IMPACT OF ANTI-RESORPTIVE TREATMENT ON RECOVERY OF BONE AFTER DISUSE

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Impact of Anti-Resorptive Treatment on Recovery of Bone After Disuse. (May 2015)

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Bisphosphonates (BP), drugs that inhibit bone resorption, are used to minimize bone loss in long-duration spaceflight, extended bed rest, and acute spinal cord injury; however, the long term impact of BP use on recovery of bone after disuse is not well understood. This experiment tests the hypothesis that the BP zoledronic acid (ZOL) will protect against loss of bone mass during 28 d hindlimb unloading (HU) while diminishing the ability of cancellous bone formation rate (BFR) to recover following HU. Male Sprague Dawley rats (6 mo) were assigned to aging control (AC), HU, and HU+ZOL groups and subjected to 28 d HU, then to 56 d weight-bearing recovery (REC). Rats were given 2 fluorescent labels 7 days apart to measure BFR in the final week of REC. Histomorphometric analyses of the proximal tibia and distal femur revealed that cancellous BFR was lower in ZOL+HU versus AC both immediately after HU (-96.6%) and after the recovery period (-99.9%) ($p<0.05$). However, quantitative computed tomography measures of cancellous volumetric bone mineral density (CN-vBMD) at the proximal tibia revealed that CN-vBMD was higher in ZOL+HU versus AC after HU (+120.0%) and after the recovery period (+125.5%) ($p<0.05$). These data indicate ZOL is a potent suppressor of bone formation as well as resorption. While ZOL effectively inhibited disuse-induced bone loss, the

prolonged suppression of BFR by ZOL after administration may be detrimental in long-term recovery of bone after disuse.

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CHAPTER I

INTRODUCTION

The effects of disuse on the skeletal system have been a topic of interest for many research studies. Disuse-induced bone loss occurs when bones in one specific area or throughout the entire body are not getting a regular amount of stress or pressure. Bone health is dependent on weight bearing physical activity [1]. With increased loading on the bone (i.e., resistance exercise), bones increase in mass and strength. On the other side of the spectrum, when not subjected to the proper amounts of stress, bones undergo atrophy, lose bone mass, and become brittle and increasingly susceptible to fractures. Disuse-induced bone loss occurs in many scenarios, including injuries requiring prolonged bed rest, broken bones that require casting, spaceflight, and even spinal cord injury.

Bone is one of the main constituents of the musculoskeletal system, along with skeletal muscle. Because of its rigidity and hardness, it enables the body to maintain its shape while protecting internal organs and serving as the primary load-bearing organ in the body. In addition, the bones provide attachment points for skeletal muscle allowing for locomotion. The chemical composition of bone, a combination of organic and inorganic components, is a major contributor to its great strength. The organic component of bone is composed of water, cells, and organic matrix, a system of collagenous proteins (90% of the organic matrix) and non-collagenous proteins. The inorganic component of bone is composed of hydroxyapatite, a combination of calcium carbonate, calcium phosphate, and calcium hydroxide. Bone tissue is divided into two types depending on its structure and location (Figure 1). Cortical bone is the dense, hard layer

that makes up the outer wall of bones and provides support and protection to the skeletal system. Cancellous bone, also referred to as spongy or trabecular bone, is the inner porous network of tiny rod-like structures, termed trabeculae, that provides support under compressive loads.

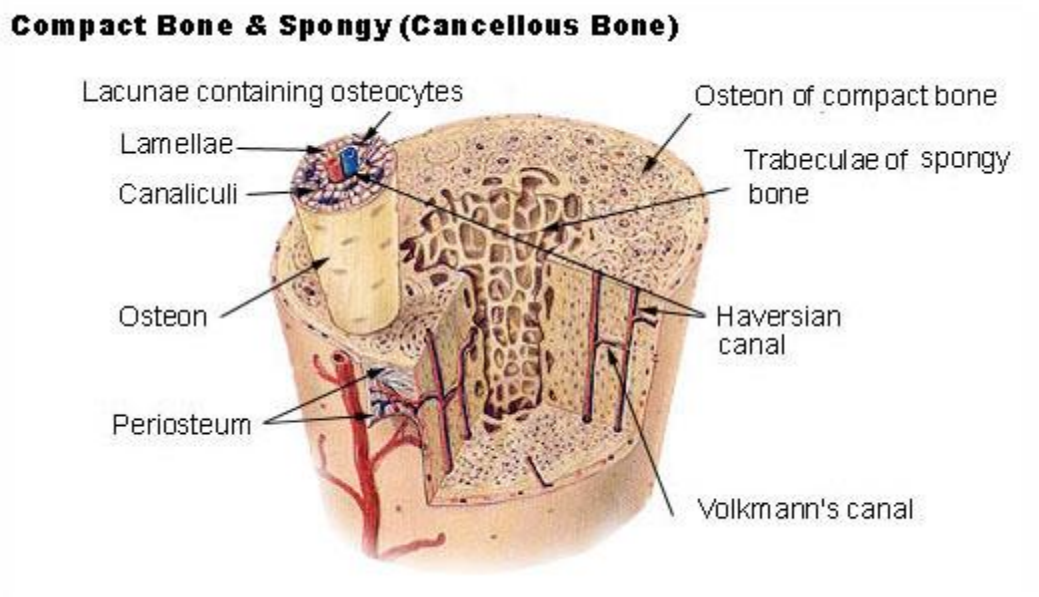


Figure 1. Illustration of the cortical and cancellous regions of bone [2].

