The Effects of Bisphosphonates on Bone Remodeling: Analysis of Microdamage Targeting by BMUs, BMU Velocity and Crack Surface Density

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By
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TITLE: The Effects of Bisphosphonates on Bone Remodeling: Analysis of Microdamage Targeting by BMUs, BMU Velocity and Crack Surface Density

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Abstract

The Effects of Bisphosphonates on Bone Remodeling: Analysis of Microdamage Targeting by
BMUs, BMU Velocity and Crack Surface Density

By Daniel Wayne Hale

Studies have indicated that microdamage in the bone matrix both activates and “steers” BMUs (Basic Multicellular Units, the groups of osteoclasts and osteoblasts that resorb and form bone respectively) toward the damage in order to replace damaged bone with new, mechanically sound bone. Also, bisphosphonate drugs have been shown to greatly suppress the remodeling process and, with such effects, are commonly used in the treatment of osteoporosis. It was hypothesized that BMUs do indeed target microdamage around them and tunnel away from the dominant lines of force in bone in order to remove the damage. Additionally it was believed that bisphosphonates would have a suppressive force on the BMU’s ability to seek out and remove microdamage. This study explored a further hypothesis that bisphosphonates would decrease BMU velocity and osteonal area while increasing crack surface density in a dose-dependent manner. Thirty-six rib bone samples were obtained from the Indiana University School of Medicine from a three year canine study in which skeletally mature beagles were administered the bisphosphonate Alendronate in doses matching, on a body weight basis, those used to treat postmenopausal osteoporosis. Control, clinical dose (Alendronate 0.2 mg/kg body weight), and five times clinical dose (Alendronate 1.0 mg/kg body weight) treatment groups were created with 12 dogs in each group. Before harvesting rib bones, the dogs were injected with calcein at two different time periods to mark areas of new bone formation. For this study, data for mean crack length, resorption space density, osteonal area, and crack surface density were obtained from

Matt Allen at the Indiana University School of Medicine. BMU velocity was determined by measuring the distance between fluorescent labels in longitudinal sections of bone and dividing by the time between injections. Statistical analysis supported the hypothesis that BMUs target microdamage and bisphosphonates suppress that ability in a dose dependent manner. Also, bisphosphonates have a significant suppressive effect on BMU velocity. Analysis shows that there is no difference in osteonal area or crack surface density among the three treatment groups (CON, ALN0.2, and ALN1.0) leading to the conclusion that bisphosphonates seemed to have little effect on osteonal area or crack surface density over the three year study.

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1. Introduction and Literature Review

Bone Tissue

The skeletal system of the human body is a highly adaptable and constantly changing structure. Bone density is under continuous optimization to produce the highest strength with the lowest amount of weight. There are two main types of bone, called compact (cortical) bone and trabecular (cancellus) bone respectively, which comprise a majority of the long bones that make up the skeleton (Figure 1).

Figure 1. Important features of a typical long bone including compact and trabecular bone (1)

Compact bone resides as the outer layer of a bone and is characterized by circular structures called osteons (Figure 2).

Figure 2. Osteons in compact bone (1)

A blood vessel runs through the central canal of each osteon, called a Haversian canal, and is responsible for providing nutrients and removing wastes from the surrounding bone cells [1]. Compact bone has a high fracture resistance and toughness due to the geometry of the osteon. Osteons are built up as oppositely oriented, concentric, circular matrices called lamellae. This geometry provides a high level of fracture resistance since the energy of approaching cracks is dissipated around the edge of the circular osteon. As the forces acting on an osteon increases, the circular layers begin to pull apart and delaminate before they begin to crack. A large amount of the energy contained in a crack is dissipated as an osteon “unravels”. This behavior gives cortical bone a fracture toughness of $2.2\text{-}6.3 \text{ MPa}\cdot\text{m}^{1/2}$, elastic modulus of 17.4 GPa , and a high tensile ultimate stress of 133 MPa [1]. Trabecular bone is found near the ends of long bones and is characterized by a sponge-like geometry with many interconnected struts (Figure 3).

Figure 3. Trabecular bone structure (1)

Trabecular bone is specialized for absorbing and dissipating the energy of impact on bone. The geometry of trabecular bone is optimized to support large loads and spread forces acting on bones. Due to their geometry, trabecular bone has an elastic modulus of 272 ± 195 MPa and an ultimate stress of 2.54 ± 0.62 MPa [1]. Through the center of long bones runs the medullary canal. This area is filled with bone marrow which contains undifferentiated stem cells that form components of the immune system, red blood cells, and bone cells.

Bone Cells

The arbitrators of bone turnover are osteonal bone cells; primarily osteoclasts, osteoblasts, osteocytes, and bone lining cells. Osteoclasts are multinucleated cells originating in the bone marrow. They arise from the fusion of multiple differentiating bone marrow cells. Their main function is to absorb bone through the use of enzymes. Existing bone is absorbed at the interface between the cellular membrane and the bone surface (brush border) created by numerous infoldings of the osteoclasts' plasma membrane [1] (Figure 4).

Figure 4. Multinuclear osteoclast (upper right) resorbing bone (lower left). CZ labels clear zones where the cell is sealed to the bone surface; RB labels the ruffled border where enzymes are released to break down bone; B labels the calcified bone matrix. (1)

Enzymes are released at the brush border that de-mineralize the bone matrix and then dissolve the bare collagen scaffold. In this way osteoclasts are similar to macrophages in that they absorb and consume bone. Osteoblasts are mononucleated cells that originate in the bone marrow. Growth factors influence the differentiation of bone marrow progenitor cells into osteoblasts [1]. Their main function is to generate osteoid tissue (un-mineralized matrix of Type I collagen) as they follow bone absorbing osteoclasts (Figure 5).

Figure 5. Osteoblast forming bone. The dark area at the bottom is mineralized bone. Lighter material is osteoid produced by the rough endoplasmic reticulum of the cell. A portion of a process of the cell protrudes into the lighter osteoid material. (1)

Osteoblast activation is contingent on osteoclast activation and their relative activities may be linked or unlinked. As more osteoid tissue is produced, osteoblasts become trapped in the developing matrix and become osteocytes. Osteocytes are connected to other osteocytes in the bone matrix through channels called canaliculi. Cytoplasmic processes of neighboring osteocytes form gap junctions through which nutrients and chemical signals can pass [1]. The network of communication between osteocytes is called the syncytium. Through this network, osteocytes can relay information governing apoptosis, increasing forces, and nutrient deprivation. Bone lining cells are present on the periosteal and endosteal surfaces of bone and arise from

osteoblasts on the surface of bone. They lie in a layer and regulate the movement of calcium and phosphate into and out of bone. Also, bone lining cells communicate with osteocytes and receive signals in response to high stress levels in the bone matrix. Upon signaling, the cells release hormones that activate quiescent osteoclasts and migrate from the bone surface to expose bone to active osteoclasts. In this way bone lining cells play a role in the modulation of the remodeling process. Bone lining cells are thought to inhibit reabsorption of bone as long as they cover the bone surface. The cellular unit involved in bone resorption and formation is called the Basic Multicellular Unit (BMU) (Figure 6).

Figure 6. BMU. Two multinuclear osteoclasts are visible on the right; osteoblasts are on bone surfaces on the left.
(1)

A BMU is composed of osteoclasts that are responsible for bone absorption followed by osteoblasts that deposit the calcified bone matrix. The osteoclasts form a cutting cone at the head of a BMU that creates a scalloped geometry in the bone matrix as the BMU tunnels. Behind the osteoclasts, osteoblasts form new bone in the open resorption space (Figure 7).

Figure 7. Osteoclasts (on the right) form the cutting cone of the BMU while osteoblasts (on the left) form new bone in the space created by the osteoclasts. BMU is traveling to the right. (1)

BMUs travel longitudinally through bone at a rate of $19.18 \pm 8.25 \mu\text{m}$ a day and are responsible for creating the osteonal structure of compact bone. Other studies have measured BMU velocities at about $40 \mu\text{m}$ a day [1].

Modeling and Remodeling

Bone turnover can be classified as two different processes: modeling and remodeling. During modeling, bone resorption and bone formation are unconnected and the overall morphology and shape of bone is changed. Remodeling links the actions of bone resorption and formation so that the morphology is unchanged while maintaining the mechanical integrity of bone. In remodeling, bone formation is preceded by bone resorption and the process keeps bone in constant state of homeostasis. The cellular unit of remodeling is the BMU.

Bone modeling occurs during childhood growth and allows for the “customization” of a bone’s shape with respect to the loading conditions acting on it [1]. As previously stated, modeling utilizes the disconnected processes of bone resorption and formation. During childhood growth, bones increase in length but also need to be modified to the correct geometry to support the loads placed on the skeleton. Bone may be removed or added in various locations to achieve optimal bone geometry. One example of modeling occurs in the metaphysis of bones, where bone is removed by osteoclasts to reduce bone diameter. In areas such as the proximal tibia, a widely flaring metaphysis is required so osteoclasts resorb bone on the periosteal surface of the

metaphysis to cut the shaft of bone direction under the growth plate down to size [1]. Another example of modeling is the adjustment of the curvature of bones during growth. Specific bones require a certain degree of curvature which is achieved by bone removal and formation on different sides of bone such that the cross-section “drifts” sideways relative to the ends of the bone [1]. The modeling process drastically decreases once skeletal maturity is reached while remodeling continues throughout life and becomes the main process through which bone is altered.

