OSTEOGENIC EFFECT OF ELECTRIC MUSCLE STIMULATION AS A COUNTERMEASURE DURING HINDLIMB UNLOADING

A Thesis

by

JUSTIN DOW ALCORN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

May 2006

Major Subject: Mechanical Engineering

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Approved by:

Co-Chairs of Committee,	Harry Hogan
	Susan Bloomfield
Committee Members,	Arun Srinivasa
Head of Department,	Dennis O'Neal

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ABSTRACT

Osteogenic Effect of Electric Muscle Stimulation as a Countermeasure during Hindlimb Unloading. (May 2006)

Justin Dow Alcorn, B.S., The University of Texas at Austin

Co-Chairs of Committee: Dr. Harry Hogan
Dr. Susan Bloomfield

Rats that undergo hindlimb unloading (HU) as a simulation for space flight experience bone changes similar to astronauts in microgravity. The purpose of this research was to assess whether an exercise countermeasure would be effective in preventing or mitigating bone degradation during HU. Controlled electrical muscle stimulation was applied to the lower left hindlimb to simulate resistive exercise.

Adult 6-mo. old male rats were assigned to 3 groups of 12 each: hindlimb unloaded (HU), aging cage control (CC), and baseline (BL). The CC group was pair-fed to match the nutritional intake of HU animals during the 28 days of the study. The left leg was exercised 3 days a week for the duration of the study, with the unexercised right leg serving as a contra-lateral control. Mechanical tests were conducted to assess the strength of cancellous bone in the proximal tibia metaphysis. Although isolated specimens of cancellous bone are not feasible, reduced platen compression (RPC) was employed to directly load only the cancellous core region of each specimen.

There was no significant difference in ultimate stress or elastic modulus between BL, CC, and HU-Ex (exercised). However, HU-Ex results were dramatically and significantly higher than HU-No Ex (contra-lateral unexercised control) for both ultimate stress (68%) and elastic modulus (81%). It is also notable that ultimate stress was 32% higher (but not statistically significant) for HU-Ex compared to CC. The total bone mineral density in the tibial metaphysis was significantly larger, 11%, in the HU-Ex compared to the HU-No Ex group's values. The results clearly demonstrate the efficacy of the exercise protocol in preventing the substantial mechanical deterioration induced by HU.

ACKNOWLEDGEMENTS

I'd like to thank Jan Stallone for teaching proper lab technique and procedures. Brent Vyvial was tremendously important as a constant figure during sacrifice. Thank you Mats Nelson and Josh Swift for help harvesting tibia. Dr. Harry Hogan and Dr. Susan Bloomfield, thank you for your guidance and patience.

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1. INTRODUCTION

1.1 Problem

Skeletons adapt to environmental changes such as exposure to microgravity. The smaller applied loads during long-term space flight cause the skeleton to become less robust. Varying levels of bone loss have been observed in astronauts and cosmonauts after space flight. Soviet crew members on the MIR space station experienced an average loss of 0.3% bone mineral density (BMD) per month, primarily lost from the pelvis and legs [(1)]. Urine calcium levels increase 33% after 84 days of space flight [(2)]. Combating bone degradation is a concern when planning a long term space mission.

Subjects exposed to prolonged periods of microgravity develop osteoporosis. Osteoporosis is defined by the World Health Organization as a BMD of 2.5 standard deviations or more below the young adult mean [(3)]. The American College of Sports Medicine has summarized the effects of physical activity on bone mass. The effects of weight bearing exercise are localized to the loading site and the training stimulus must exceed the loading experienced in daily activity [(4)]. For a mechanical force to have an osteogenic effect, the stress must be unique, variable, and dynamic [(5)]. A study following post menopausal women observed a change in whole body BMD percentage of 1.6-2 percent and the change in lumbar BMD percentage of 1.5-1.8 percent over one year of weight bearing exercise when compared to the control [(6)]. The positive affects of weight bearing exercise are reversible. Positive effects on BMD are observed with high intensity resistance exercise, the gains are not preserved after the exercise is discontinued [(5)]. The development of an anabolic exercise protocol may attenuate bone loss due to disuse. The mechanical integrity of bone decreases during disuse [(7)]. The mechanical integrity of bone is characterized by changes in BMD, elastic modulus, and ultimate strength.

This thesis follows the style of the Journal of Bone and Mineral Research.

1.2 Objectives

Rats that undergo hindlimb unloading (HU) as an animal model for space flight experience bone changes similar to astronauts in microgravity [8]. The purpose of this research is to explore the potential beneficial effects of resistance exercise as a countermeasure to bone loss during HU in adult male rats. In this study, "exercise" is provided by electrical stimulation of the lower hind limb muscle using an established protocol to produce high force eccentric contractions. A microgravity environment affects cancellous bone more than cortical bone in rats [9] and in humans [10]. Thus the primary region of interest is cancellous bone in the proximal tibial metaphysis which exhibits significant decrements in BMD of 28 days in adult rats [9]. The main hypothesis is that exposing animals to high force muscle contractions three days a week during 28 days of hind limb unloading will mitigate losses in total BMD, elastic modulus, and ultimate strength of the proximal tibial metaphysis when compared to the non-exercised HU contra-lateral limb control.

The cancellous bone mineral density (BMD) was measured by peripheral quantitative computer tomography (pQCT). In previous studies [9], pQCT analysis of HU animals demonstrated a 21% decrease in cancellous BMD at the proximal tibia when compared to cage control (CC) after 28 days. Reduced platen compression (RPC) was used to test mechanical properties of cancellous bone that is still attached to the cortical shell. Does the addition of high force eccentric muscle contractions affect the BMD, elastic modulus, and ultimate stress of bone in the tibial metaphysis during hind limb unloading, when compare to the non-exercised contra-lateral limb control? This quantitative assessment of material properties (elastic modulus and ultimate stress) coupled with the BMD measurements by pQCT provides a comprehensive characterization of bone properties.

2. BACKGROUND

2.1 Bone Structure

The skeleton provides protection for the body's organs and load transmission by the body's long bones. The bones of interest are the long bones, such as the tibia or femur, because of the increase in fractures associated with osteoporosis. Long bones consist of trabecular (spongy bone) at the bulbous ends and cortical(hard bone) as the shell (Fig.2.1). The cancellous bone behaves like a shock absorber, absorbing energy from impacts. The trabeculae latticework of rods and plates are between 100-150 μm thick. The trabeculae align themselves in the direction of the loading. The cancellous bone provides an interface between the marrow cavity and the endocortical surface. The Cortical bone provides the majority of the structural support and load transmission from the muscles and makes up 80% of the skeletons mass.