Bone as an adaptive tissue changes in accordance with the loads placed on it and the local environment. Bone tissue has the ability to break down, rebuild, and heal itself. This process of breaking down and rebuilding bone is known as the bone remodeling cycle (Figure 2). This process is enabled by the activity of two cell types: osteoblast and osteoclast. Osteoblasts build bone by depositing new bone matrix that later mineralizes, while osteoclasts break down bone by resorbing existing mineralized bone matrix. The combined functions of osteoblasts and osteoclasts promote the formation and maintenance of healthy bone. The mechanical loading of bone can directly affect the remodeling cycle, allowing the bone to respond to changes (increases or decreases) in mechanical loading.

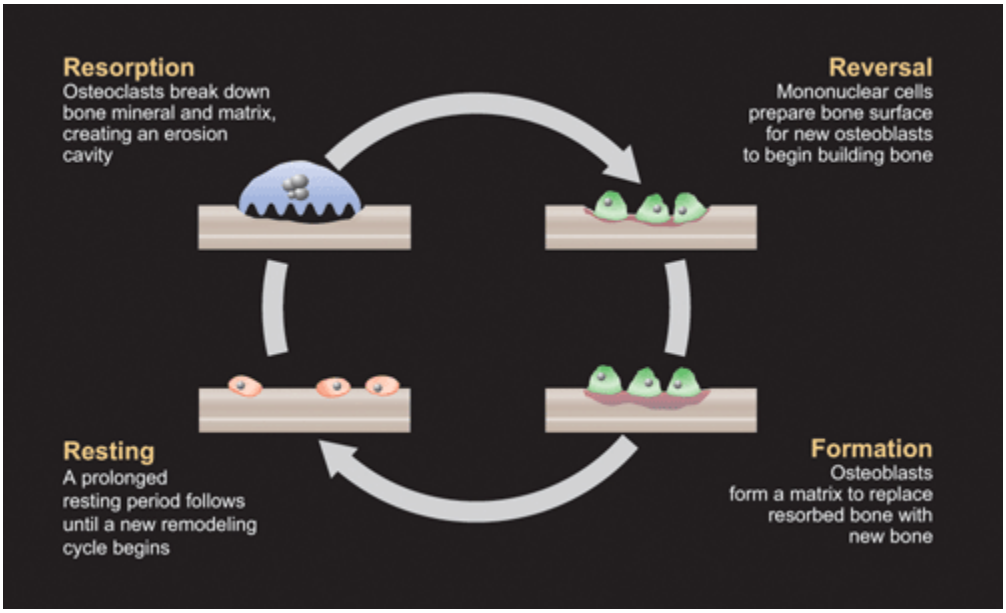


Figure 2. Illustration of the bone remodeling cycle [3].

Disuse-induced bone loss is a prominent concern in astronauts who undergo a significant loss of bone during long-duration space flight. While a lower bone mass is not dangerous while in the low gravity environment of space, this loss of bone becomes problematic once returning to the gravity of the earth. On earth we heavily depend on the force of gravity to develop and maintain strong and healthy bones. In space, astronauts cannot rely on the force of gravity to produce the mechanical loading necessary for the promotion of healthy bones. Even with vigorous daily exercise routines, astronauts still experience significant acceleration of bone resorption. Upon return from space flight, astronauts can have a higher risk for fracture than elderly individuals. International Space Station crew members experience a 1-2% decrease in bone mineral density (BMD) per month while in space [4]. This is a drastic loss of bone mass when compared to elderly post-menopausal women, the population with the highest prevalence of low bone mass and high fracture risk, who experience a decrease in BMD of less than 1% per year [5]. With the

increasing occurrence of astronauts making multiple trips to space and the potential for even longer missions to space, it is imperative to find effective methods to combat the deleterious effects of microgravity on the skeletal system.

While spaceflight provides a unique population of individuals, application of disuse-induced bone loss has practical implications on Earth as well, in patients such as fallen elderly or people suffering from chronic illnesses, paralyzed limbs, or spinal cord injury. Falls are the greatest cause of accidental death in the elderly population with a third of community-dwelling elderly and sixty percent of nursing home residents experiencing a fall yearly [6]. Fallen elderly become less mobile and bone loss becomes prevalent. Such bone loss only leaves subjects more susceptible to recurrent falls and fractures. In 2012, the medical costs for injuries sustained after falls in the elderly reached just over $30 billion [7]. With a rapidly aging US population, the prevalence of falls in the elderly, as well as the associated medical costs, will increase exponentially.