Remodeling can be initiated by increased forces and loads acting on bones. One theory of remodeling is that bone lining cells, located on the surface of bone, sense stress and strain during bone loading and communicate the increase in forces to osteocytes. Osteocytes in the location of increasing forces recruit osteoclasts to begin bone resorption at a specific location. Osteoclasts then begin to absorb bone following the major lines of force (stress and strain) followed by increased bone formation by osteoblasts. The increased forces on bone guide the osteoclasts by activating osteocytes present in the bone matrix which then signal to osteoclasts to begin bone resorption. In this way, bone is built up along the principle stress directions altering the bone’s morphology.

Remodeling is also believed to be initiated by the activity of osteocytes [1, 2, 4]. In this case, remodeling initiation relies on fractures and crack propagation in the bone matrix. Osteocytes are either disturbed by propagating cracks or sense the changes in stress distribution around them [1, 4, 5]. When a crack is formed, osteocytes in the area surrounding the crack undergo apoptosis and release a multitude of chemical signals as they die. A network of apoptotic osteocytes forms around the damaged bone and this network attracts active BMUs. The recruited osteoclasts begin to absorb bone in the area containing the cracks and fractures,

ultimately removing a majority of those structures from the bone matrix. Following the resorption performed by osteoclasts, osteoblasts deposit bone in the newly created cavity. As osteoblasts continue to generate osteoid tissue they become trapped in the bone matrix and become osteocytes. In this way, old, weak bone is replaced with new, mechanically sound bone complete with a syncytium of interconnected osteocytes.

Another activator of the remodeling process is when bone is in a state of disuse. In this case, bone experiences a low mechanical stress environment and BMU initiation occurs to remove unneeded bone. The skeletal system, like the rest of the body, attempts to operate at the highest efficiency possible and will remove metabolically demanding yet mechanically unnecessary bone.

The process of bone remodeling can be divided into six stages. The first stage is called the *activation stage* and it involves the recruitment of osteoclasts from precursor cells. Progenitor cells in bone are triggered to become osteoclasts and gather at a specific area to form the resorption cone or cutting cone of the BMU. The next stage of remodeling is called the *resorption stage*. Newly created osteoclasts begin to resorb bone by traveling longitudinally at a rate of $19.18 \pm 8.25 \mu\text{m}$ a day. Other studies have measured BMU velocities at approximately $40 \mu\text{m}$ a day [1]. The area of resorption is an ellipsoidal shape with an approximate diameter of $200 \mu\text{m}$. This diameter can vary between different BMUs. The third stage is called the *reversal stage*. In this stage the transition from osteoclastic bone resorption to osteoblastic bone formation takes place. The length of this stage is dependent on the lag time between osteoclast activation and osteoblast activation. Typically, the reversal and resorption stages together take about 30 days in humans. In a completed secondary osteon the cement line is called the reversal line because it denotes where bone formation began to take place in the BMU. The cement line is the boundary

around an osteon that separates the newly formed bone of the osteon and the surrounding, older bone. The fourth stage of bone remodeling is called the *formation stage*. In this stage, osteoblasts around the outer edge of the tunnel formed by osteoclasts begin to create the collagenous framework that bone is built upon. Osteoblasts build concentric lamellae at a rate of about 1-2 μm per day [1]. As the tunnel is filled, bone formation slows and a canal is left in the center of the tunnel. This central area, called a Haversian canal, is approximately 40-50 μm in diameter and contains an arterial blood vessel. The Haversian canal is necessary because osteoblasts and osteoclasts are living cells and need nutrients and a waste disposal system. Also, the blood vessel brings phosphate and calcium ions where they are needed in the bone matrix and provides nourishment for osteocytes. The next phase is called the *mineralization stage* and follows the formation of bone. Up to this point in the remodeling process, organic, unmineralized osteoid tissue is laid down. Within the first few days after the osteoid tissue is formed approximately 60% of the matrix is mineralized with hydroxyapatite [1]. This stage of mineralization happens quickly and is called primary mineralization. Secondary mineralization occurs for the next six months and mineralizes the remaining osteonal tissue. Because of the drawn out mineralization process, osteons comprised of new bone display different mechanical properties than osteons made up of older bone. The last phase of the remodeling process is called the *quiescence stage*. Once the bone resorption and formation processes are finished, the BMU “disbands” and osteoclasts and osteoblasts separate. Approximately 10-20% of the osteoblasts involved in the BMU transitioned into osteocytes while some become bone lining cells in the Haversian canal and others disappear entirely [6]. Osteoclasts leave the completed osteon and may join another BMU. This stage represents the normal activity of an osteon as it functions as a component of the bone matrix.

Osteoporosis

The process of bone replacement is fundamental in many life functions. Fracture healing is a major occurrence that initiates both bone modeling and remodeling. At the crux of osteoporosis lies a malfunctioning case of bone remodeling. Osteoporosis commonly affects postmenopausal women over the age of 50 [7]. The disease is characterized by the increasing porosity of bone and a decrease in bone's fracture toughness. As bone is remodeled, less bone is deposited after the resorption phase [7]. Osteoblasts exhibit decreased functionality while osteoclasts continue to function normally. This uneven activity causes the amount of bone present to continually decrease. Decreasing bone integrity is also seen in older individuals without osteoporosis. As people age, the number of active BMUs decreases and damage begins to accumulate [7]. Fracture risk increases due to decreases in the elastic modulus and toughness of bone and the increase of microdamage [1, 7]. Older individuals experience increasing levels of bone degeneration and daily activities are impacted.

Current treatment for osteoporosis includes supplementing the diet with vitamin D and calcium [7, 8]. Increasing specific vitamins and minerals in the diet helps to decrease bone loss by providing a wealth of available building blocks for bone. Another treatment method involves exercise utilizing resistance training. Exercise with weights increases the loading on bones and stimulates the modeling and remodeling processes to increase bone density. Care must be taken by individuals to avoid injury during weight training. A further method of treating osteoporosis involves the use of nitrogen-containing bisphosphonate drugs [8].

Bisphosphonates

The bisphosphonate class of drugs penetrates the bone matrix and contains chemical functional groups that inactivate, disrupt, and kill osteoclasts. Decreasing the action of osteoclasts also reduces the processes of bone resorption and bone formation. Initially after treatment with bisphosphonates, an overall increase in bone density is witnessed as the osteoblasts that were previously functioning continue to deposit bone [8]. The tradeoff to bisphosphonate use is that microdamage is allowed to accumulate in the bone structure due to the reduction of the remodeling process. Recall that remodeling replaces old, damaged bone with new, mechanically sound bone. As microdamage accumulates, the risk for fracture may increase as well. Alendronate, a common nitrogen-containing bisphosphonate, is given as osteoporosis medication under the commercial name Fosamax.

Nitrogen-containing bisphosphonates have a strong affinity for bone and not for other tissues due to their chemical makeup. Bisphosphonates have a characteristic structure seen in Figure 8.

Figure 8. Characteristic structure of bisphosphonates. All have a hydroxyl group on the carbon atom and vary only at the R group, which always contains a nitrogen atom. (8)

Bisphosphonates have a similar chemical structure to pyrophosphate which accounts for their attraction to calcium ions. The hydroxyl group on the central carbon atom provides a high

affinity for calcium circulating in the blood and at the bone surface. Also, the phosphorus-carbon-phosphorus backbone is highly resistant to biological enzymes that usually degrade foreign chemicals [8]. Bisphosphonates are metabolically inactive which allows them to circulate the blood stream and exit the body as the original molecule. Variation between bisphosphonates occurs at the R group attached to the carbon atom which always contains a nitrogen atom in either an alkyl or heterocyclic structure [8].

Nitrogen-containing bisphosphonates work by interfering with the enzymes utilized by osteoclasts during differentiation and bone absorption. Specifically, bisphosphonates inhibit farnesyl diphosphate synthesis (FPP synthase) [8]. FPP synthase is a crucial part of the metabolic pathway that activates small GTPases such as Rab, Rac, Ras, and Rho. These GTPases are signaling proteins that when activated regulate important processes and structural properties for osteoclast function, including morphology, cytoskeletal arrangement, vesicular trafficking, and membrane ruffling [8]. Vesicular trafficking and membrane ruffling are two main osteoclast functions that are essential to bone resorption. The ruffled border produced by osteoclasts at the bone surface is the site of demineralization and disintegration of the collagen matrix. A disruption in osteoclasts' vesicular movement means that the enzymes required for bone degradation will not be concentrated at the bone surface and thus will be ineffective. Also, the formation of an inadequate ruffled border will inhibit the ability of osteoclasts to resorb bone and may dislodge them from the bone surface [8]. The concentration of bisphosphonate medication given also plays a role in the degree of osteoclast activity suppression. Low concentrations inhibit the functional osteoclastic activities that involve the cytoskeleton such as vesicular trafficking and membrane ruffling [8]. Higher concentrations inhibit osteoclast differentiation and at concentrations nearing 100 μM osteoclast apoptosis is induced [8]. The majority of BMU

suppression occurs during the first few months of bisphosphonate treatment. In a study by Kimmel [8], osteoclast function was seen to decrease by 70-80% during the first months of treatment and then remain at that level for the entirety of the treatment. Once bisphosphonate treatment is stopped, the bone resorption rate increases back to pre-treatment levels.