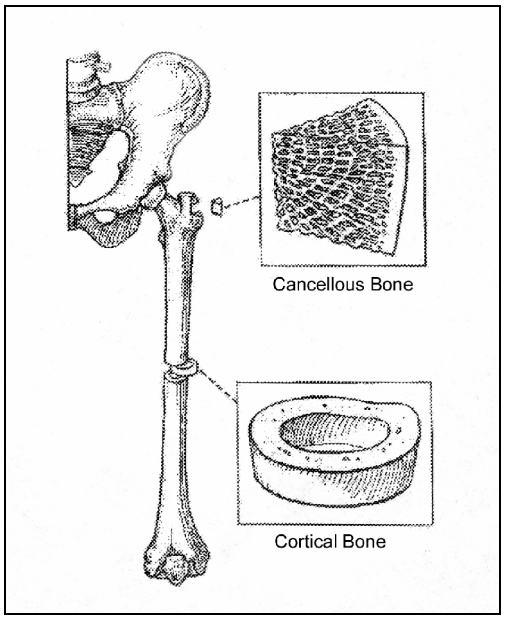

Figure 2.1: Examples of cortical and cancellous bone[11].

2.2 The Steps of Bone Remodeling

Bone remodeling is a multiple step process responsible for the adaptations of the skeleton. Remodeling is quit remarkable because of the involvement of several cell lineages cooperating in a basic multi-cellular unit (BMU). Remodeling may be thought of as a self organizing process. The steps on bone remodeling are quiescence (resting phase), activation, resorption, reversal, formation, and back to quiescence (Fig. 2.2).

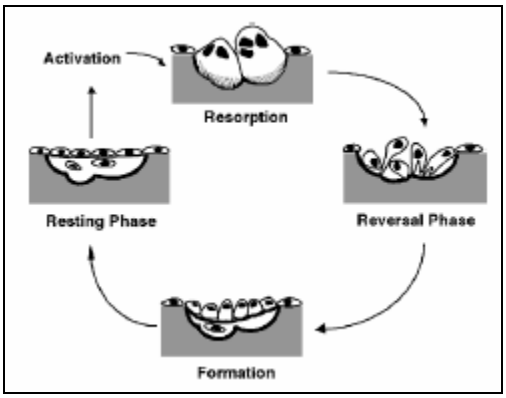

Figure 2.2: Steps of bone remodeling[12].

In quiescence, the resting phase, the bone lining cells are flat and inactive. The first event is osteoclast activation. When the bone lining cells, members of the osteoblast lineage, are exposed to a resorptive agent they retract. Osteoclasts begin populating the area. The activation frequency is increased by PTH, thyroid hormone and vitamin D. Decreases in activation are caused by gonadal steroids and cacitonin. The osteoclast is activated by membrane bound signals, the ligand for RANK, expressed by cells of the osteoblast lineage [13].

In the resorption phase the osteoclasts attach to the bone. At the attachment point a seal is formed, where the ruffled border of the osteoclast secretes acid. The mineral dissolves in this environment and enzymes digest the collagen matrix. The rate of resorption is 20 μm/day and forms a lacuna, or hole, 40-60 μm deep [12].

During reversal the osteoclasts, the multinucleated osteoclasts are replaced by mononuclear cells that are capable of some resorption. These cells smooth the resorption cavity. A layer of proteoglycans, glycoproteins and acid phosphatase, called the cement line, is deposited.

Osteoblasts are recruited to the site and begin the formation state. The coupling of reversal and formation is unclear. A proposed mechanism of coupling is that an osteoblast simulating factor such as transforming growth factor (TGF) – β is released from the bone matrix during resorption, stimulating the activity of osteoblasts [13]. Osteoblasts precursors proliferate as they are attracted to the lacuna. The osteoclast precursors differentiate and mature. Layers of osteoid, unmineralized bone, is deposited by the osteoblasts. As these layers mature the collagen crosslinks form and gradually mineralizes. When the formation stage is complete the osteoblast become bone lining cells once again in a stage called quiescence [13].

2.3 Bone Degenerative Effects of Microgravity Exposure in Humans

The skeleton is a dynamic organ that adapts to most effectively suit its environment. The mature skeleton constantly adapts to its changing habitat. One such environmental change is microgravity exposure. The smaller applied loads during long-term space flight cause the skeleton to become less robust. Trabeculae align themselves with the direction of principal stress. The orientation of trabeculae changes in response to alterations in mechanical stress directions. Therefore decreases in loading patterns due to immobilization or microgravity reconfigure the bone to its new requirements. Varying levels of skeletal deterioration have been observed in astronauts and cosmonauts. Soviet crew members on the MIR space station experienced an average loss of 0.3% BMD per month, primarily lost from the pelvis and legs [1]. Urine calcium levels increase 33% after 84 days of space flight [2]. Combating bone degradation is a concern when planning a long term space mission.

Bone loss, in space flight, increases with flight duration. Loss is evident in cancellous bone after 1 month of exposure and cortical losses are seen in the second

month. At six months the mean cancellous BMD in the tibia of 11 subjects decreased 5.4% [8]. It is difficult to draw conclusions from these studies because of the small sample size, differences in exposure, and nutritional inconsistencies.

2.4 Hind Limb Unloading vs. Space Flight in Rats

Hindlimb suspension is developed as a microgravity simulation because of similar head down bed rest studies in humans. The head down position of the animal keeps the forelimbs free, allowing them to maintain normal activities. A cephalic fluid shift is seen in the hindlimb suspension model, which is consistent with fluid shifts in space flight.

Similar observations of skeletal changes exist with a HU model in comparison to space flight. However, differences in the model remain. During HU the forelimbs of the animal remain loaded. The stress during space flight reentry and the time delay before harvest also complicates the comparison to the HU model.

Hind limb suspension of 6 month old rats shows that the percent surface of cancellous bone occupied by osteoblasts decreased by 66% ($P<0.05$) after 7 days of unloading. The percent surface of cancellous bone occupied by osteoclasts did not change. The cancellous bone volume was also unaffected [14]. The bone formation rate is decreased while resorption rates are unaffected in the HU model of mature rats.