Fall-related fractures, spinal cord injuries, and chronic illness can all lead to extended periods of bedrest. Bedrest models in research studies are often used to simulate the microgravity conditions experienced during space flight. Patients experiencing extended bedrest experience a significant loss of bone in the spine and lower extremities [8]. Pharmaceutical treatment of bedrest patients can be critical as many bedrest patients are unable to perform exercise to help combat their loss of bone.

When bones are fractured, the disuse that occurs during the healing process causes a loss in bone as well as muscle mass. In adults, tibial fractures result in a significant loss of bone mineral density (BMD) at the fracture site and distal segment for at least 11 years [9]. This decreased BMD makes a subject more likely to suffer multiple repeat fractures in the future. In women recovering from hip fractures, there is a significant loss in BMD and lean body mass while there is an increase in body fat. These conditions lead to a higher chance of new fractures [10].

The use of pharmaceutical agents to treat the effects of osteoporosis, a condition with greatly decreased bone mass, on the skeletal system has been well documented; however, there has not been much research on the effects of these agents to prevent disuse-induced bone loss. Bisphosphonates, a class of osteoporosis drugs that inhibit bone turnover, have proven to be a useful treatment method. These bisphosphonates work by inactivating osteoclasts. The use of bisphosphonates, specifically alendronate and zoledronic acid, has relevance in any type of disuse, whether that is from extended bed rest, acute spinal cord injury, or prolonged space flight. As early as 1997, it was confirmed that alendronate decreased the amount of turnover in trabecular bone in patients in a disuse study [11]. In space life science research, alendronate has been used to combat the effects of microgravity on the skeletal system of astronauts during spaceflight; however, zoledronic acid, a bisphosphonate with greater affinity for bone than alendronate, has yet to be used during spaceflight. Preliminary reports presented at the American Society of Bone and Mineral Research (ASBMR) suggests that alendronate is effective in slowing bone loss in International Space Station astronauts. Clinical use of these drugs has been important for treatment of patients with acute spinal cord injuries. Alendronate given soon after

an acute spinal cord injury has been found to prevent the rapid loss in bone associated with such an injury [12].

It is well established that zoledronic acid is effective in the suppression of bone turnover, but there has yet to be a study to look at its effects during recovery from disuse. We aimed to examine whether or not the effects of zoledronic acid continue beyond a period of disuse. We hypothesized that zoledronic acid would protect against the loss of bone mass during a period of disuse and diminish the recovery of bone formation rate (BFR).

CHAPTER II

METHODS

Data from two different experiments was used for this thesis. The experiments had identical designs and treatments but had different end points. The first experiment (Exp 1), terminated animals immediately after a single bout of hindlimb unloading. The second experiment (Exp 2) terminated animals after the 56 day recovery period following hindlimb unloading. All animal procedures were approved by the Texas A&M Institutional Animal Use and Care Committee.

Animal and Experimental Design

Adult Sprague-Dawley rats were randomly assigned to 3 different groups: aging control (AC), hindlimb unloaded (HU), and hindlimb unloaded plus zoledronic acid (ZOL+HU). In Experiment 1, rats were anesthetized and terminated after 28 days of HU. In Experiment 2, rats were anesthetized and terminated after 56 days of recovery following the 28 day HU period (Figure 3).

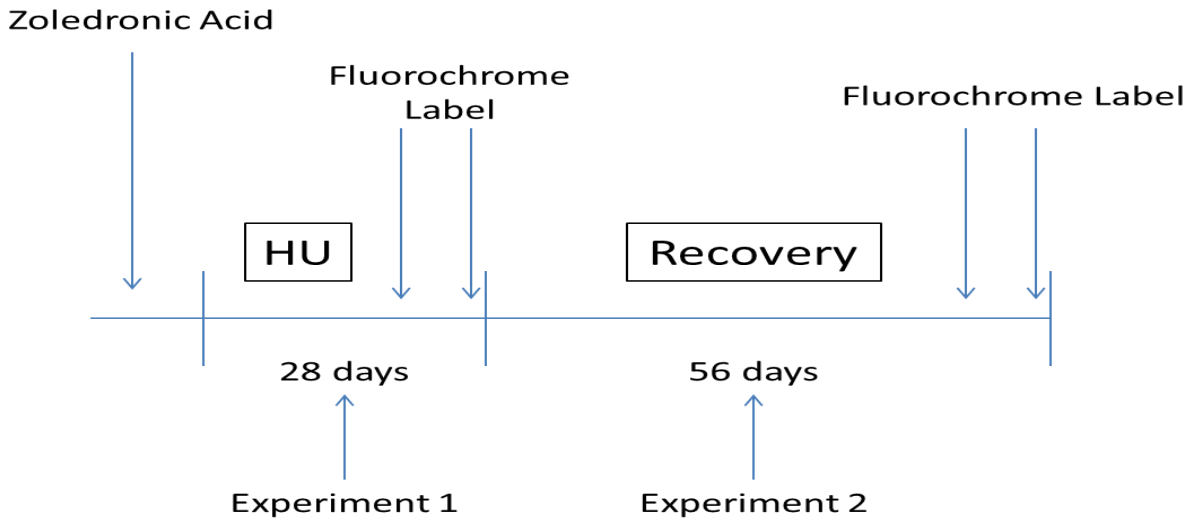


Figure 3. Timeline of experimental treatment for AC, HU, and ZOL+HU. This figure displays the experimental design throughout the duration of the project.

