher percentages of apoptotic osteocytes than those of both the 5 week unloaded samples and the 2 week loaded samples, when averaged across all regions, averaged across all quadrants and when isolated to each individual region or quadrant. Including p-values of .0565 or less, 5 week loaded samples showed statistically higher densities of apoptotic osteocytes than 5 week unloaded control samples for each quadrant, when averaged across all regions, and for each region, when averaged across all quadrants and when looking at the cranial and caudal quadrants individually. For 2 week loaded versus 5 week loaded samples, all 5 week loaded quadrants, except the cranial quadrant, showed higher densities of apoptotic osteocytes, when averaged across all regions. Also, densities of apoptotic osteocytes were higher in 5 week loaded midshafts, when averaged across all quadrants and when analyzed in the caudal or lateral quadrants individually. The percentage of viable osteocytes in 5 week loaded bones was

significantly higher than in 2 week loaded bones for all quadrants, when averaged across all regions, and in all regions, when averaged across all quadrants.

Fitting with this study's hypothesis, 5 week loaded samples showed the biggest differences when compared with all other samples, especially in terms of the density and percentage of apoptotic osteocytes present. Statistically significant values, indicating that each quadrant and region in 5 week loaded samples had a higher percentage of apoptotic osteocytes than any other sample group, provides strong evidence in support of increased apoptosis and regional variations in samples that are cyclically loaded. 5 week loaded samples also showed an increase in the density of apoptotic osteocytes, but not to the same magnitude that the percent of apoptotic osteocytes data showed. There were also many statistically significant regions and quadrants showing increased viable osteocytes in 5 week loaded samples compared to 2 week loaded samples. This difference was not also observed between 5 week loaded samples and the controls because the difference appears to be due to the specific time points in which the two difference loading groups were loaded and subsequently sacrificed. As previously discussed, the 2 week loaded samples may have shown slightly more apoptotic osteocytes and less viable osteocytes than 2 week unloaded samples because, as described by Noble, samples exhibited less apoptotic and viable osteocytes as well as the first signs of remodeling at 14 days after the initial damage stimulus from loading. In the same study by Noble et al., at 7 days after loading, more osteocytes with DNA fragmentation and similar viable osteocytes, when compared to the control samples, were seen (Noble, et al. 2003). These two observations, from Noble's study, help shed light on why statistically significant differences in the amount of viable osteocytes were only observed between 5 week and 2

week loaded samples. All of these previously discussed trends involving the 5 week loaded samples, showing increased values apoptotic and viable osteocyte values in these samples, are further strengthened by the fact that no significant trends were observed when comparing the 2 week unloaded samples against both the 2 week loaded and 5 week unloaded samples, thus agreeing with the rationalizations given in this discussion as well as the observations presented here from other studies; only 5 week loaded samples, in this study, showed multiple statistically significant differences to other samples.

The results from this study were focused on quantifying variations in the amount of apoptotic osteocytes present in loaded mice hindlimbs, unloaded mice hindlimbs and any differences there may be between the two, in an attempt to better explain the mechanisms and factors in which bone remodeling is initiated. Bones repair and replace themselves in response to the specific mechanical loads that they experience throughout each region (Noble, et al. 2003). How bones specifically use this information has been a question that has attempted to be answered by various theories, one of which involves naming apoptotic osteocytes as the signaling mechanism in which osteoblasts and osteoclasts are targeted, as necessary. The data from this study did in fact support the hypothesis that osteocyte apoptosis was increased and regional variations were enhanced due to cyclical loading applied to murine tibias. Other research has similarly made observations about bone remodeling and osteocyte apoptosis. One study, by Noble et al., demonstrated a U-shaped correlation with osteocyte apoptosis and bone loading, with disuse and fatigue loading microdamage showing increased apoptosis (Noble, et al. 2003). A study by Verborgt et al. showed that microdamage caused by fatigue loading showed increased levels of apoptotic osteocytes, especially concentrated around the