A rat's loss of cancellous bone (BV/TV) is 55% in spaceflight compared to 29% in the suspended model, over to a 7 day period. The cancellous density, defined as trabeculae per millimeter, was seen to decrease 22% in the HU animals versus 40% in space flight animals. The number of osteoclasts observed in space flight animals after seven days was unaffected [8]. Thus it appears that space flight causes an inhibition of bone formation with little effect on resorption.

Differences between HU and spaceflight exist but formation rates are suppressed in both HU and spaceflight. An increase in osteoclast activity has not been consistently shown with hind limb unloading [15]. The mechanisms governing bone metabolism in the HU model closely mimic space flight.

2.5 Resistive Exercise as a Countermeasure to Disuse

Exercise protocols may attenuate bone loss by means of a mechanical signal. Julius Wolff observed that trabeculae align themselves with the direction of principal stress. He proposed that the orientation of trabeculae could change in response to alterations in mechanical stress directions [16].

Disuse models in humans such as bed rest have been used to mimic microgravity. Typical male astronauts exposed to 17 weeks of bed rest lose 3.9% total hip BMD and 1.7% lumbar spine BMD when compared to baseline values[8]. To attenuate this loss, a resistive exercise countermeasure was developed for use during bed rest. The bed rest and exercise group has an increase of 0.3% total hip BMD and a 4.4% increase in lumbar spine BMD [10]. The application of an anabolic exercise regime nullifies decreases BMD in the hip and lumbar spine during to bed rest.

Rats exposed to hindlimb unloading as a disuse model have a 45% decrease in mineral apposition rate (MAR) when compared to the cage control. The trabecular bone formation rate over bone volume (BFR/BV) is suppressed by 72% in the HU group when compared to control. The application of an exercise regime, a low magnitude vibrating plate at 90Hz and 0.25*g* for 10 min a day, is seen to attenuate the loss of MAR and BFR/BV caused by disuse. The MAR of the HU + Exercise group decreases by 7% and the MAR is 1% below that of control [17].

The implementation of an anabolic exercise protocol has been observed to attenuate changes in BFR/BV and MAR in rats. In humans, BMD values have been seen to increase at the hip and lumbar spine when exposed to exercise during disuse [10].

2.6 Osteogenic Loading Methods

In vivo bone deformation is preformed in a variety of novel methods. Some methods do not utilize the subject's muscles to deform the bone. While the direct application of load allows more accurate measurements, the level or direction of loading my not be physiological.

2.6.1 Four Point Bending

In vivo four point bending of a tibia has been observed to simulate bone formation. Load is applied on the lateral side. A load of 31 N for 36 cycles at 2 Hz is applied to the right leg of the animal. The lower supports are placed 22 mm apart and the upper supports are 10.5 mm apart, see Fig.2.3. The load was applied on alternate days for three weeks.

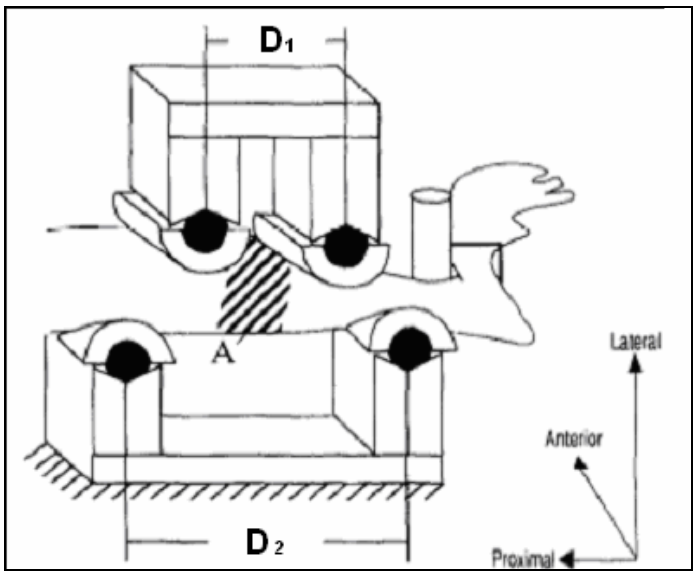

Figure 2.3: Four point bending diagram[18].

Strains between 1200-1600 με on the medial side are seen form the application of 31N. The mineral apposition rate is observed to increase on the lateral and medial periosteum in the loaded limb, when compared to the unloaded contra-lateral limb. The tibial area at midshaft is observed to increase significantly in the loaded limb, when compared to the unloaded contra-lateral limb (4.27 ± 0.37 mm^2 vs. 4.43 ± 0.39 mm^2) [19].

Osteogenic changes are seen in the tibia with the exposure of dynamic loading. Four point bending creates strains at the osteogenic threshold. However the loading pattern is not physiological. A loading regime which employs a subject's muscles would address the affect of weight bearing exercise on bone formation more fully.

2.6.2 Ulna Loader

Loading the ulna axially creates a force distribution which is more natural than other methods. This method compresses the ulna in a physiologically relevant manor. The loads applied to the ulna are greater than those seen during animal activity. The applied loads observed during exercise are hyper-physiological [19]. A diagram of the placement of the loading cups appears below (Fig.2.4).

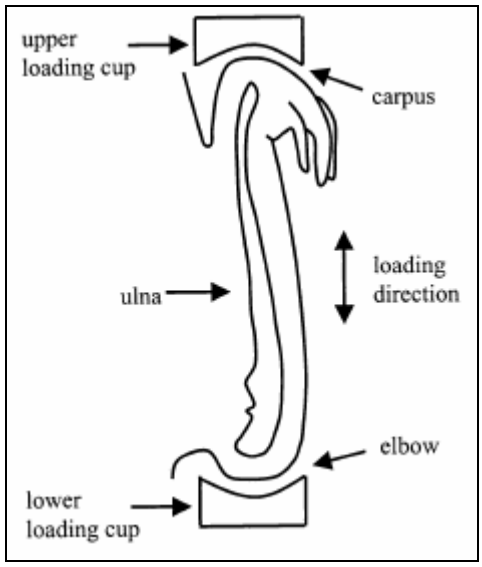

Figure 2.4: Schematic of ulna loading [19].

As the upper and lower cups are brought together the ulna deforms. A load of 17 N applied at 2 Hz for 90 cycles for 4 bouts with a 3 hour recovery between each bout has shown osteogenic strain levels of -3500 με on the medial surface. The load is applied 3 days a week for 16 weeks.