The pharmacokinetics of nitrogen-containing bisphosphonates focuses on the drug's interaction with body systems. Upon oral administration of the drug, bisphosphonates begin to circulate in the bloodstream within a few hours. The drug is then partitioned, approximately equally, to the kidneys and skeletal system [8]. The hydroxyl group contained on the bisphosphonate has equal affinity for calcium ions circulating the blood and found on the bone surface. Bisphosphonates reaching the kidneys are eliminated from the body un-metabolized through the urine whereas the remainder of the drug is deposited to a certain extent on the skeleton.

Different skeletal areas show different affinities for nitrogen-containing bisphosphonates depending on the bone-remodeling activity occurring at the bone surface. The three bone regions to be discussed are resting, resorbing, and forming which are associated with the cellular activity of bone lining cells, osteoclasts, and osteoblasts respectively. At the resting surfaces, bone is covered with bone lining cells and thus has a relatively low affinity and retention rate for bisphosphonates [8]. Since the nitrogen-containing bisphosphonates are bound loosely there is a chemical gradient that favors their re-uptake into the vascular system within hours to days [8]. A large portion of bisphosphonates are processed through the resting surfaces due to the large area of bone classified as resting. The resorbing surfaces make up a smaller portion of the total bone surface area but process a large amount of bisphosphonates because calcium is being released from the bone surface into the bone fluids by the activity of osteoclasts. Osteoclasts liberate

calcium ions as they dissolve the bone matrix and produce a high local concentration of calcium that is available for chelating by bisphosphonates. As more bisphosphonates are attracted to the areas of bone resorption, the concentration of bisphosphonates at these surfaces increases to levels sufficient to deactivate osteoclast functions [8]. Once FFP synthase is inhibited, the osteoclasts cease functioning. If bisphosphonate treatment is halted the concentration of bisphosphonates at the resorbing surfaces will be removed within days to weeks ultimately reaching full removal to the blood and then the kidneys [8]. Forming surfaces have a high affinity for bisphosphonates too due to the high levels of calcium being affixed to osteoid tissue during mineralization. All the bisphosphonate at forming surfaces chelates with calcium ions and is then buried in the bone matrix [8]. The bisphosphonates will remain in the bone matrix until they are uncovered by resorbing osteoclasts and released into the bone fluid which circulates back into the bloodstream. Nitrogen-containing bisphosphonates buried in the bone matrix are considered biologically inert and have no interaction with any bone cells [8]. When bisphosphonates are released by resorbing BMUs they return to the bloodstream and are equally partitioned to the kidneys and skeletal system. In this way, bisphosphonates released from the bone matrix are able to affect osteoclast function, albeit at lower concentrations than the previous dose. Bisphosphonates uncovered during the remodeling process may also affect osteoclast function if their local concentration around the osteoclasts is high enough to disrupt FFP synthase [8].

Even though bisphosphonates specifically target bone tissue and are metabolically inactive, there are some side effects associated with their use. Acute inflammation of the esophagus and stomach lining is the most prevalent, yet easy to prevent side effect seen with bisphosphonate use. Standing or sitting for 30-60 minutes after orally taking the medication is

enough to avoid stomach and esophageal irritation. Another side effect is hypocalcemia or decreased blood calcium levels. This disease arises due to bisphosphonates' high affinity for calcium ions. Bisphosphonates circulating in the blood chelate calcium and render it unavailable for other functional uses. In some cases of bisphosphonate use renal impairment occurs due to the drugs passage through the kidneys as it is excreted.

Microdamage Targeting by BMUs

As previously mentioned, BMUs are initiated by cracks and damage within bone (Figure 9).

Figure 9. Microcracks in compact bone. Arrows point to cracks formed between osteons. (1)

This damage is caused by normal, everyday activities including walking and lifting objects. One theory is that BMUs reabsorb and deposit bone along the lines of force within bone. Another theory involving BMU movement during remodeling is that reabsorbing osteoclasts target microdamage within bone and shift their movement toward damaged areas [2]. Once the damaged bone has been reabsorbed, the BMU tracks back to the nearest dominant line of force in

the bone. In this way, bone is strengthened as cells deposit bone along the prominent lines of force and repair cracks and damage within bone.

Studies proposed that osteonal BMUs tunnel in alignment with the local principal stress directions [9]. The reasons for this are as follows. Osteocytes need a stress-driven fluid flow across their processes to survive and remain active [9]. In the absence of a sufficient stress field osteocytes become apoptotic and release a variety of signaling hormones to surrounding osteocytes and bone lining cells. These messenger chemicals are a combination of “come eat me” and “death announcement” signals that attract nearby osteoclasts and communicate with nearby osteocytes [2]. When BMUs are aligned with the principle stress directions, a region of very low stress is created slightly ahead of the BMU. This low stress region causes apoptosis of nearby osteocytes which then release the signaling chemicals to osteoclasts. The BMUs osteoclasts follow the changes in load direction as the apoptotic signal shifts. In this way the BMU stays aligned with the dominant stress directions in the bone.

Focusing on BMU activation and steering by microcracks, remodeling activities and BMU movement will be discussed. It can be assumed that a resorbing BMU will remove at least a portion of the crack that initiated it and potentially, portions of other cracks encountered during its movement. Even though only a portion of the crack will be removed, this is extremely beneficial in reducing the probability of crack extension because the stress required to propagate a crack is inversely proportional to the square root of its length [10]. With this assumption in place, the value of microcrack “targeting” in reducing fracture risk is apparent. In a study performed by Martin, mean microcrack lengths were plotted as a function of resorption space density for a group of male and female human femur specimens to calculate the average cross-sectional area of a BMU [2]. The value calculated for average cross-sectional area of a BMU was

$1.66 \times 10^6 \mu\text{m}^2$ which correlates to an osteonal diameter of $1400 \mu\text{m}$ (assuming circular osteons) [2]. This value is much larger than osteonal diameters found for human femurs by Moyle and Bowden [11] which ranged from 150 to $220 \mu\text{m}$. An osteonal diameter of $220 \mu\text{m}$ corresponds to an osteonal area of $0.038 \times 10^6 \mu\text{m}^2$ which is only 2.3% of $1.66 \times 10^6 \mu\text{m}^2$. From these calculations it can be observed that BMUs are resorbing bone as if they were much larger than they really are. Martin's calculations represented an "effective BMU area" and not the actual BMU area [2]. The effective area surrounding the trajectory of a BMU provides a region through which the BMU can steer in order to remove microdamage.

Martin [2] discussed the possibility of a larger effective BMU area by focusing on two questions:

- 1) How do a BMU's osteoclasts sense microdamage through several hundred μm of intervening bone matrix and direct their resorption toward it?
- 2) When signals for steering toward microdamage and steering in alignment with principle stress direction compete, how are they integrated or resolved?

In response to the first question, focus is placed on the signaling molecules Bax and Bcl-2. Bax is a molecule commonly expressed in cells undergoing apoptosis [2]. Bcl-2 is a molecule with a chemical structure similar to Bax but with the function of aiding in cell survival. Due to their similar chemical structure, Bax and Bcl-2 can form homodimers and heterodimers that play important roles in regulating osteocyte apoptosis. Homodimers of the Bax molecule result in mitochondrial structures that produce apoptosis and heterodimers of Bax and Bcl-2 protect against apoptosis [2]. The following figure, generated by Verborgt et al. [4], shows the distribution of cells exhibiting Bax and Bcl-2 with respect to their relation to microcracks (Figure 10).

Figure 10. Schematic diagram approximating osteocyte expression of Bax and Bcl-2 as a function of distance from a microcrack. The gray area represents the hypothetical $\sim 700\ \mu\text{m}$ radius of the effective osteonal area calculated by Martin [2]. Bax diminishes to zero over a distance of about 4 mm. Bcl-2 approximately doubles over a distance of 1.5 mm away from the crack and then decreases to zero at 4 mm. The solid line suggests a net expression of Bax and Bcl-2, i.e., the difference between the Bax and Bcl-2 signals. (4)

By examining the graph it can be seen that cells exhibiting Bax, apoptotic cells, reside closest to the microcrack with the density of such cells decreasing linearly toward zero over a distance of about four mm. Conversely, the density of cells exhibiting Bcl-2 starts low nearer to the microcrack and then doubles at a distance about 1.5 mm away from the crack. Then the concentration of Bcl-2 drops linearly to zero at about 4 mm away from the crack. It was concluded by Verborgt [4] that “targeting and guidance of bone resorption to sites of microdamage may be carried out not only by signals from apoptotic cells near damage sites, but also by signals from surrounding cells expressing apoptosis-inhibiting gene products”. These observations show that healthy cells residing significant distances away from microcracks are protected from apoptosis, and thus resorption, while cells nearer to microcracks are influenced to undergo apoptosis and thus amplify the signal for resorption. The solid line in the figure shows the relative values of Bax and Bcl-2 illustrating the protective and shielding nature of Bcl-2 as one moves further from a microcrack. The shaded region in the figure depicts the $700\ \mu\text{m}$

distance from a microcrack that can be accessed by a steered BMU. In this region the solid line increases in magnitude as distance to the microcrack decreases, showing that Bax is the dominant molecule influencing apoptosis of surrounding cells and signaling of BMUs.