regions with microdamage or resorption (Verborgt, et al. 2000). Another study, by Cardoso et al., showed similar results indicating how apoptotic osteocytes are temporally and spatially linked to fatigue induced microdamage and the intracortical bone remodeling that follows (Cardoso, et al. 2009). A study by Hedgecock et al. showed a strong correlation between apoptotic osteocytes and remodeling parameters (Hedgecock, et al. 2007). In the same study, the researchers stated that their approximation of viable osteocytes showed no linear correlation with any of the remodeling or modeling factors studied (Hedgecock, et al. 2007). Although there is still much information lacking, especially in terms of the specific cellular interactions and communication networks at play from the beginning initiation of the remodeling process to the end of it, it is the hope of this study that the data analyzed and discussed within joins the previously mentioned studies in providing evidence in support of the role that apoptotic osteocytes must play in the remodeling process.

4.1 Limitations

Despite being a continuation of a previous study (Chan 2011), there are many limitations to the current study. Firstly, efforts were put forth to maintain all mice under the same day to day conditions by giving them similar living, dietary and loading conditions. However, as the mice were allowed to move around day to day, when not being loaded, there may have been some differences in the amount of exercise that each mouse received. Secondly, even though care was taken to ensure consistency from sample to sample when taking cross-sectional slices from the distal, midshaft and proximal regions of each sample, the location within each region that sample was taken from may have been different from subject to subject, which may cause unwanted

differences when analyzing the data. In regards to the TUNEL and methyl green staining, best efforts were put forth to use the same staining procedure with all samples for equivalency, however, some samples showed clearer color, indicating that the staining in some samples may have been better than others; TUNEL staining may also be subject to false staining which may have affected some of the counted data. In order to perform the TUNEL and methyl green staining, two adjacent samples were cut and the TUNEL stain was applied to one sample while the methyl green was applied to the other. Although this eliminated some of the problems encountered with co-staining, the two adjacent samples used may not have been entirely similar, which could have caused more unwanted differences or discrepancies in the data analysis. Also, by not co-staining the two methods together, the TUNEL stain may have incorrectly stained non-apoptotic osteocytes that contain DNA breaks, which was not accounted for in this study (Hedgecock, et al. 2007). When counting the amount of apoptotic and viable osteocytes present in each sample, there may have been some error in the actual values counted as this was done one by one by a single person, which leaves room for potential error.

4.2 Applications and Future Work

Experiments such as the current study provide more information surrounding apoptotic osteocytes and their role in the bone remodeling process. The results from this study give support to the idea that apoptotic osteocytes play an important role in bone remodeling and give data showing differences between unloaded and loaded bones in terms of apoptotic osteocytes. Future work should be performed to further look at the effects that apoptotic osteocytes have on neighboring cells and communication networks. This may include looking deeper into any signaling, an example being osteoclastogenic

signaling, that may be occurring between other neighboring osteocytes, both apoptotic and non-apoptotic, as well as any spatial or temporal patterns between the cells present while various types of remodeling communication is occurring.

4.3 Conclusion

This study set out to demonstrate regional and quadrant differences, namely increases in the number of apoptotic and viable osteocytes, in cyclically loaded murine tibiae when compared with unloaded bones in attempt to provide evidence in support of apoptotic osteocytes playing a role in the remodeling process. The results of this study showed increased apoptotic osteocytes, both in percentage and density, for the 5 week loaded samples when compared against the unloaded controls as well as the 2 week loaded bones. An increased percentage of viable osteocytes was also observed in the 5 week loaded bones when compared with the 2 week loaded samples. Individually, 5 week loaded samples also showed the most enhanced regional variation. Overall, 5 week samples showed an increase in apoptotic osteocytes as well as the most regional and quadrantal variation when compared against all other samples, thus, supporting our initial hypothesis and providing evidence in support of apoptotic osteocytes playing a role in the remodeling process. Further research that delves into the specific mechanisms of how apoptotic osteocytes help initialize bone remodeling as well as other potential localized interactions or communication that apoptotic osteocytes provide will further our understanding of the human body and the bone remodeling process.