Robling statistically shows that in a right to left comparison that the treated right limb has an ultimate load of 53± 0.8 N vs. 27.6 ±1.0 N in the untreated limb. A paired t-test was used to compare right vs. left and had a $p<0.001$. The stiffness while elevated in the treatment group was not statistically significant when compared to the untreated

limb (51±1.8 N/mm vs. 46.3±2.1 N/mm, respectively). The affect of the countermeasure causes more bone to be deposited on the medial side. The following figure illustrates the geometric change (Fig.2.5) [20].

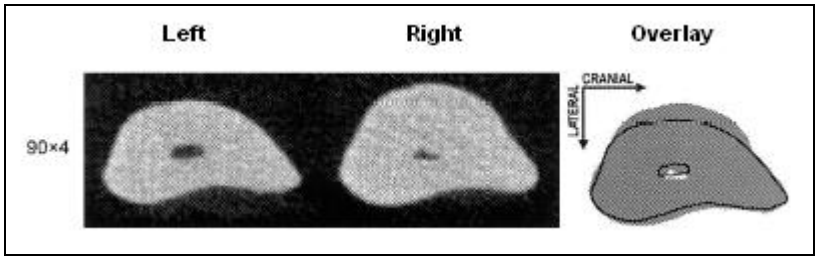

Figure 2.5: Illustration of the in cortical area along the vertical axis of the ulna [20].

The application of load is not physiological because it is not applied at the flexed carpus and olecranon. Due to the shape of the bone, the strain and stress distributed to the bone approximate levels seen during normal activity. This is due to the bending moments incurred through the axial compression of a curved bone [19].

2.6.3 Electrical Muscle Stimulation

Muscle stimulation causes the bone to be loaded by the animal. The advantage to this loading pattern is physiological. The peak strain level observed in this method during an eccentric contraction at 120% of peak isometric torque is 1100 με with strain rates are between 7000 and 10,000 $\mu\varepsilon s^{-1}$. The following figure is diagram of the exercises setup (Fig.2.6).

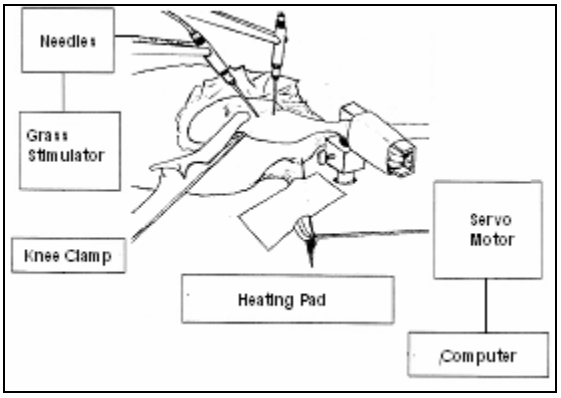

Figure 2.6: Diagram of electrical muscle stimulation unit [21].

Seen in the diagram are the insertion points of the fine wires, which attach to the grass stimulator. The fine wires are placed on opposite sides of the sciatic nerve. The knee is clamped at a 90 degree angle. The foot is taped into the footplate with an ergonomically shaped pad to create robust interface between the foot and servo motor. The servo motor measures the torque applied to the foot plate.

An electrical muscle stimulation protocol that produces eccentric contractions at 150% of isometric max has shown small affects on cortical bone. Ovariectomized (OVX) rats were trained with 10 contractions every third day over an 8 week period. Three point bending was used to determine the mechanical properties of the tibia. The bones were broken lateral side down. The training protocol was able to show a statistically significant ($p = 0.013$) increase of 15% in the stiffness of the OVX + training group when compared to control. The ultimate load was not shown to change, nor did the CSMI [21].

The marginally positive effect of electrical stimulation on cortical bone, encourages the exploration of electrical muscle stimulation's effect on cancellous bone. The loading pattern and loads supplied to the bones in this method are physiological. The use of this model represents the affect of weight bearing exercise on bone health more adequately than others.

2.7 Compression Testing of Trabecular Bone

Correlations between compressive mechanical properties and the density of cancellous bone may create a clinical diagnostic tool to determine bone integrity. Current *in vivo* assessments of bone density insufficiently evaluate osteoporosis in patients. There is a strong correlation between BMD and mechanical properties but a 30-50% unaccounted for variance exists when using BMD to predict mechanical properties [(22)]. Destructive testing is necessary because there is not a sufficient relationship between bone imaging methods and mechanical properties. The only way to accurately asses the material properties of cancellous bone is through RPC testing.

The loads that cortical bone can sustain under compression are larger than cancellous bone. However cancellous bone can sustain strains of 75% before failing *in vivo*, but cortical bone will fracture when strain exceeds 2%. Cancellous bone has a greater capacity to store energy compared to cortical bone [(12)]. The difference in cancellous and cortical material properties necessitates the isolation of cancellous bone during compression. The investigation of cancellous bone's contribution to the overall strength of the bone is less transparent when cancellous and cortical bone is tested together. The use of reduced platen compression as opposed to whole bone testing allows the cancellous bones material properties to be isolated.

Intrinsic cancellous bone properties use a compression test that directly loads only cancellous bone. The specimen wedged between two flat, parallel platens of similar diameter that are smaller than the surrounding cortical shell. A uniaxial compressive load compresses the sample until a desired displacement. Platen selection must test as much cancellous bone as possible without loading any cortical tissue. The appropriate location in the bone must be consistently selected to maximize the amount of cancellous bone available while not including the primary or secondary spongiosa in the specimen.

Platens of varying sizes will be used to test different bone specimens because of the greater variability in bone sizes between treatment groups as well as the difference in ages. In this case, the values of the extrinsic properties do not give any meaningful

comparisons between bones. On the other hand, intrinsic properties such as compressive stress, strain, and modulus take into account the size of the platens and thus, exhibit meaningful information concerning the quality of the bone in each specimen.

3. MATERIALS AND METHODS

3.1 Introduction to the Experimental Design

A general overview of the experiment is provided here, specific details are provided in subsequent sections. Thirty six, 5.5 month old male Sprague-Dawley rats were randomly assigned three groups; base line (BL), cage control (CC) and hind limb unloaded (HU). The groups were normalized to body weight and total BMD. The duration of the study was 28 days. The left leg of all HU rats experiences electrical stimulation to imitate weight bearing exercise three times a week over a 28 day period. The right leg of the HU animals is the contra-lateral control for the countermeasure. The design advantage of a contra-lateral limb control the same animals are used in your groups of interest. Therefore, any unaccounted for differences in individual animals will be symmetric within the two groups. The BMD of the tibial metaphysis determined using pQCT. Separate scanning session for the right and left legs aid in consistent limb positioning. pQCT scans occur at day 0 and 28. Sacrifice is on day 28, followed by tibial extraction, then stored in saline, and frozen. Reduced platen compression (RPC) testing determines the mechanical properties (elastic modulus and ultimate stress) of the trabecular bone.