The second question looks at the resolution between conflicting steering mechanisms of microcracks and the dominant lines of force in bone. As previously discussed, an area of low force develops in front of a tunneling BMU resulting in apoptosis of those osteocytes residing in that area. The apoptosis of these osteocytes attracts osteoclasts which home in on the region of apoptotic cells. When this apoptotic area in front of a BMU merges with the apoptotic region surrounding a microcrack, the osteoclasts are attracted to the center of the denser region of apoptotic osteocytes. The BMU is drawn closer to the center of this area where lies the microcrack. Thus, the target of the BMU shifts from the dominant line of force to the microcrack, its associated array of apoptotic osteocytes, and the chemical gradient produced by signaling molecules [2]. Once the BMU passes through the microcrack it will track back to the local stress field as its osteoclasts are attracted to the dominant loading patterns in the bone.

Martin concluded that the direction of BMU advancement is dependent on alignment with the principle stress directions and targeting the removal of damage [2]. The degree to which the trajectory of a BMU is altered to seek out and remodel microdamage is dependent on the ability of signals to attract a BMU's osteoclasts. Osteocytes and their messenger chemicals released during apoptosis are integral to this observation.

Hypothesis

The goal of this thesis is to investigate the relationship between bisphosphonate medications and the remodeling process to see if the remodeling-suppressing drugs have an effect on BMU steering and movement of BMUs. Measurements will be made on longitudinal

sections of canine rib bones to determine the distance between fluorescently tagged bands of new bone growth. These distances along with the known time between administrations of the fluorescent labeling will yield the velocity of the BMUs as they tunnel through bone. Data from Matt Allen's research group at the Indiana University School of Medicine along with measurements taken on the longitudinal bone sections will be utilized to determine the effects of bisphosphonates on BMU steering and BMU movement. Three hypotheses were formed from the general objectives:

1. Data will lend support to Martin's theory [2] that BMUs are steered towards microcracks by demonstrating that tunneling BMUs have an effective resorption area that is much greater than the actual area of an osteon.
2. Bisphosphonates will have the effect of decreasing the calculated effective resorption area of tunneling BMUs, and thus decrease their ability to steer, and the decrease in the effective resorption area is correlated to dose concentration.
3. Bisphosphonates will have the effect of reducing BMU tunneling velocity and measured osteonal resorption area (in a dose dependent manner) thus effecting a new equilibrium crack surface density, under the assumption of steady state damage formation and removal.

2. Materials and Methods

Experimental Design

Bone samples came from a 3-year study performed by the Indiana University School of Medicine involving the daily administration of bisphosphonate drugs to skeletally mature beagles [12, 13, 14]. All experimental procedures were approved by the Indiana University School of Medicine Animal Care and Use Committee. Female beagles (1–2 years old, $n = 36$) were confirmed to be skeletally mature (closed proximal tibia and lumbar vertebral growth plates on X-ray) prior to the start of the study [12]. Treatment involved daily, oral doses of vehicle (VEH, 1mL/kg saline) or Alendronate (ALN, 0.2 or 1.0 mg/kg; Merck, Rahway, NJ) administered by syringe. The lower dosage (0.2 mg/kg) corresponds to clinical levels of bisphosphonates used to treat postmenopausal osteoporosis while the larger dosage (1.0 mg/kg) corresponds to bisphosphonate levels used to treat Paget's disease [13]. Both dose sizes were prepared by mixing Alendronate in saline to produce a 0.05% (lower dose) and a 0.2% solution (higher dose). In each preparation, a correction was used to account for the 16.4% moisture content of Alendronate [12]. Dosing was performed each morning after an overnight fast and at least two hours prior to feeding.

To label newly formed bone, calcein (5 mg/kg as a 3% solution) was intravenously administered on a 2-12-2-5 labeling schedule preceding necropsy. The animals were killed by intravenous injection of sodium pentobarbital (0.22 mg/kg Beuthanasia-D Special; Schering-Plough, Union, NJ[AU1]) [12]. Immediately after death, the midpoint of the ninth rib was removed and saved in 70% ethanol for evaluation of microdamage. Also, an adjacent section of

the ninth rib was saved in 10% neutral buffered formalin for dynamic histomorphometric measurements.

Microdamage Analysis

In order to evaluate specimens for microdamage, bone samples were stained en bloc with 1% basic fuchsin and then embedded undecalcified in methyl methacrylate [12]. Two bulk stained samples per animal were used to assess microdamage using a semiautomatic analysis system (Bioquant OSTEO 7.20.10; Bioquant Image Analysis, Nashville, TN) attached to a microscope equipped with an ultraviolet light source (Nikon Optihot 2; Nikon, Tokyo, Japan). The possibility of finding no microdamage in any given specimen was reduced by analyzing two specimens per animal. Ultraviolet fluorescence was used to measure crack length (Cr.Le), crack number (Cr.N), calculations of crack density (Cr.Dn, Cr.N/bone area), and crack surface density (Cr.S.Dn, $[\text{Cr.N} * \text{Cr.Le}]/\text{bone area}$) in the cortex of the rib [18]. All variables were measured and calculated in accordance with American Society for Bone and Mineral Research–recommended standards [15]. Analysis was performed by Matt Allen’s group at Indiana University and results were obtained for subsequent calculations.

Sample Preparation

Bone samples were received embedded undecalcified in methyl methacrylate and stained en bloc with 1% basic fuchsin. Longitudinal sections were cut from each sample using a high precision bone saw (Isomet 1000; Buehler Ltd., Lake Bluff, IL). After cutting, the sections were sanded to a thickness of 100 micrometers using a sequence of 400 and 600-grit sandpaper and then mounted on microscope slides (Eukitt Mounting Medium; Electron Microscopy Sciences, Hatfield, PA).

BMU Velocity Measurements

The velocity of resorbing BMUs, V_{BMU} , was calculated by measuring the distance between fluorescent calcein labels and dividing that distance by the time between subsequent injections of calcein (labeling schedule). Slides were viewed using a fluorescent microscope (Olympus BX41; Olympus, Center Valley, PA) and ultraviolet filter (31001 FITC; Chroma, Rockingham, VT). The excitation and emission wavelengths of calcein are 495 and 520 nanometers respectively [16]. Calcein labels of new bone formation appear as parallel bright, yellow lines under ultraviolet light. Pictures of labeled BMUs were taken using imaging software (QCapture Pro; QImaging, Surrey, BC, Canada) and imported into image processing and analysis software (ImageJ; <http://rsb.info.nih.gov/ij/>). A ronchi ruling was used to calibrate the length measurements taken using ImageJ. Measurements were made using the method of Jaworski and Lok [17] on bone specimens from dogs receiving treatment for three years (Figures 11 and 12).

Figure 11. Double labeled BMUs under fluorescent microscope. In BMUs B and D the first and second labels are marked as I and II respectively. (17)

Figure 12. Schematic representation of a longitudinally sectioned BMU. The distance Q represents the distance the BMU traveled between tetracycline labeling injections. (17)

Jaworski and Lok viewed BMUs under fluorescent microscopy and calculated the distance between the end of the first fluorescent label and the end of the second fluorescent label (distance

Q in Figure 12). This method was repeated for the current study as can be seen in Figures 13 and 14. Figures 15 and 16 show two more examples of double-labeled BMUs that were measured in this study.

Figure 13. Double-labeled, longitudinally sectioned BMU. The BMU was traveling to the left. Labeling injections were given 12 days apart, where I represents the first label and II represents the second label.

Figure 14. BMU in Figure 12 with arrows representing the distances measured to determine BMU velocity.

Figure 15. Double-labeled longitudinally sectioned BMU with arrows representing the distance measured between labels for BMU velocity calculation.

Figure 16. Double-labeled longitudinally sectioned BMU with arrows representing the distance measured between labels for BMU velocity calculation.

The three treatment categories were control, low dose bisphosphonate (Alendronate 0.2 mg/kg body weight), and high dose bisphosphonate (Alendronate 1.0 mg/kg body weight). At least three double-labeled BMUs were located and measured for each longitudinally sectioned specimen and the measurements were averaged to yield the average distance for each specimen. The average distances for each specimen were divided by 12 (the number of days between labeling injections) to determine BMU velocity. The average velocities for each of the 12 specimens in each treatment group were averaged to determine the overall average velocity for each treatment group.