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APPENDICES

Appendix A

Mice Loading Protocol

Equipment needed:

Bose Enduratec 3220

Load cell

Wintest PCI control system

Enduratec signal box

Mouse fixture with weights

Surgical tape

Q-tips

Procedure:

- 1) Turn on the Enduratec signal box, Wintest PCI control system and the computer.
- 2) Open the program: WinTest
- 3) File→ Open Project → 05-177 Cal poly pchang test.prj
- 4) Setup → Limits→ Display check limits (ensure that fixture will not hit bottom of the plunger)

Channel	Minimum	Maximum	Time	Action	Group
Disp	-2.500	6.000	0.0002	Abort	Group 1
Load	-200.00	200.00	0.0002	Abort	Group 1
Ch 3	-8.00	5.00	0.0002	Abort	All
Ch 4	-10.00	10.00	0.0002	Undefined	All
Null	-10.00	10.00	0.0002	Undefined	Group 1
UserCalc	-10.00	10.00	0.0002	Undefined	All
Axial cmd	-44.00	44.00	0.0002	Undefined	Group 1
Axial err	-100.00	100.00	0.0002	Undefined	Group 1

OK Cancel ☒ Include Mover Count

5) Setup → Channels → Autotare

6) Feedback → Set axial feedback to displacement

7) Local → High (means displacement can be adjusted live)

8) Click position button → raise so that the mouse can fit underneath the plunger

- 9) Ensure that nose cone is fasten securely with the chemistry stand and that it is not touching the fixture. Chemistry stand should be secured to the table with C-clamps.
- 10) Set up the isoflurane anesthesia machine so that it is connected to the anesthesia chamber but readily available to be connected to the nose cone tubing.
- 11) In the chamber, 4.5% isoflurane should be release at 3L/min.
- 12) Place a piece of surgical tape on the forelimbs of the mouse.
- 13) Once anesthetized in the chamber, the chamber tubing should be switched with the nose cone tubing and the anesthesia machine should be set to 2% isoflurane at 1L/min.
- 14) The mouse is then placed on top of the ramp fixture.
- 15) Ensure that the nose of the mouse is secured in the nose cone (use the pieces of the tape on the forelimbs to help secure the nose location).
- 16) Put left heel of the mouse into the groove on the ramp fixture. Use a piece of surgical tape to secure the foot down.

17) Lower the plunger position slowly until a force of -0.04 N is achieved. Use the q-tip to push the top of the femur so that the knee joint is fully constrained by the plunger cup indentation.

18) Click waveform → block signal tab → peak/valley → browse → name the file.

19) Click the scope for group 1 eraser icon

20) Hit the disk icon→ save scope→ export file (turns yellow)

21) Click on run → zero start

22) Once it finishes, click the disk icon again and name the file.

23) Click the position icon → raise the plunger until the mouse

Appendix B

Microtome Protocol

- 1) Put paraffin embed sample block(s) in the refrigerator for at least 30 minutes.
- 2) Wash hands and water bath with soap (try not to get the front of the water bath wet).
- 3) Dry with paper towels.
- 4) Put gloves on.
- 5) Fill water bath with distilled water.
- 6) Write name in the logbook.
- 7) Use the lever to secure wax block in the holding mechanism.
- 8) Insert the blade into the designated slot and lightly tighten until secure.
- 9) Release the handle lock on the right side of the microtome.
- 10) Push the base, holding the blade, up to the tissue block and lock in place.
- 11) Set the microtome to 20 μm and face the block a few times to take off the top layers of wax and reveal the bone.
- 12) Set the microtome to 7 μm and cut sections as needed.
- 13) Use the tweezers to carefully carry and place the sections in the water bath
- 14) Use blunt dissection cuts to separate individual sections so they fit on a microscope slide.
- 15) Angle the microscope slide into the water below the sections and raise it slowly to the surface ensuring that the section gets carefully placed onto the microscope (be sure that the microscope slide is oriented properly so that it can still be written on and labeled).