3.2 Experimental Outline

Thirty six, 5.5-month old, male Sprague-Dawley rats were divided into three sets and run in two cohorts containing 6 rats in each group; BL, CC and HU rats. The determination of the initial weights for the animals uses a Mettler PC 440 Balance (Mettler Instrument Corp, Hightstown, NJ). All rats were monitored daily and weighed weekly in order to detect weight variances that may have occurred during the study. Performance of pQCT scans of the left leg of all rats determines the BMD of the proximal tibial metaphysis prior to the study. The rats were divided into groups with the equivalent average body weights and BMD of the left tibial metaphysis.

Each rat was individually housed and maintained in an animal care facility at Texas A&M University accredited by the American Association for the Accreditation of Laboratory Animal Care. The animals were individually housed in cages in a temperature-controlled (72^{o} F) room with a 12:12 light/dark cycle.

The hind limb suspension of a rat uses a U shaped sling constructed from medical tape. The tape adheres to the lateral sides of the tail for 3 inches starting a centimeter above the base of the tail. The adhesive's commercial name is marine glue. At the trough of the U a paperclip attaches to the sling. The paperclip attaches to an overhead wire. The wire elevates the animal's hind regions roughly 30 degrees. The forelimbs allow the animal to ambulate freely (Fig.3.1). The animal is able to freely groom, eat, and drink.

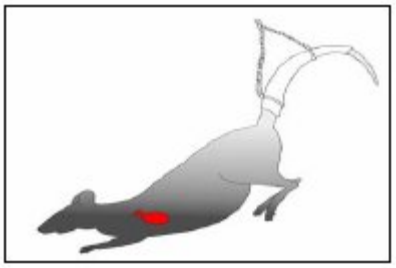

Figure 3.1: Example of hind limb suspension [9].

BL rats are sacrificed on day zero of the experiment. HU rats receive standard Harlan Teklad 4% rodent chow (Teklad Premier, Madison, WI) and water ad libitum. Pair feeding of the CC rats restricts the food level of the CC group to that of the HU group. Every individual in the HU group has their food intact recorded everyday. The average of this food intake determines the amount that the CC group receives. Pair feeding the CC group accounts for nutritional effects of bone health. The study lasts for 28 days. The left leg of all HU rats undergoes electrical stimulation to imitate resistance exercise three times a week over a 28 day period. The untreated right leg serves as the contra-lateral control for the countermeasure in the HU group. The determination of the

total and cancellous BMD uses pQCT. Separate scanning session for the right and left legs aid in consistent limb positioning. Scanning occurs on day 0 and 28. Sacrifice happens on day 28, followed by tibial extraction, stored in saline, and frozen. RPC testing determines the intrinsic mechanical properties (elastic modulus and ultimate stress) of the trabecular bone.

3.3 Electrical Stimulation Protocol

The exercise protocol utilizes high intensity low frequency mechanical loading as a countermeasure during hindlimb unloading. As the use of high intensity exercise, increases, high force and strain, the number of cycles required to initiate bone formation decreases [(23)]. Bone response is known to be dependent upon the intensity of the load and frequency of cycles.

Electrical impulses are created using a Grass S48 stimulator. The stimulator control uses a 16 bit D/A and A/D board (KPCI-3108; Keithley Instruments, Cleveland, Ohio, USA), an 866 , a PC and custom software written in Test Point [(24)].The stimulation probes are inserted on opposing sides of the sciatic nerve. Voltage optimization occurs before every session to yield the maximum isometric torque at 175 Hz. An isometric contraction is when the muscle does not stretch or contract. Nerve stimulation at the prescribed frequency yields eccentric contractions at 120% of the maximum isometric torque for 0.5 seconds for 40 stimulations during the exercise protocol. Sweeping through a range of frequencies, 30-60 Hz, allows the determination of a frequency which creates an eccentric contraction at 120% of the previous obtained maximum isometric torque. An eccentric contraction is when the muscle lengthens while it contracts. The foot plate is rotated 40 degrees during stimulation at an angular velocity of 100 degrees per second, from -20 degrees planter to 20 degrees dorsal flexion. The rotation of the foot plate during muscle contraction produces an eccentric contraction on the posterior muscle. The exercise regimen consists of 4 sets of 10 reps. The animal's muscles contract every 10 seconds with a 2 minute rest between each set, yielding a total of 40

contractions a day. The rats are exercised 3 days a week on alternate days for four weeks, a total of 12 exercise sessions.

The rat is anesthetized and laid on its right side. The left foot is attached to the footplate with tape. The knee is clamped to form a 90^{o} angle between the longitudinal axis of the tibia and the foot. The servo motor shaft connected to the footplate measures the torque, displacement and angle versus time during the contraction. The Aurora Scientific 305B servomotor controller is used to synchronize the contraction of the hindlimb with the movement of the servo shafts movement. The Grass Stimulator output and footplate movement are controlled by Testpoint software, using accustom designed program (courtesy of Dr. Gordon Warren III, Georgia State University). Data is sampled at 10Hz.

The HU group's left and right tibias comprise the disuse plus exercise and disuse groups. Harvesting these bones answers shows the affect of the countermeasure applied to a disuse model. Collection of the left tibia of the CC group accounts for nutritional and age related affects. The left tibia of the BL group illustrates the previous three groups starting point.