The AC group was allowed normal weight bearing activity in standard rat cages where food and water were provided *ad libitum*. The HU group was subjected to one 4 week (28 days) period of HU. In Experiment 1, animals were terminated immediately after. In Experiment 2, a 56-day recovery period of normal weight bearing followed the single bout of HU. The ZOL+HU group followed the same protocol as the HU group, with the addition of a single dose of zoledronic acid (60 µg/kg bodyweight) 7 days before HU. The drug was administered by intraperitoneal injection. The animals from Experiment 2 were subjected to a second HU exposure and euthanized on day 112; however, the data obtained for this study did not include any measures beyond the 56 day recovery period.

The fluorochrome label calcein was injected twice, 9 and 2 days before the animals were euthanized, to label the mineralizing surfaces in bone. Calcein has a high affinity for circulating calcium, hence it binds to newly mineralizing bone surfaces. The labels are used for histomorphometric analyses of bone formation rate after euthanasia of the animals. At the

experiment end point, the animals were anesthetized and euthanized by decapitation. The right tibia bones were cleaned of soft tissue and saved in 70% ethanol and stored at 4^0 C for histomorphometric analysis.

Hindlimb Unloading

HU was accomplished using the established tail suspension method [13]. By the use of a harness, the animal is suspended by the tail to prevent the animal from bearing weight on its hindlimbs. The model prevented any contact of the animal's hindlimbs with the floor or sides of the cage. The harness system creates approximately a 30^0 downward tilt to simulate the headward fluid shift achieved during space flight and bed rest studies. Through a harness attached to a pulley system above the cage floor, the animals could move about their cage and had free access to food and water. HU animals were monitored 2-3 times daily to ensure health, adequate food intake, and clean cages.

In Vivo pQCT

The proximal tibial metaphysis (4 slices distal to the growth plate to avoid primary spongiosa) was measured *in vivo* using a Stratec XCT Research-M device while the animals were anesthetized via inhaled isoflurane. Scans were performed using a voxel size of 100 μm and a scanning beam thickness of 500 μm. Scans were made at baseline and then every 28 days until the conclusion of the experiment. Cancellous volumetric bone mineral density (vBMD) was obtained; this does not include the more dense cortical shell of the metaphysis, but isolates cancellous bone, which is more quickly lost with reduced weight bearing.

Histomorphometry Analysis

Cancellous histomorphometry was performed on the harvested bones to determine mineral apposition rate (MAR), mineralized surface (MS/BS), and bone formation rate (BFR) from both Exp 1 and Exp 2 and all groups - AC, HU, and ZOL+HU. Undermineralized proximal tibia were serially dehydrated and embedded in methyl methacrylate. Eight micron sections were made and mounted unstained on slides. The histomorphometric analyses of fluorochrome labels were performed using an epifluorescent light-equipped microscope and the OsteoMeasure Analysis System, Version 1.3. Analysis of a defined region of interest was established within the endocortical edges approximately 500 μm distal to the growth plate creating an approximate area of $8mm^2$ at 20x magnification [14]. Single-labeled surface (sL.S/BS) and double-labeled surface (dL.S/BS; the interlabel width) were measured at 20x magnification. Each sample was verified for presence of label somewhere in the bone section (cancellous or cortical) to ensure proper delivery of the label in that particular animal. Therefore, the absence of label in the cancellous bone signified that there was no bone formation following binding of the label. Samples without any MS/BS were given the value 0 while samples with an MS/BS, but without an MAR were given the MAR value of 0.05 μm/d. This value was chosen because it is the lowest MAR seen in multiple studies with rats the same age and sex. Therefore, BFR values were calculated for samples with an MS/BS but lacking an MAR (BFR=MS/BS x MAR). Assigning this imputed MAR value created a bias toward underestimating the MAR and BFR for the imputed groups [15]. Histomorphometry nomenclature follows standard usage [16].

Statistical Analyses

A One-way ANOVA with repeated measures was performed on pQCT measures of cancellous vBMD (Exp 1 – pre- and post-HU, Exp 2 – post-HU and end of recovery). A Tukey post hoc test was used to measure differences between groups. The histomorphometry data was analyzed using a one-way ANOVA. If the $p<0.05$, an orthogonal contrast was performed to make direct comparisons between AC versus ZOL+HU and HU versus ZOL+HU. Statistical difference was set at $p<0.05$.