Histomorphometric Analysis

Measurements of mean crack length, Cr.Le, and mean BMU resorption cavity density, Rs.Ar, for the rib sections were made by Allen et al. and provided for further analysis. Using a model created by Martin [2] to describe BMUs traveling through bone containing microcracks, the cross-sectional area of a BMU can be calculated. According to Martin, in a section of bone with area A_T containing a crack with length L_0 , a BMU, with area A_{BMU} , tunneling at random through this section of bone will remove a fraction of the crack's length, ΔL . The portion of the

crack removed by the BMU can also be defined as $\Delta L/L_0 = A_{BMU}/A_T$ and the total length of the crack is then expressed by the following equation:

$$L = L_0 - \Delta L = L_0(1 - \Delta L/L_0) = L_0(1 - A_{BMU}/A_T) \quad [2] \quad (1)$$

If multiple BMUs, N_R , all with the same area, A_{BMU} , tunnel independently through the section of bone at random locations so that none overlap, then the crack length absorbed by the BMUs would be N_R times as great and the above equation can be re-written:

$$L = L_0\{1 - (N_R A_{BMU})/A_T\} \text{ or } L = L_0 - A_{BMU}L_0(N_R/A_T) \quad [2] \quad (3)$$

Changing to standard histomorphometric notation and noting that N_R/A_T is equal to the resorption spaces per unit area, $Rs.Ar$, the above equation can be written in the following form:

$$Cr.Le = iCr.Le - (On.Ar \times iCr.Le)Rs.Ar \quad (4)$$

Where $Cr.Le$ is the mean crack length, $iCr.Le$ is the mean initial crack length, and $A_{BMU} = On.Ar$, the cross-sectional area of individual osteons [2]. Taking values from multiple bone specimens and graphing mean crack length ($Cr.Le$) with respect to mean resorption space density ($Rs.Ar$) should yield a linear relationship for bone specimens where the slope divided by the intercept is equal to the mean osteonal area, $On.Ar$, of a resorbing BMU. $On.Ar$ represents the “effective” osteonal area and may differ from actual calculated osteonal areas if the BMUs travel toward and resorb microdamage that they wouldn’t normally pass through. It is assumed that a normally tunneling BMU will follow the predominant lines of force in a bone specimen and will resorb whichever microcracks the BMU happens to encounter along that path. If BMUs target microdamage then they will veer off of their normal path to pursue microdamage lying within a certain distance away from the guiding line of force before returning back to their original heading. As the BMU constantly weaves through the bone matrix resorbing microdamage and following the principal loading directions, its calculated effective osteonal resorption area will

appear larger than its measured osteonal area. Three graphs were created in order to calculate effective On.Ar for the three year study data consisting of a control bone treatment group, low dose (0.2 mg/kg) of Alendronate treatment group, and high dose (1.0 mg/kg) of Alendronate treatment group. Mean crack length and mean resorption space density data needed to perform these calculations were provided by Allen et al. and can be seen in Table 1.

Analyzing Effect of Bisphosphonates

The effects of bisphosphonates on microdamage targeting by BMUs were analyzed by statistically comparing their calculated effective osteonal resorption areas. As discussed above, the effective osteonal resorption areas were calculated for a control group, low dose bisphosphonate group (Alendronate 0.2 mg/kg body weight), and a high dose bisphosphonate group (Alendronate 1.0 mg/kg body weight). Significance between the groups was calculated to determine if there is a relationship between bisphosphonate dose size and the size of the effective osteonal resorption area. It is hypothesized that an increase in bisphosphonate dose corresponds to a decrease in the effective osteonal resorption area because as bisphosphonates suppress the action of BMUs the ability of BMUs to steer towards microdamage will also be suppressed.

The effects of bisphosphonates were also explored in an analysis of BMU tunneling velocity, osteonal area, and total crack length per unit area of bone cross-section. Assuming that BMUs do not target microcracks, they will remove whatever damage they encounter and their damage removal rate can be found by the following equation:

$$[d(\text{Cr.S.Dn})/dt]_{\text{removal}} = V.\text{BMU} \times \text{On.Ar} \times \text{Cr.S.Dn} \quad (5)$$

Where Cr.S.Dn is the total crack length per unit area of a bone cross-section (crack surface density) and V.BMU is the velocity of a tunneling BMU. This equation predicts that the crack removal rate is directly proportional to V.BMU, On.Ar, and Cr.S.Dn and will increase or

decrease with corresponding changes in the aforementioned variables. As the tunneling velocity of a BMU increases, it logically follows that more microdamage will be encountered and removed by the BMU. Introduction of bisphosphonates is assumed to suppress the BMU activation rate and subsequently decrease damage removal causing a decrease in tunneling velocity of BMUs and mean osteonal area. When damage formation and damage removal reach equilibrium in bone, equation 5 implies that damage formation is equal to $V.BMU \times On.Ar \times Cr.S.Dn$. This means that when $V.BMU$ and $On.Ar$ decrease due to the presence of bisphosphonates, $Cr.S.Dn$ may increase until a new equilibrium is reached. Increased $Cr.S.Dn$ ($Cr.S.Dn^E$, where the E stands for the new equilibrium value) may be expressed as:

$$Cr.S.Dn^E = \frac{[d(Cr.S.Dn) / dt]_{formation}}{V.BMU^T \times On.Ar^T}$$

Where $V.BMU^T$ and $On.Ar^T$ are the decreased BMU velocity and mean osteonal area respectively, caused by the administration of bisphosphonates. $V.BMU$ data was measured following the methods described by Jaworski and Lok [17]. Cross-sections of bone specimens were used to calculate mean $On.Ar$ and $Cr.S.Dn$. Osteonal area and microcrack length and number data were provided by Allen et al.

Statistics

To evaluate dependence of mean BMU velocity, mean osteonal area, and total crack length per unit area of bone cross-section on bisphosphonate treatment, a one-way ANOVA utilizing a Tukey posthoc comparison was performed using Minitab (Minitab Inc.; State College, PA). P-values less than 0.05 were considered statistically significant and represented significant difference between the treatment groups. The ANOVA was verified by performing a test for equality of variance and normality for each data set. Equality of variance testing utilizing

Levene's test statistic showed significance of equality for $p > 0.05$. Normality testing analyzed the data set's residuals using the Anderson-Darling method which showed normality for $p > 0.05$.

T-test analysis was performed for the equilibrium crack surface density values to look for equality between the equilibrium values and the measured values where the null hypothesis was that the equilibrium crack surface density was equal to the measured crack surface density.

Similar analysis was also performed for the measured On.Ar values for the CON, ALN0.2 and ALN1.0 treatments to see if the mean measured area was similar to the calculated effective osteonal resorption area. For each test, the null hypothesis was rejected for $p < 0.05$.

3. Results

Effective Osteonal Area

Using the equation developed by Martin [2]:

$$\text{Cr.Le} = \text{iCr.Le} - (\text{On.Ar} \times \text{iCr.Le})\text{Rs.Ar} \quad (1)$$

where Cr.Le equals mean crack length, iCr.Le equals mean initial crack length, On.Ar equals mean osteonal area, and Rs.Ar equals mean resorption space density, data was taken from Allen et al [12] and analyzed. See Table 1 for three year values of crack length and resorption space density organized by treatment category. Twelve samples for each of the three treatment categories – control, 0.2 mg/kg Alendronate, and 1.0 mg/kg Alendronate (CON, ALN0.2, and ALN1.0 respectively) – were measured by Allen et al. [12] to provide the data in Table 1.

Table 1: 3 year data by treatment for crack length and resorption space density.

3 year			
Sample #	Treatment	Cr.Le (mm)	Rs.Ar (mm²)
59210	CON	0.04487	0.77256
59211	ALN1.0	0.09284	0.67596
59219	ALN0.2	0.07609	0.40838
59230	ALN0.2	0.06811	0.71990
59231	ALN1.0	0.09103	0.47557
59234	CON	.	0.12996
59235	ALN0.2	0.06926	0.61030
59236	ALN1.0	0.06561	0.77620
59238	ALN1.0	0.06971	0.67416
59239	ALN0.2	0.06490	0.65326
59244	ALN1.0	0.05488	0.59295
59256	ALN0.2	0.06485	0.61265
59257	CON	0.05847	0.55057
59258	ALN0.2	0.07192	0.88063
59315	CON	0.06880	1.28135
59536	ALN1.0	0.06955	1.02281
59677	CON	0.04754	0.82046
60592	ALN0.2	0.06769	0.38463
60593	ALN1.0	0.07078	0.69665
60628	CON	0.06950	0.15427
60629	ALN0.2	0.07447	0.57732
60630	CON	.	0.68077
60631	ALN0.2	0.08154	0.65338
60632	CON	0.06957	0.29406
60634	CON	0.07113	0.13583
60636	ALN0.2	0.06506	0.15357
60641	ALN1.0	0.07902	0.12191
60642	ALN0.2	0.08264	0.22093
60643	ALN1.0	0.07534	0.46634
60644	CON	0.04548	0.74438
60645	ALN0.2	0.08629	0.14696
60657	ALN1.0	0.08190	0.63245
60667	CON	0.06471	0.73417
60707	CON	0.04920	0.00000
60750	ALN1.0	0.05774	0.25834
60809	ALN1.0	0.07237	0.25422

Using the data in Table 1 and Martin's equation, crack length (Cr.Le) was graphed with respect to resorption space density (Rs.Ar) for each treatment category as seen in Figures 16 - 18.

Figure 17. Crack length graphed with respect to resorption space density for control (CON) treatment. Effective osteonal resorption area is calculated by dividing the magnitude of the slope by the y-intercept.

Figure 18. Crack length graphed with respect to resorption space density for Alendronate 0.2 mg/kg (ALN0.2) treatment. Effective osteonal resorption area is calculated by dividing the magnitude of the slope by the y-intercept.

Figure 19. Crack length graphed with respect to resorption space density for Alendronate 1.0 mg/kg (ALN1.0) treatment. Effective osteonal resorption area is calculated by dividing the magnitude of the slope by the y-intercept.