3.4 pQCT Procedure

Measures of tibial bone density and geometry were taken *in vivo* using a peripheral quantitative computed tomography (pQCT) device (XCT Research M Stratec; Norland Corp., Fort Atkinson, WI). Animals were anesthetized with a ketamine/ medetomidine cocktail (1 mg/kg BW given IP) and placed prone on a scanning platform with the left leg extended into the CT scanner gantry and taped securely into position. Right and left leg scans preformed separately. An initial scout view taken of the entire tibia to determine scan slice positions. With a reference line placed at the proximal tibial plateau, slices taken at both the metaphyseal (5, 5.5, and 6mm from reference) and a single slice taken diaphyseal (50% total bone length) regions. All slices thickness are 0.5mm. Due to the diameter of the leg, a scanning diameter of 45 mm is necessary thus requiring a minimal voxel size of 100μm. Total scan time is approximately 45 minutes

from time of scout view until scanning completes. Data from the three metaphyseal region slices are averaged to get a single value for each variable. The cortical section of bone is compact is the cortical shell of bone. The cancellous section of bone is spongy, or comprised of trabeculae. A peeling algorithm defined by a density threshold defines the two regions. The total section is the sum of both the cancellous and the cortical sections. Key outcome variables include total, cancellous, and cortical bone mineral density (BMD); total and cortical area, total bone mineral content (BMC); and cross-sectional moment of inertia (CSMI) and section modulus, which combine CSMI and BMD data to provide an estimate of bone strength. The BMC is the amount of bone mineral present. The BMD is the BMC divided by the volume of bone present in the 3 scans. Machine precision (based on manufacturer data) is ± 3mg/cm^3 for cancellous bone and ± 9mg/cm^3 for cortical bone. *In vivo* CV's from our laboratory using this method at the proximal tibia (with repositioning between scans) are ±2 .13% for cancellous BMD, ± 0.23% for cortical BMD, ± 1.95% for total area. Corresponding CV's at mid-diaphysis for the tibia are ± 0.86% for cortical BMD, ± 1.09% for cortical area, and ± 2.42% for marrow area [(9)].

3.5 Bone Removal and Preservation

The anesthetized rats were euthanized by decapitation and the tibias from all groups and legs were collected at necropsy. Tibiae were thoroughly cleaned of adhering soft tissue, separately wrapped in gauze wetted with Ringers solution, sealed in labeled plastic bags, and frozen at –80^{o}C. It should be noted that in 1984, Pelker showed that methods of preservation including freezing do not substantially affect the mechanical properties of bone [(25)].

3.6 Reduced Platen Compression (RPC)

It is of interest to determine the properties of trabecular bone. The cortical shell affects the properties of the trabecular bone. Mechanically loading the trabecular bone independently of the cortical shell reveals the *in vivo* properties of

cancellous bone. Intrinsic properties are the materials behavior at the tissue-level, found by normalizing the bone, or specimen, size and shape. "Intrinsic properties from the RPC tests provide measures of the mechanical behavior of the cancellous bone material (as a porous material at the macroscopic level, not at the trabecular tissue solid phase level)." [26]

The basic goal is to cut out specimens 2 mm in thickness from within the metaphysis just distal to the growth plate as shown in Fig. 3.2. The top of the sample is located distal to a tibial tuberosity. Figure 3.2 contains an x-ray of an actual RPC sample.

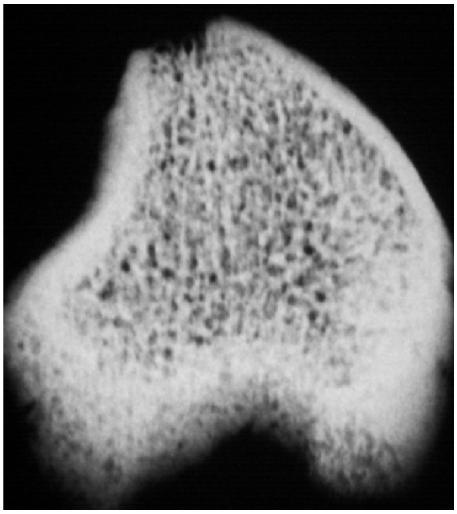

Figure 3.2: Contact X-ray of a RPC sample proximal side up.

Bones were allowed to thaw reach room temperature before mechanical testing. The specimens were kept moist by spraying with saline solution throughout the testing process. After handling the bones were immediately re-wrapped in gauze saturated with saline solution and returned to their appropriate containers.

Identifying the location for each sample cut was determined on each bone by visual inspection. The distance between the top of the epiphysis and the landmark was measured 3 times and averaged, determining the distance to the proximal side of the sample, see Figure 3.3. The landmark is the indention where the growth plate and

proximal metaphysis meet or where the tibial tuberosity is, see Figure 3.3. The sample was mounted in plastic grippers. The top of the epiphysis was aligned parallel to the blade of a Buehler Isomet low speed diamond wafer saw blade model 11-4244. The first cut is made at the predetermined distance, see Figure 3.3. A visual inspection of the bone was also conducted in order to verify that the growth plate was cleared. The second cut made 2.3 mm distal to the first cut, yielding a 2mm sample. After the second transverse cut, the thickness of the RPC specimen was measured and recorded with the digital calipers.

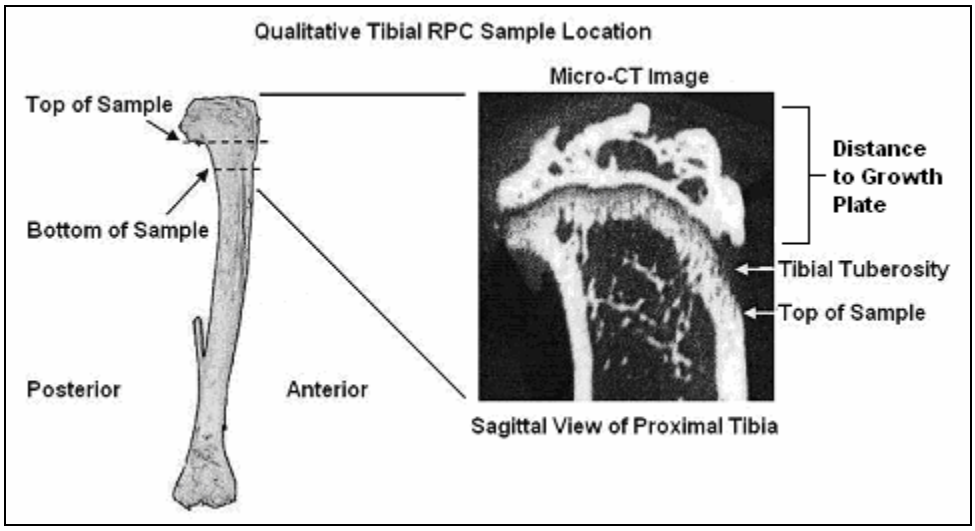

Figure 3.3: Illustration of landmark and RPC sample location.