CHAPTER III

RESULTS AND DISCUSSION

Body Weight

The initial animal response to HU was seen as a change in body weight. During the first couple of days of HU, animals tend to eat less food until they acclimate to the treatment. An expected initial decrease in body weight was seen in the HU group and the ZOL+HU group as the animals acclimated to HU. Body weight in the HU group and the ZOL+HU group decreased during HU but was subsequently able to recover during the recovery period following HU in Exp 2. Treatment with ZOL had no effect on body weight or the animal's ability to recover during the period following HU (Figure 4).

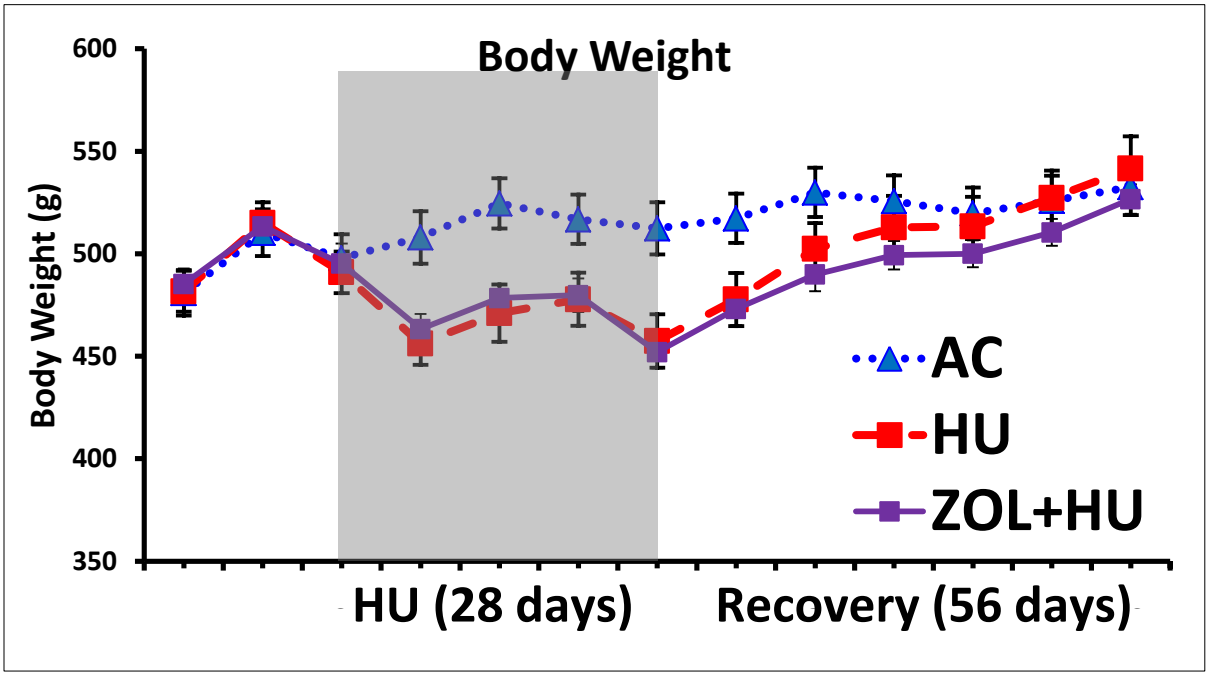


Figure 4. Body weight during hindlimb unloading (HU) and recovery therefrom. The experimental groups are as follows: aging control (AC), hindlimb unloaded (HU), and hindlimb unloaded plus zoledronic acid (ZOL+HU). Values represent the mean ±SE.

Cancellous vBMD is preserved during recovery with zoledronic acid before HU

Pre- and post-HU measurements of CN-vBMD at the proximal tibia were obtained using the tissues from Experiment 1 and % change was calculated. These results demonstrated that CN-vBMD in the HU group declined significantly over 28 days of HU ($p < 0.05$) (Figure 5). Following HU, the absolute CN-vBMD in the HU group was lower than both the AC and ZOL+HU groups ($p<0.05$). The data suggest that HU negatively affected cancellous bone mass. With the treatment of ZOL, bone cancellous mass was maintained throughout HU. ZOL prevented the negative impact of HU on cancellous bone mass.