Martin's equation assumes that the data follows a linear relationship, the slope of the line of best fit through the data represents the product of osteonal area and initial crack length, and the y-intercept of the line of best fit represents the initial crack length. By dividing the slope of the line of best fit by y-intercept and converting units to μm in Figures 1 - 3, the effective osteonal area is obtained. The effective osteonal area is then compared to the measured osteonal area for each sample as displayed in Table 2.

Table 2: Effective osteonal resorption area and actual osteonal area by treatment

3 year		
Treatment	Effective On.Ar (μm^2)	Measured On.Ar (μm^2)
CON	1.783×10^5	1.392×10^4
ALN0.2	1.441×10^5	1.135×10^4
ALN1.0	2.022×10^4	1.542×10^4

The three year data shows an effective osteonal area of 1.783×10^5 , 1.441×10^5 , and 2.022×10^4 μm^2 for the CON, ALN0.2, and ALN1.0 treatments respectively. Measured osteonal areas for the three year samples are 1.392×10^4 , 1.135×10^4 , and 1.542×10^4 μm^2 for the CON, ALN0.2, and

ALN1.0 treatments respectively. These effective areas correspond to osteonal diameters of 476.46, 428.34, and 160.45 μm for the CON, ALN0.2, and ALN1.0 treatments respectively while the measured osteonal areas correspond to osteonal diameters of 133.14, 120.23, and 140.11 μm with respect to the CON, ALN0.2, and ALN1.0 treatments. The three year data shows an effective osteonal area approximately 12.8 times as large as the measured osteonal area for the CON group and an effective osteonal area approximately 12.7 times as large as the measured osteonal area for the ALN0.2 treatment. The effective osteonal area is nearly similar to the measured osteonal area for the ALN1.0 treatment. The effective osteonal areas for the CON and ALN0.2 treatments are an order of magnitude larger than the effective osteonal area for the ALN1.0 treatment.

BMU Velocity

BMU velocity calculations were made for the three year samples by measuring the distance between double-labeled BMUs in longitudinal sections following the method of Jaworski and Lok [17] and then dividing the length measurements by 12 – the number of days between labeling injections. Velocity data for the three different treatments (CON, ALN0.2, and ALN1.0) can be seen in Table 3.

Table 3: BMU velocity, measured osteonal area, and crack surface density by treatment.

Sample Number	Treatment Group	V.BMU ($\mu\text{m}/\text{day}$)	Measured On.Ar (μm^2)	Cr.S.Dn ($\mu\text{m}/\text{mm}^2$)
59210	CON	21.8000	12671.280	10.074
59211	ALN1.0	23.1500	.	15.485
59219	ALN0.2	16.6400	12529.640	84.403
59230	ALN0.2	14.1000	.	14.251
59231	ALN1.0	4.9700	9261.140	67.963
59234	CON	26.8500	17875.933	.
59235	ALN0.2	20.6200	.	37.743
59236	ALN1.0	7.3400	.	29.717
59238	ALN1.0	14.8500	.	32.326
59239	ALN0.2	23.9500	6723.558	34.229
59244	ALN1.0	.	.	7.933
59256	ALN0.2	11.9800	.	19.804
59257	CON	34.5400	.	26.958
59258	ALN0.2	8.4500	9918.595	41.581
59315	CON	12.7400	.	38.130
59536	ALN1.0	4.3900	11499.070	50.578
59677	CON	10.0900	17174.090	9.740
60592	ALN0.2	8.3600	9570.580	74.703
60593	ALN1.0	5.4700	.	62.150
60628	CON	24.4100	.	83.539
60629	ALN0.2	10.8700	.	23.732
60630	CON	26.4500	.	.
60631	ALN0.2	15.9900	19004.800	91.402
60632	CON	12.1100	12011.710	5.515
60634	CON	21.9700	13245.650	66.902
60636	ALN0.2	6.1200	.	10.352
60641	ALN1.0	7.8800	15310.070	80.388
60642	ALN0.2	18.0700	.	48.916
60643	ALN1.0	4.8300	25273.850	34.993
60644	CON	12.4600	.	15.682
60645	ALN0.2	14.8000	10365.970	57.832
60657	ALN1.0	11.3500	15748.200	48.304
60667	CON	18.6000	.	4.614
60707	CON	7.4000	10553.058	11.845
60750	ALN1.0	21.9100	.	28.786
60809	ALN1.0	18.4600	.	30.544

Average BMU velocity is 19.12, 14.16, and 11.33 $\mu\text{m}/\text{day}$ for the CON, ALN0.2, and ALN1.0 treatments respectively (Table 4).

Damage Formation

Table 3 also contains measured osteonal area (On.Ar) and crack length per unit area of bone cross-section (Cr.S.Dn) data. Osteonal area and crack density measurements are provided by Matt Allen et al. BMU velocity, osteonal area and crack density are related to BMU damage removal by the following equation:

$$[d(\text{Cr.S.Dn})/dt]_{\text{removal}} = V.\text{BMU} \times \text{On.Ar} \times \text{Cr.S.Dn}. \quad (2)$$

Using the values from Table 3, the rate of damage removal for the control specimens is found to be $6605.66 \mu\text{m}^2/\text{day}$. Making the assumption that in a steady state situation damage removal is equal to damage formation, damage formation is also equal to $6605.66 \mu\text{m}^2/\text{day}$. Under treatment, with changing values of V.BMU and On.Ar, a new equilibrium crack surface density will be reached that is represented by the following equation:

$$\text{Cr.S.Dn}^E = \frac{[d(\text{Cr.S.Dn})/dt]_{\text{formation}}}{V.\text{BMU}^T \times \text{On.Ar}^T}. \quad (3)$$

Average values for the V.BMU, On.Ar, and Cr.S.Dn data present in Table 3 are shown below in Table 4.

Table 4: Average BMU velocity, average measured osteonal area, and average crack surface density by treatment.

Treatment Group	Ave. V.BMU ($\mu\text{m}/\text{day}$)	Ave. Measured On.Ar (μm^2)	Ave. Cr.S.Dn ($\mu\text{m}/\text{mm}^2$)
CON	19.118	13922	24.818
ALN0.2	14.163	11352	44.912
ALN1.0	11.327	15418	40.764

Using equation 3 and the damage formation rate determined for the control group, an equilibrium crack surface density was calculated for both the ALN0.2 and ALN1.0 groups. For the ALN0.2 and ALN0.1 treatments, the equilibrium crack surface density is 41.086 and $37.822 \mu\text{m}/\text{mm}^2$

respectively. Measured Cr.S.Dn values are 44.912 and 40.764 $\mu\text{m}/\text{mm}^2$ for the ALN0.2 and ALN1.0 treatments respectively as seen in Table 4.

Statistics

An ANOVA was performed to explore the relationship between actual osteonal area, BMU velocity, and crack surface density with respect to the three treatment groups. Results are found in Table 5 for the ANOVA using a Tukey posthoc comparison.

Table 5: ANOVA results for BMU velocity, measured osteonal area, and crack surface density by treatment

	CON	ALN0.2	ALN1.0	P
V.BMU ($\mu\text{m}/\text{day}$)	19.18 $\pm$ 8.25	14.16 $\pm$ 5.3	11.33 $\pm$ 7.13*	0.036
Measured On.Ar (μm^2)	13922 $\pm$ 2940	11352 $\pm$ 4185	15418 $\pm$ 6134	0.338
Measured Cr.S.Dn ($\mu\text{m}/\text{mm}^2$)	24.82 $\pm$ 27.38	44.91 $\pm$ 27.27	40.76 $\pm$ 21.56	0.157

Data are mean $\pm$ SD. P values refer to a one-way ANOVA among the three groups: * vs. CON

Individual p values for each Tukey comparison for measured On.Ar and Cr.S.Dn were all greater than 0.05 which is reflected in the overall p value reported in Table 5. The p values for the individual Tukey comparisons performed for BMU velocity are presented in Table 6.

Table 6: P values for individual Tukey comparisons for BMU velocity one-way ANOVA

Comparisons	P
CON and ALN0.2	.0922
CON and ALN1.0	.0119
ALN0.2 and ALN1.0	.3387

The ANOVA was verified by checking for equality of variance and normality for each data set. Equality of variance testing utilizing Levene's test statistic showed significance of equality for $p > 0.05$. Normality testing analyzed the data set's residuals using the Anderson-Darling method

which showed normality for $p > 0.05$. See Figures 19, 20, and 21 for graphical representation of the tests for equality of variance and normality.

Figure 20. Test for equality of variance and normality for V.BMU ANOVA.

Figure 21. Test for equality of variance and normality for measured On.Ar ANOVA.

Figure 22. Test for equality of variance and normality for Cr.S.Dn ANOVA.