The development of a method for consistent identification of the first transverse cut below the growth plate is problematic. The challenge with this cut is that the distribution of secondary spongiosa within the marrow cavity is quite limited. One must be below the growth plate, in order to avoid the primary spongiosa. Also the trabecular spacing increases with increasing distance from the growth plate. The sample must fall on a region close enough to the growth plate to yield a testable sample, while not containing primary spongiosa. Biological variations disallow the use of a standard percentage of the overall length for the first cut distance insufficient. Therefore, the

current adopted method is to define the distance to the landmark for the proximal cut on an individual basis for the tibia of rats.

To determine the appropriate platen size for each specimen, a radiograph was made computer-aided image analysis. The specimens were placed with the proximal surface flat against the film. Each set of bones radiographed at 20 kV, 1 mA, and 70 seconds. A second set of x-rays was taken of the RPC specimen where the distal portion of the metaphysis faces upward, used for platen sizing. Radiographs were developed on Kodak X-Omat TL Film (Eastman Kodak Company, Rochester, NY) and radiated with a General Electric Industrial Radiograph Machine (General Electric, Lexington, MA) set at 25 kilovolts (kV) and 1 milliampere (mA). The focal film distance was fixed at 30 inches. The radiographs were digitized using a Nikon Coolscan 5000, at 4000 dpi.

Platen size was determined by superimposing several circles with different diameters onto the RPC sample in Adobe Illustrator while not overlapping the cortical shell, see Figure 3.4.

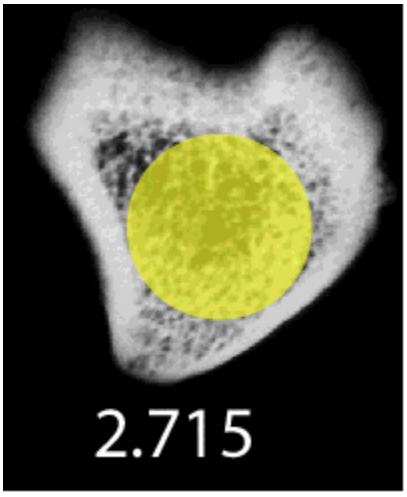

Figure 3.4: Sample radiograph with the maximum endocortical circle.
Diameter of the circle in millimeters.

Focal film distance was fixed at 30 inches and the exposure time was set at 80 seconds. The platen size was found by taking 70% of the largest superimposed circle. Once a nominal platen size was selected, the platen was held against the actual test specimen to determine if any adjustments needed to be made. Some specimens required a platen that was one size larger or one size smaller than the one prescribed. The bones were developed at the Texas A&M College of Veterinarian Medicine. The platens are available in 0.05 mm increments of diameter. The bones were then grouped according to platen size and tested from smallest to largest.

The proximal tibia specimens were tested in compression with an Instron model 1125 mechanical testing machine, with a 1000 lb. load cell. Quasi-static loading was applied with the upper platen being lowered at a rate of 0.51 mm/min (0.02 in/min) while the lower platen remained stationary. Displacement was measured via a linear variable differential transformer (LVDT) mounted to the cross-head. For the RPC test, the LVDT was calibrated to have a total stroke of 0.05 mm between −10 V and 10 V. Load-deflection data (force vs. displacement) were collected digitally at a sampling rate of 10 Hz using Labtech Notebook Pro Software Version 8.01 (Laboratory Technologies Corporation, Wilmington, MA) on a desktop IBM compatible PC.

Raw data obtained from the mechanical tests reported recorded the test time in seconds, displacement in inches, and the load in pounds. The manipulation of this data produces values of the intrinsic properties of interest. This section describes the parameters that were calculated from the raw data for each mechanical test. An idealized example of the force vs. displacement curve appears in Figure 3.5.

Assuming uni-axial compression of the cancellous bone crushed between the platens, the volume of the material comprising the loaded specimen was taken to be the product of the specimen thickness (t) and the cross-sectional area of the platen (A_{pl}). The main parameters determined from the force-displacement data recorded during each test were:

- Maximum Force (F_M) in N
- Displacement at Maximum Force (D_M) in mm
- Stiffness (k) in N/mm

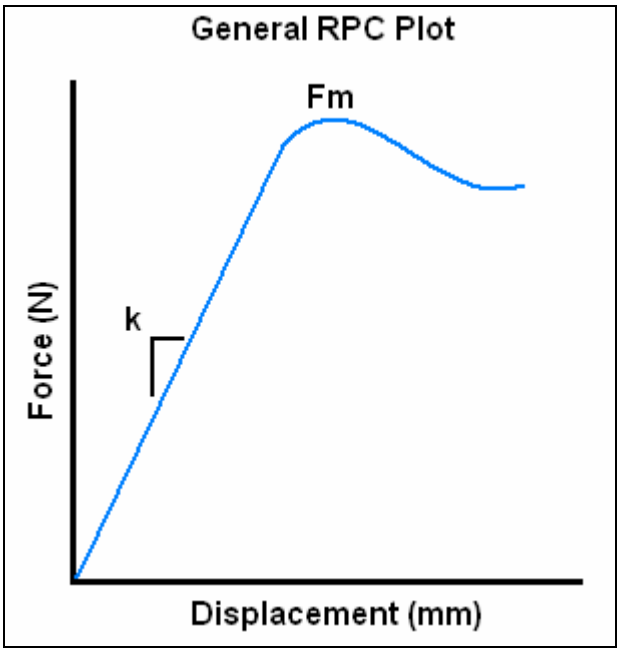

Figure 3.5: Schematic of raw test data.

These parameters were calculated using Microsoft Excel 97 and TableCurve 2D v.2.03 (Jandel Scientific, San Rafael, CA) and represent the response of each specimen as a structure and thus depend upon the size and shape of the specimen. In this case, these extrinsic properties are not useful or of direct interest. Therefore the intrinsic or tissue level properties were estimated. The determination of stiffness uses Table Cure 2D v.2.03. This program produces mathematical expression for the linear region of the data by excluding data points outside the linear region.

Assuming a pure uni-axial stress state the following intrinsic properties were found:

Ultimate stress (σmax), in MPa, is the strength of the material at maximum force and is determine by:

$$\sigma_{max} = \frac{F_M}{A_{pl}} \tag{3.1}$$

F_M is the maximum force and A_{pl} is the cross-sectional area of the platen used to compress the cancellous bone.

Elastic Modulus (E), in MPa, is the stiffness of the trabecular bone core at the tissue-level that was compressed and is determined by:

$$E = \frac{\sigma_{yy}}{\varepsilon_{yy}} = \frac{F_M t}{\Delta t A_{pl}} = \frac{kt}{A_{pl}} \tag{3.2}$$

k is the stiffness (from the linear region of the force-displacement curve), t is the thickness of the specimen, and A_{pl} is the cross-sectional area of the platen.