Appendix C

TUNEL and Methyl Green Staining Procedures

TUNEL Staining Procedure

1. Deparaffinize the tissue sections

Immerse the slides in xylene for 5 minutes

2. Immerse in 100% ethanol for 8 minutes (wash)

3. Rehydrate through graded ethanol washes

95% ethanol for 3 minutes

85% ethanol for 3 minutes

70% ethanol for 3 minutes

50% ethanol for 3 minutes

4. Air dry for 20 minutes

5. Immerse in PBS for 5 minutes (wash)

6. Immerse in HistoChoice for 15 minutes (fix)

7. Immerse in PBS for 10 minutes (wash)

8. Remove liquid from tissue and place slides on a flat surface

Prepare a 20 µg/ml proteinase k solution:

1 part 10 mg/ml proteinase k stock solution (see Appendix for procedure)

to

499 parts PBS

Add 100 µL of the proteinase k solution to each slide to cover the tissue section

Incubate slides for 10 to 30 minutes (set out to dry)

Use shorter incubation times because of the thinner sections.

9. **Immerse** in PBS for 5 minutes (wash)

10. **Immerse** in HistoChoice for 5 minutes (refix)

11. **Immerse** in PBS for 5 minutes (wash)

For positive control: treat sample with DNase I → causes DNA fragmentation

12. **Tap the slides to remove excess liquid** (use Kimwipes)

Cover the cells with 100 μ L of equilibration buffer

Equilibrate for 5 to 10 minutes

13. **Thaw biotinylated nucleotide mixture on ice**

Prepare rTdT reaction mixture, keep on ice

100 μ L of the reaction mixture per slide will be enough to cover the section

For positive control:

Combine: 98 μ L of equilibration buffer

 1 μ L of biotinylated nucleotide mixture

 1 μ L of rTdT

14. **Blot around equilibrated areas with tissue paper**

Add 100 μ L of the rTdT reaction mixture to the sections on each slide

Do not allow the sections to dry out

15. **Cover the sections with plastic coverslips to evenly distribute the reagent**

Incubate at 37 degrees C for 60 minutes inside of a humidified chamber (wet paper towel over dish)

This step allows the end-labeling reaction to occur

16. Dilute: 1 part 20x SSC solution

to

10 parts deionized water

Remove coverslips

Immerse in the 2x SSC solution for 15 minutes

This step terminates the end-labeling reaction

17. Immerse in fresh PBS for 15 minutes (wash)

This step removes unincorporated biotinylated nucleotides

18. Dilute: 1 part 3% hydrogen peroxide

to

10 parts PBS

Immerse in the 0.3% hydrogen peroxide solution for 3 to 5 minutes

Do not use the 20x hydrogen peroxide

This step blocks endogenous peroxidases

19. Immerse in PBS for 15 minutes (wash)

20. Dilute streptavidin HRP solution 1:500 in PBS

Add 100 μ L to each slide

Incubate for 30 minutes (set out to air dry)

21. Immerse in PBS for 15 minutes (wash)

22. Combine DAB components right before use

Add 50 μ L of DAB substrate 20x buffer to 950 μ L of deionized water

Add 50 μ L of DAB chromagen and 50 μ L of hydrogen peroxide 20x

Add 100 μ L of this DAB mixture to each slide and develop until there is a light brown background, approximately 20 minutes

Keep DAB components and mixture away from light and use mixture within 30 minutes

23. Rinse several times in deionized water

24. Mount slides with permount

Methyl Green Staining Procedure

1. Do steps 1 through 4 of TUNEL procedure

To do simultaneously with the TUNEL procedure, offset the Methyl Green slides by 8 minutes behind.

2. Immerse in deionized water for 5 minutes

3. Immerse in 2% Methyl Green for 45 seconds

4. Rinse in distilled water

5. Rinse in deionized water

6. Mount slides with permount

Proteinase k Reconstitution Procedure

Proteinase k comes in a powdered form and needs to be reconstituted with a proteinase k buffer solution.

Proteinase k Buffer:

100mM Tris-HCl (pH 8.0)

50mM EDTA

1. Micropipette 0.100 mL of 1M Tris-HCl (pH 8.0) into a 1 mL aliquot.
2. Micropipette 0.100 mL of 0.5M EDTA (pH 8.0) into the aliquot.
3. Bring the volume to 1 mL with deionized water.
4. Mix 10 mg of the proteinase k powder with 1 mL of the buffer.

This reconstituted proteinase k will be a 10mg/mL solution.