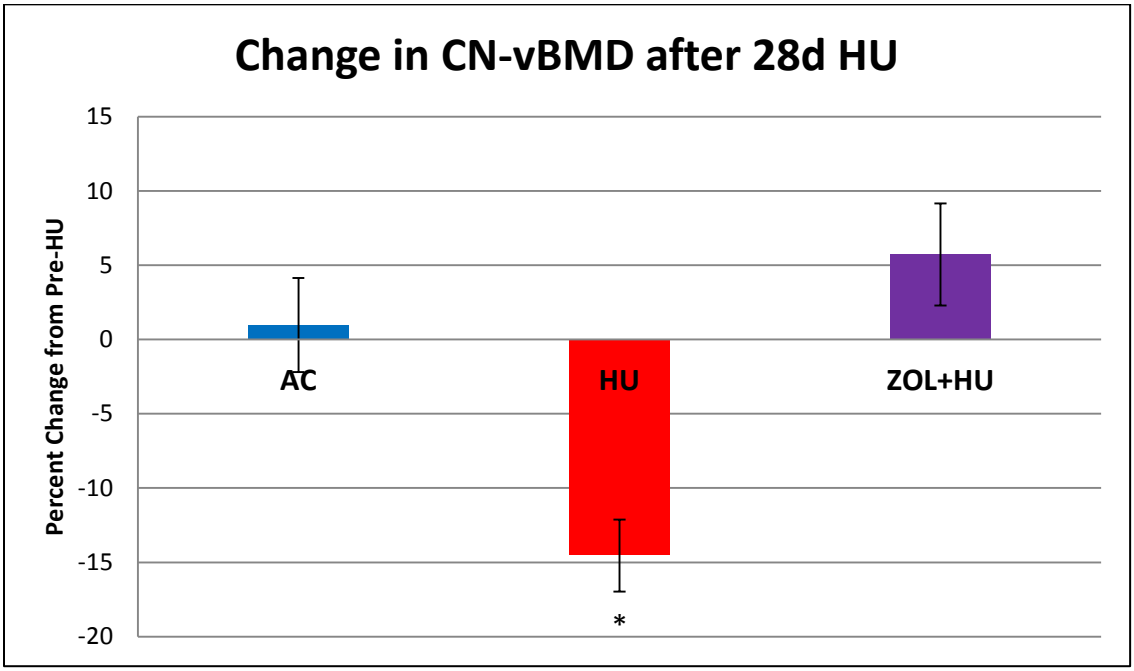


Figure 5. Change in cancellous volumetric bone mineral density (CN-vBMD) after 28 days of hindlimb unloading (HU). The experimental groups are as follows: aging control (AC), hindlimb unloaded (HU), and hindlimb unloaded plus zoledronic acid (ZOL+HU). Values represent the mean ±SE. Statistics were done on raw data. *Indicates a significant difference from the age-matched control and ZOL+HU, $p<0.05$.

Post-HU (start of recovery) and post-recovery CN-vBMD measurements were obtained using the tissues from Experiment 2. Post-recovery measurements at the proximal tibia found that absolute CN-vBMD in ZOL+HU was greater than both the AC and ZOL+HU groups ($p<0.05$) (Figure 6). Because BMD is strongly associated with mechanical strength of bone at the proximal tibia, we can surmise that ZOL continued to have effects on bone strength throughout the duration of the recovery period. Specifically ZOL treatment preserved cancellous bone during the recovery period.