The normality test for Cr.S.Dn has $p < 0.05$ which would usually signify a failure of normality but the deviations from normality, in this case, won't interfere with the conclusions drawn from the ANOVA of the Cr.S.Dn data. A clustering of data points in the Cr.S.Dn data set is causing the failure, but this clustering can be overlooked as inconsequential due to the nature of the data thus the ANOVA is valid [19]

T-test analysis was performed for the equilibrium crack surface density values to look for equality between the equilibrium values and the measured values. Similar analysis was also performed for the measured On.Ar values for the CON, ALN0.2, and ALN1.0 treatments to see if the mean measured area was similar to the calculated effective osteonal resorption area. Beginning with the ALN1.0 treatment area calculations, it was found through a one-sample t-test analyzing the measured On.Ar data, that the mean of the On.Ar data was similar to the calculated effective area with $p = 0.155$. The null hypothesis, that the mean of the On.Ar data was the same as the calculated effective area, was not rejected because $p > 0.05$ and the effective osteonal area is statistically similar to the measured osteonal area for the ALN1.0 treatment. T-tests for the CON and ALN0.2 groups showed that there was a significant difference between the effective osteonal area and the measured actual osteonal area. P values for these t-tests were both less than 0.001 resulting in a rejection of the null hypothesis. With respect to equilibrium crack surface density, the null hypothesis being tested was that the mean measured crack surface density was the same as the calculated equilibrium crack surface density for the ALN0.2 and ALN1.0 groups. T-test results yield p values greater than 0.05 for both the ALN0.2 and ALN1.0 groups. Thus it can be assumed that the equilibrium crack surface density values are equal to the mean measured crack surface density for both the ALN0.2 and ALN1.0 treatments.

4. Discussion

The objective of this study was to investigate the relationship between long-term bisphosphonate use (3 years), microdamage targeting by BMUs, BMU velocity, and crack density in canine rib bones. The canine model was selected for this study because the remodeling dynamics appear to be similar between human and canine cortical bone [17]. Bisphosphonate doses used in this research were administered on a milligram per kilogram of body weight basis in order to parallel common dosage levels used to treat postmenopausal osteoporosis in the clinical setting. Canines are also suitable for studies involving bone remodeling dynamics because they show evidence of osteocyte apoptosis and microcrack accumulation. The hypotheses under investigation are as follows: 1) BMUs are drawn toward microdamage in the bone matrix and effectively steer towards microcracks as they tunnel through bone along the dominant lines of force present in bone, 2) Bisphosphonates will have the effect of decreasing the calculated effective resorption area of BMUs, and thus decrease their ability to steer, in a dose dependent manner, and 3) Bisphosphonates will reduce the tunneling velocity and actual osteonal area of BMUs thus effecting a new equilibrium crack surface density, under the assumption of steady stage damage formation and removal. Data comes from samples supplied by Allen et al. [12] from the Indiana University School of Medicine. This study is unique in that it combines the canine rib model with bisphosphonate treatment spanning a three year time period. Previous long-term studies focused on vertebral remodeling or lasted one year [13, 14, 20, 21, 22, 23].

Microdamage Targeting

The three year study data supports the hypothesis that BMUs target microdamage and are steered by microcracks in the bone matrix. For two of the treatments (CON and ALN0.2) the calculated effective osteonal area is greater than the measured osteonal area. Following Martin's study [2], the results support his assertion that BMUs are drawn towards microcracks as they tunnel through bone following the dominant lines of force. In the case of the control group, the effective osteonal area is approximately 12.8 times as large as the measured osteonal area. At $1.39 \times 10^4 \mu\text{m}^2$, the measured osteonal area is 7.8% of the $1.78 \times 10^5 \mu\text{m}^2$ value derived from Martin's equations. The effective osteonal area corresponds to an effective osteonal diameter of $476 \mu\text{m}$, which is significantly larger than the $120 \pm 28 \mu\text{m}$ range predicted by Jaworski and Lok [17] for BMUs in canine cortical bone and the 150 to 220 μm range predicted by Moyle and Bowden [11] for BMUs in human cortical bone. The ALN0.2 treatment group also shows an increased effective osteonal area compared to the measured value. The lower dose Alendronate treatment (ALN0.2) exhibits an effective osteonal area of $1.44 \times 10^5 \mu\text{m}$ while the measured osteonal area is $1.14 \times 10^4 \mu\text{m}$, and the higher dose Alendronate treatment (ALN1.0) exhibits an effective osteonal area of $2.02 \times 10^4 \mu\text{m}$ while the measured osteonal area is $1.54 \times 10^4 \mu\text{m}$. For the ALN0.2 treatment, the measured osteonal area is 8% of the effective area value and yields an effective osteonal area that is greater than the measured osteonal area. Statistical analysis shows that the calculated effective osteonal area is significantly similar to the measured osteonal area for the ALN1.0 treatment indicating that microdamage targeting does not occur for the high dose Alendronate treatment. Only the CON and ALN0.2 treatment groups support the hypothesis that BMUs target and steer towards microdamage as they tunnel through bone during the remodeling process. These findings also support the assertions made by Burr that about 30% of bone

remodeling is targeted to the repair of microdamage [24]. The larger-than-measured effective osteonal areas demonstrate that BMUs are straying from their trajectories along the principle loading directions in bone to seek out microdamage. Although Burr's hypothesis regarding targeted repair of microdamage has yet to be experimentally or computationally proven, the present data lends support to the idea that remodeling targets microdamage.

The second hypothesis is addressed by observing the relationship between the effective osteonal area and bisphosphonate dosage. The presence of bisphosphonates seems to have a suppressive effect on the effective osteonal area. Li et al. [20] found that bisphosphonate treatment suppressed both targeted and nontargeted (stochastic) remodeling along with targeted repair of microdamage. Li's study administered both Risedronate and Alendronate bisphosphonates to skeletally mature beagles and assessed the association between cracks and resorption spaces. The study observed that there were fewer cracks associated with resorption spaces than expected in the Risedronate and Alendronate groups indicating that both targeted and non-targeted remodeling are suppressed. Their findings are supported by current results in that the calculated effective osteonal area of the ALN1.0 group is observed to decrease from that of the control group's. As stated above, the effective osteonal area of the CON group was 12.8 times larger than the measured area while the effective osteonal area of the ALN1.0 group was statistically similar to the measured area. In addition, the effective osteonal area for the CON group was a factor of 10 larger than that for the ALN1.0 treatment group. Bisphosphonate treatment at the high dose level caused a decrease in the ratio of effective osteonal area to actual osteonal area with respect to the CON and ALN1.0 groups. The difference in the ratio of effective osteonal area to actual osteonal area with respect to the CON and ALN0.2 groups was less decisive. The effective osteonal area calculated for the ALN0.2 group was 12.7 times as

large as the actual osteonal area while the effective area for the CON group was 12.8 times as large as the actual osteonal are. Statistical analysis shows that for both the CON and ALN0.2 groups, the calculated effective area is significantly different that the measured osteonal area. The ALN0.2 treatment seems to have little suppressive effect on the effected area while the ALN1.0 seems to have a large suppressive effect on the effective area. This observation was most likely the result of the dosage level of the bisphosphonate treatment. The data seems to indicate that suppression of the effective osteonal area doesn't occur at the lower bisphosphonate dose level but requires a dose approximately five times higher than clinical osteoporosis treatment levels to achieve the suppressive effect. This suppressive action is expected since the action of bisphosphonates is to inhibit BMU activation and the ability of osteoclasts to absorb bone. If the ability of osteoclasts to tunnel through bone is inhibited, then they won't be as able to steer towards microdamage. Suppressing BMUs in such a way decreases their effectiveness during remodeling and explains the decrease in effective resorption area seen in this study. These results support the hypothesis that bisphosphonates suppress the microdamage targeting ability of BMUs in a dose dependent manner. As mentioned above, one-sample t-test statistical analysis comparing the effective osteonal area to the measured osteonal area determined that the effective area for both the CON and ALN0.2 differ from the measured, actual osteonal area. The null hypothesis for the one-sample t-test is that the mean measured osteonal area is equal to the effective osteonal area. P values for the analysis involving the CON and ALN0.2 treatments were below 0.05 resulting in the rejection of the null hypothesis. T-test results for the ALN1.0 treatment led to the conclusion that the effective and measured areas do not different from each other. These results support the second hypothesis of this study and show that the effective osteonal resorption area is diminished with increased dose of bisphosphonate treatment. In

regards to BMU targeting of microdamage, it is implied that a decreased effective osteonal resorption area results in decreased ability to steer towards microdamage. The data showing that the effective area of the ALN1.0 treatment doesn't differ from the measured area further illustrates the decreased ability of BMUs to steer towards microdamage while under bisphosphonate treatment. If bisphosphonates didn't have an effect on BMU steering, it is expected that the effective osteonal area would match the CON group value in experimentation involving any dose of bisphosphonate treatment.

Actual Osteonal Area

Contrary to this observation of suppressed effective osteonal area, the measured osteonal area is similar between all three experimental groups. Even though targeted remodeling is being suppressed by bisphosphonate treatment, the actual resorption area of active osteons remains at normal values. Statistical analysis reveals that the mean measured osteonal areas are statistically similar for the CON, ALN0.2, and ALN1.0 treatments. While it was hypothesized that actual osteonal area would decrease with bisphosphonate use this is clearly not the case. The hypothesis was formed based on research examining the suppressive affects of bisphosphonates on the remodeling process. It was thought that inhibited BMUs would be unable to form osteons of normal size due to the inability of osteoclasts to properly resorb bone. Osteoclasts would be less effective at creating normal sized resorption cavities which would result in osteons with lower than normal areas after bone formation by osteoblasts. The analysis performed in this study refutes the hypothesis that bisphosphonate treatment reduces actual osteonal area in a dose dependent manner since the measured osteonal areas were shown to be statistically significant among all three treatment groups.