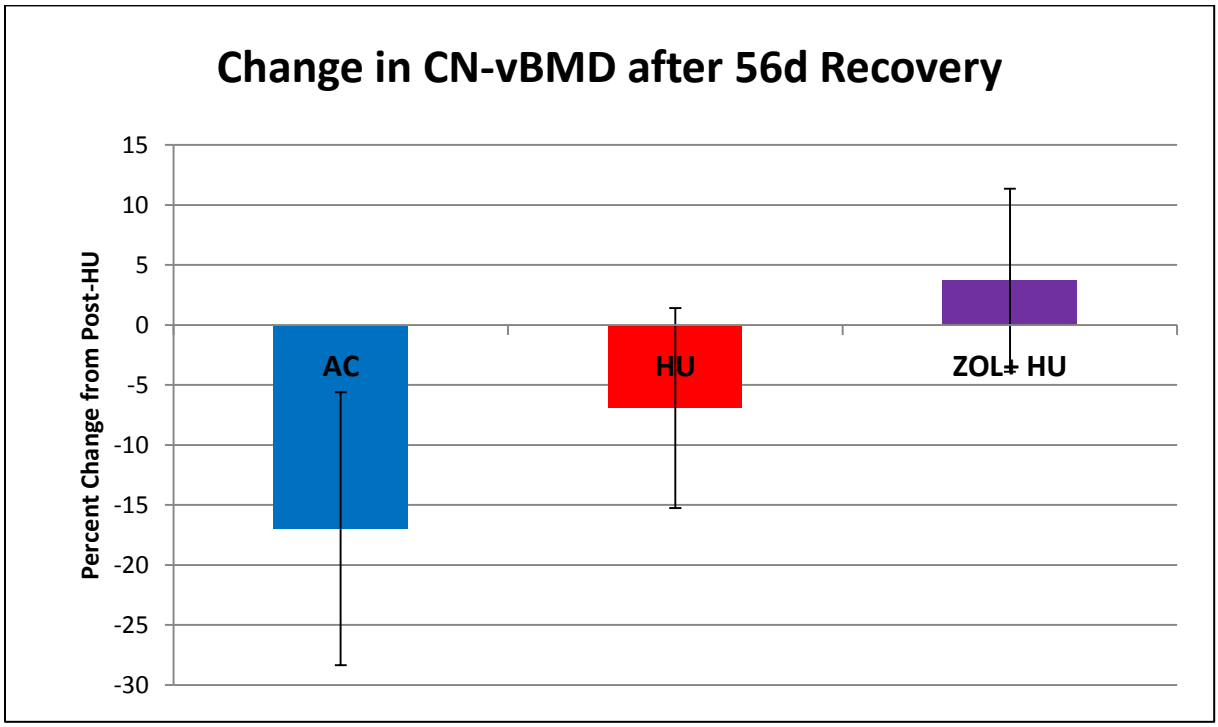
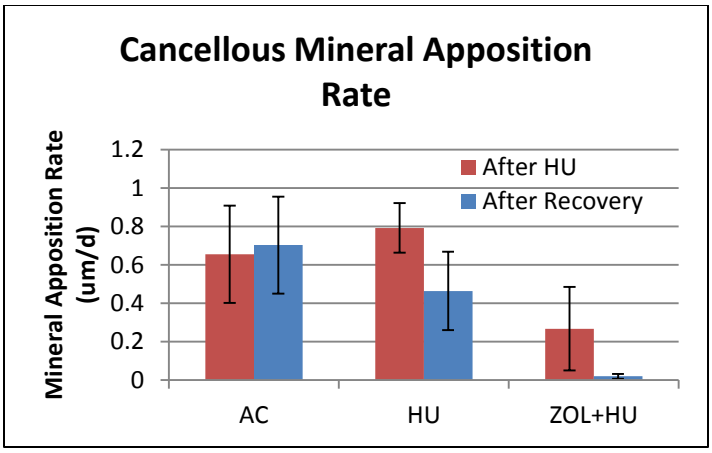
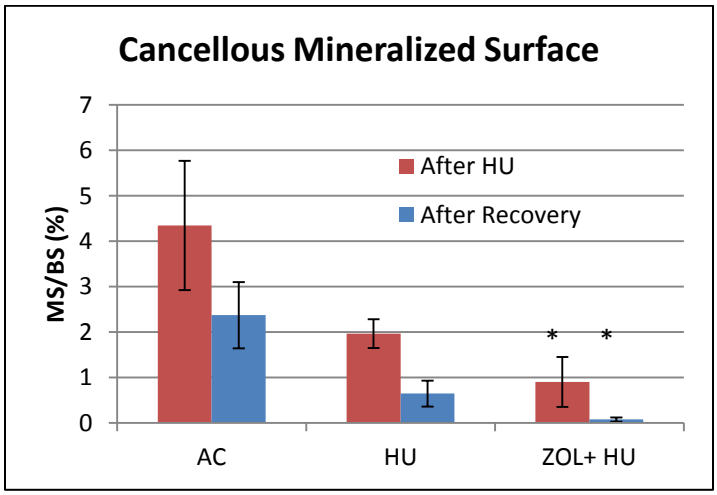


Figure 6. Change in cancellous volumetric bone mineral density (CN-vBMD) after 56 days of weightbearing recovery. The experimental groups are as follows: aging control (AC), hindlimb unloaded (HU), and hindlimb unloaded plus zoledronic acid (ZOL+HU). Values represent the mean ±SE. Statistics were done on raw data.

Bone formation activity is depressed during recovery with zoledronic acid before HU

Cancellous mineralized surface, mineral apposition rate, and bone formation rate were measured at the end of HU in Experiment 1 and at the end of the recovery period in Experiment 2. Measurements at both time points found that mineralized surface in ZOL+HU showed a significant decrease as compared to the AC (Figure 7). This suggests that ZOL caused a decrease in osteoblast number. Mineral apposition rate trended to show a decrease in ZOL+HU as compared to the AC ($p=0.06$). Since mineral apposition rate is a measurement of the average osteoblast activity, this trend might suggest that ZOL caused a decrease in osteoblast activity. Likewise, ZOL+HU trended to be lower than HU ($p=0.09$). Bone formation rate at both time points was significantly lower in ZOL+HU animals compared to the age-matched controls ($p< 0.05$). Zoledronic acid more potently inhibited bone formation rate than in the HU group. This significant inhibition of BFR was seen at both the end of HU and the end of recovery.