BMU Velocity

In a three year study by Allen et al. the toughness of bone was analyzed in relation to bisphosphonate treatment and microdamage accumulation. Under Alendronate treatment, bone toughness decreased without an overall accumulation of microdamage [12]. Bisphosphonate doses mirrored doses given in the present study, and significant losses in toughness were noted only for the ALN1.0 treatment group [12]. This result seems to parallel the decrease in the effective osteonal area calculated in the present study in that only the higher dose of bisphosphonate treatment has an effect on the scrutinized property. Across all three treatment groups in Allen's study (CON, ALN0.2, and ALN1.0) there was no significant difference in overall microdamage accumulation. Taking Allen's work a step further, the effect of bisphosphonate treatment on BMU velocity was explored in the study presented here. Average velocities for the 12 samples in the CON, ALN0.2, and ALN1.0 treatments were 19.12, 14.16, and 11.33 $\mu\text{m}/\text{day}$ respectively. Analysis revealed no significant difference in the average velocity for the CON and ALN0.2 treatments. Conversely, a significant difference in the average velocities of the CON and ALN1.0 groups was noted. Analysis comparing BMU velocity for the ALN0.2 and ALN1.0 groups reveals that they are statistically similar. It can be reasoned that high dose bisphosphonate treatment decreases BMU velocity by observing the significant difference between the CON and the ALN1.0 treatment groups. The lack of significance in velocity differences between the CON and ALN0.2 treatments may be due to a threshold effect with respect to bisphosphonate dose level. There may be a critical dosage level that once surpassed causes a significant decrease in BMU velocity from the control like that seen for the ALN1.0 treatment. The significance of the decrease in average velocity between the CON and ALN1.0 treatments shows a suppressive effect of bisphosphonate treatment at high dose levels.

Crack Surface Density

As part of the analysis of Allen et al. on the effects of bisphosphonates on bone toughness and microdamage accumulation [12], mean crack length, crack number, and crack surface density were calculated. Utilizing mean crack length, crack number, and crack surface density, Allen et al. [12] concluded that there were no significant differences in overall microdamage accumulation between the CON, ALN0.2, and ALN1.0 treatments although mean crack length was higher in both the ALN0.2 and ALN1.0 groups compared to the CON group [12]. The present study furthers the analysis of Allen et al. [12] by validating a model to predict equilibrium crack surface density values in an assumed steady state condition with respect to damage formation and removal. The third hypothesis is evaluated using the average values of BMU velocity, actual osteonal area, and crack surface density for the CON group to calculate the steady state value of damage formation and removal following the equations:

$$[d(\text{Cr.S.Dn})/dt]_{\text{removal}} = V.\text{BMU} \times \text{On.Ar} \times \text{Cr.S.Dn} \quad (1)$$

$$\text{Cr.S.Dn}^E = \frac{[d(\text{Cr.S.Dn})/dt]_{\text{formation}}}{V.\text{BMU}^T \times \text{On.Ar}^T} \quad (2)$$

In equations (1) and (2), V.BMU equals BMU velocity, On.Ar equals measured osteonal area, Cr.S.Dn equals crack length per unit area of bone cross-section, Cr.S.Dn^E equals equilibrium crack surface density, V.BMU^T equals the BMU velocity under treatment, On.Ar^T equals the osteonal area under treatment, and $[d(\text{Cr.S.Dn})/dt]_{\text{removal}}$ and $[d(\text{Cr.S.Dn})/dt]_{\text{formation}}$ equal damage removal and formation respectively. At steady state with respect to the remodeling process, it is assumed that due to normal BMU activity, the amount of damage formed in the bone matrix is equal to the amount of damage removal by the remodeling process. Using the steady state damage formation value and dividing by the treatment values of BMU velocity and actual osteonal area an equilibrium crack surface density was calculated for both the ALN0.2 and

ALN1.0 treatments. Equilibrium crack surface density values were 41.086 and 37.822 $\mu\text{m}/\text{mm}^2$ for the ALN0.2 and ALN1.0 treatments respectively. The measured values of crack density were 44.912 and 40.764 $\mu\text{m}/\text{mm}^2$ for the ALN0.2 and ALN1.0 treatments respectively. Statistical analysis comparing the calculated equilibrium values to their corresponding measured values showed no significant difference in the values. This conclusion supports the third hypothesis by validating the steady state crack density model. The equilibrium crack surface density values are equal to the measured crack surface density values as predicted, thus verifying equation (2). As V.BMU decreases, On.Ar and $[d(\text{Cr.S.Dn})/dt]_{\text{formation}}$ remain the same under treatment and the equilibrium crack surface density should increase above the control value. Statistical analysis determined that there is no significant difference in measured mean crack surface density between the control and Alendronate treatments. Current crack surface density data fails the Anderson-Darling normality test ($p < 0.05$), but the deviations from normality, in this case, won't interfere with these conclusions because of the insignificant clustering effect of the data [19]. It follows logically that as bisphosphonate treatment inhibits the remodeling process, the crack surface density will increase as damage accumulates until a new equilibrium is reached between damage formation and damage removal. This conclusion is supported by research performed by Allen and colleagues in a study where crack surface density was seen to increase over control values with the administration of bisphosphonates for a one year time frame [14]. Allen's one year study [14] analyzing the microdamage accumulation in canine vertebral bone found that crack surface density increased across all the Alendronate doses (ALN0.1, ALN0.2, ALN1.0) compared to the control group. The doses of bisphosphonates used in the study correspond to clinical treatment levels for postmenopausal osteoporosis, half that value, and five times that value (used to treat Paget's disease). Allen also noted that although bisphosphonate

treatment allowed significant microdamage accumulation in the vertebra, the detrimental effects were offset by increases in bone volume and mineralization such that there was no significant impairment of mechanical properties [14]. A later study by Allen and Burr examined the damage accumulation effects of bisphosphonates on canine vertebra over a three year period and compared the data to one year findings [13]. They concluded that while three years of Alendronate treatment resulted in higher vertebral microcrack density ($\#/mm^2$) than for one year of treatment, the amount of microdamage accumulation (determined by crack surface density, $\mu m/mm^2$) was not significantly different than the one year Alendronate treatment [13]. Allen and Burr reasoned that damage accumulation due to bisphosphonate use occurs early in treatment so there is little further damage seen after this period. Crack surface density in vertebral bone reported by Allen and Burr was not significantly different among the treatment groups. The current study's results seem to parallel Allen and Burr's findings as the steady state model predicts that equilibrium crack surface density is similar to measured values for both the ALN0.2 and ALN1.0 treatments over a three year period. Present data also supports and confirms Allen and Burr's results that there was no significant difference in crack surface density among treatment groups as seen from the results of the previously mentioned one-way ANOVA showing that there is no significant difference comparing measured crack surface density between the CON, ALN0.2, and ALN1.0 treatment groups.

Conclusions

This experimental study examined the theory of microdamage targeting by BMUs and evaluated the effects of bisphosphonate treatment on remodeling dynamics. The results and analysis aid in the understanding of the effective osteonal resorption area that is a result of BMU

steering. Data analysis also shows the dose dependent nature of bisphosphonates on remodeling dynamics. The main conclusions to be drawn from this study are reported below:

1. Current research has shown that BMU steering is a highly plausible explanation for targeted remodeling. Work done by Martin [2], concerning the calculation of an effective osteonal resorption area that is greater than the measured osteonal resorption area, is supported by this experimental study.
2. Bisphosphonates are seen in multiple studies to decrease the occurrence of bone remodeling thus allowing the accumulation of microdamage [12, 13, 14, 20, 22, 23]. While suppressing the remodeling process, bisphosphonates also suppress the ability of BMUs to steer towards microdamage as seen in the dose dependent decrease of effective osteonal resorption area.
3. Bisphosphonates also have a suppressive nature in regard to remodeling dynamics and decrease BMU tunneling velocity while leaving the actual osteonal area unaffected.
4. An extrapolation of Martin's model describing the effective resorption area of BMUs successfully shows that the new steady state equilibrium condition in damage removal and formation achieved under bisphosphonate treatment is a valid predictor of crack surface density.
5. Mean crack surface density for all three treatment groups (CON, ALN0.2, and ALN1.0) is not significantly different which supports results in previous studies examining the effects of bisphosphonate treatment on the remodeling process [12, 13].

Recommendations for Future Work

Currently, postmenopausal osteoporosis is a major disease affecting a majority of older women. While predominantly diagnosed in women, osteoporosis also affects older men. The main treatment for this disease is bisphosphonate medication meant to depress the remodeling process in order to halt bone loss. To date, the effects of long term bisphosphonate treatment in humans are not fully understood and complications involving microdamage accumulation may exist that outweigh the risk of fracture encountered when not taking bisphosphonate drugs. More long term studies concerning fracture risk associated with bisphosphonates are needed to better quantify the benefits of bone remodeling suppressing drugs.

There are many present theories that offer explanations for the activation and movement of BMUs as they tunnel through bone. The model explored in this study focuses on BMU steering by microcracks present in the bone matrix. Further studies that explore the effective resorption area demonstrated by Martin [2] can be used to add support or refute his theory. After observing the effects of microcracks on the effective resorption area of BMUs it would be beneficial to correlate osteocyte apoptosis or osteocyte signaling to explore their relationship to BMU steering.

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