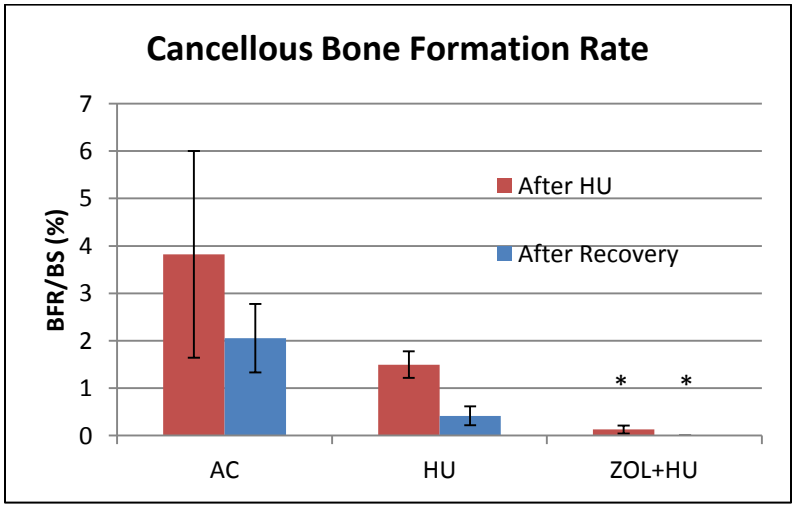


Figure 7. Indices of osteoblast activity after 28 days of hindlimb unloading (HU) and after 56 days of weightbearing recovery. The experimental groups are as follows: aging control (AC), hindlimb unloaded (HU), and hindlimb unloaded plus zoledronic acid (ZOL+HU). Values represent the mean ±SE. *Indicates a significant difference from the age-matched control, $p<0.05$.

CHAPTER IV

CONCLUSIONS

The purpose of this study was to determine the effects of the bisphosphonate zoledronic acid (ZOL) on bone formation rate at the end of a period of hindlimb unloading (HU) and following a subsequent recovery period. The hypothesis that ZOL would protect against the loss of bone mass during HU was supported by our data as the zoledronic acid-treated HU group (ZOL+HU) showed a positive percent change in cancellous vBMD during HU that was statistically different from the HU alone group. The treatment of ZOL fully preserved bone mass during HU. This suggests that ZOL may be an effective treatment to mitigate bone loss during long-duration disuse. The hypothesis that ZOL would diminish the recovery of bone formation rate (BFR) following HU was also supported by our data, as BFR at the end of recovery was significantly lower in ZOL+HU than AC. ZOL proved to be a potent suppressor of bone formation, as it had a persistent effect throughout the duration of the experiment.

Bisphosphonates suppress bone loss by inactivating osteoclasts. This inactivation causes a stop in bone resorption. Since the bone remodeling cycle is a coordinated effort, the absence of bone resorption causes a decline in osteoblast activity and, therefore, bone formation. This sequence of events stops the bone remodeling cycle. Therefore bone is not breaking down, but at the same time no new bone is being built. This was seen in the data presented, as zoledronic acid-treated group had little to no BFR both after HU and after recovery. ZOL appears to be a potent suppressor of BFR. Clinical use of ZOL has found that a single infusion can treat osteoporotic bone loss in postmenopausal woman for up to a year [17]. Though ZOL can prevent bone loss

during a period of disuse, there may be lingering effects upon the return to normal weight bearing, a situation astronauts or those recovering from bedrest would experience. The high potency of ZOL may render weight bearing activities futile while trying to restore bone health after an extended period of disuse. This may contribute to prolonged periods of no bone remodeling.

The long lasting effects of ZOL on BFR may prove to be problematic. Bone is a dynamic tissue that is constantly being broken down and rebuilt. Bone remodeling is necessary for the maintenance of strong, healthy bone. When this normal adaptive process is inhibited, there is no mechanism promoting normal turnover replacing older tissue with new mineralized bone. This may suggest a higher risk of damage to bone, or inability to repair any damage incurred, even though ZOL is protecting against bone loss. If a fracture is to occur while ZOL is in the system, the bone may be unable to heal effectively because BFR is suppressed.

There are clear positives and negatives for the use of ZOL in cases of disuse. A major beneficial effect is the preservation of bone mass during disuse. A negative influence is the nearly complete suppression of bone turnover. Future long term studies should seek to answer whether the use of ZOL has more long term benefits than detriments. A potential future study could look at the mechanical strength of bones from ZOL-treated animals that have undergone a period of disuse followed by a recovery period and examine multiple time points in the recovery to determine the time period needed for the animal to completely metabolize ZOL. This would provide more evidence about the long term efficacy and risks of using ZOL during long term periods of disuse.

In conclusion we found that zoledronic acid mitigated bone loss during a period of disuse. We also found that zoledronic acid was a potent suppressor of bone formation rate during a period of disuse and throughout a subsequent weightbearing recovery period. Although zoledronic acid protected against disuse induced bone loss, it prevented the recovery of bone formation rate in the long term. When translated to human patients, the use of zoledronic acid may prevent long-term disuse related bone loss. However, because ZOL is very potent, use of the drug may continue to suppress bone remodeling once returning to normal weight bearing. For example, a patient that was treated with ZOL to decrease bone loss during physician mandated bedrest due to a prolonged period of illness. ZOL may be effective in mitigating bone loss during disuse. However, after the period of bedrest, ZOL may continue to inhibit bone formation rate affecting the patient's ability to replace older tissue with fresh bone.

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