The extrinsic properties were calculated using the raw data; the values cannot be compared between groups because various sized platens were used for different specimens. The intrinsic properties, normalizes for the different sizes of the platen used, allows for a more meaningful comparison between bones of different sizes.

The data will be reported in the form Mean ± Standard Error. The statistical analysis of the longitudinal pQCT data uses a Two Way ANOVA with a pair wise Student-Newman-Keuls comparison method. One sorting factor was the day of the study that the pQCT scan was performed, either 0 or 28. The second sorting factor was what treatment group the bone belonged to: none, HU+ No Exercise, and HU + Exercise. The BL group is excluded from this analysis because it is not longitudinal data. The BL group only has day 0 pQCT data, which is obtainable from the CC group. The statistical analysis of the mechanical properties uses a One Way ANOVA with a pair wise Dunn's comparison method because there are not longitudinal data. The BL group is excluded from the statistical analysis because it is not the same age. However, it is shown as a reference point. Data is sorted according to the treatment received, none, HU+ No Exercise, HU + Exercise. The comparison of HU+ No Exercise and HU + Exercise illustrates if exercise mitigates the affect of disuse. The comparison of HU+ No

Exercise and CC illustrates the affect of disuse. The standard T-test is used to compare the percent difference between HU + No Exercise and CC with the percent difference between HU + Exercise and CC.

4. RESULTS

4.1 Introduction

The detected changes in the BMC, cross-sectional area, and BMD, collected using pQCT, are contained in this section. The intrinsic mechanical properties of the cancellous bone in the proximal tibial metaphysis, determined with RPC testing, are also presented. The results illustrate that the countermeasure attenuated the decrements in most parameters of BMD, BMC, area and mechanical properties due to hindlimb suspension. Bone's mechanical and geometric properties are the primary interest of the study. It is desired that the exercised HU limb has the same properties as that in the CC animal. This will mean that the losses due to HU are abated due to the exercise intervention during suspension. The data is report as Mean ± Standard Error.

4.2 Body Weight

The body weight of the rats decreases significant due to the application of a hindlimb unloading at 28 days compared to the CC group. The weight of the CC rats did not statistically increase during the course of the study (Fig.4.1). The weight of the HU rats statistically decreased during 28 days of hindlimb unloading, ($P = 0.019$; 418.7 ± 8.53 vs. 383 ± 7.32). The CC rats were pair feed to the level of HU rats during the study.

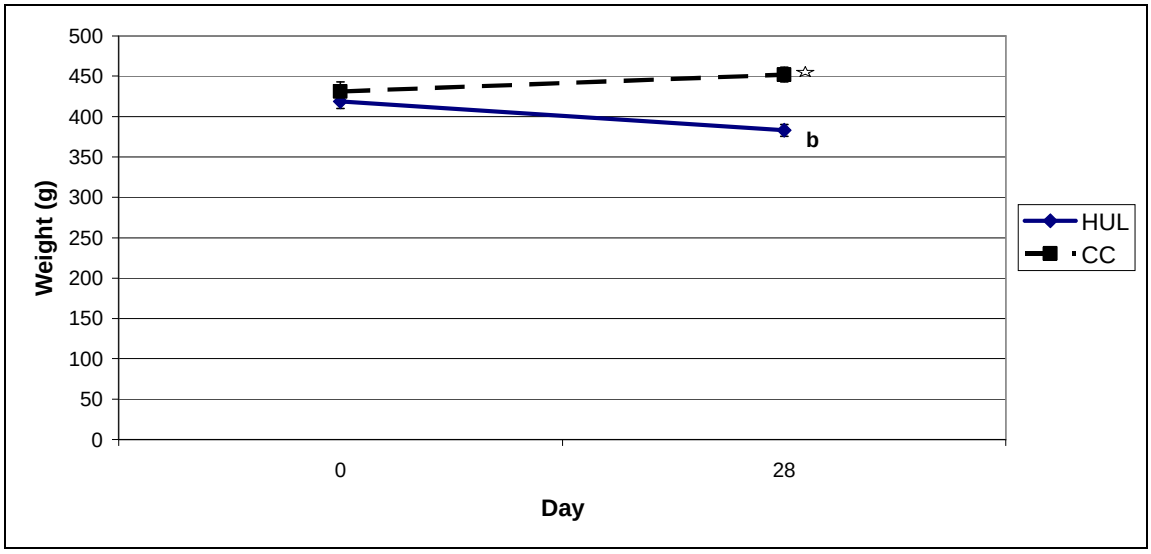

Figure 4.1: Body weight during 28 days of unloading.

*, P<0.05 vs. HU-Ex at day 28.

b, P<0.05 vs. day 0 value.

4.3 Tibial Metaphysis Bone Mineral Content (BMC)

4.3.1 Total BMC

Changes in bone mineral content (BMC) were affected with the administration of the countermeasure. BMC is reported because it is a measure of the total amount of bone mineral present, allowing one to observe actual changes to bone size. The total BMC of the HU – EX tibia is 27% larger than the HU - No EX group at 28 days (11.5± 0.3 mg vs. 9.0 ± 0.2 mg; $P < 0.001$). The countermeasure attenuated the detrimental affects of disuse (Fig.4.2). The HU-Ex tibia and the CC tibial BMC values are not statistically different at 28 days, ($P = 0.965$). The CC tibia is 20.9 % larger than the HU-No EX tibia (11.4 ± 0.2 vs. 9.0 ± 0.2 mg; $P< 0.001$). At day zero the HU- No Ex tibia's total BMC is 7.6% greater than the CC tibia (11.9 ± 0.1 vs. 10.9 ± 0.2; $P = 0.008$). There are no other statistically significant differences among groups at day zero.

Two Way ANOVA analyses expose a statistically significant difference for total BMC within the groups at day zero and day 28, $P < 0.001$. The post hoc pairwise

comparison of the HU- EX group's value at day 0 and 28 does not show a statistically significant change over time. The CC total BMC does not show a statistically significant change over time. The HU – No EX group exhibits a 24% decrease in total BMC after 28 days; (11.9 ± 0.4 mg vs. 9.0 ± 0.3 mg, P<0.001). The countermeasure maintains total BMC during disuse equal to the level seen in pair fed cage controls.

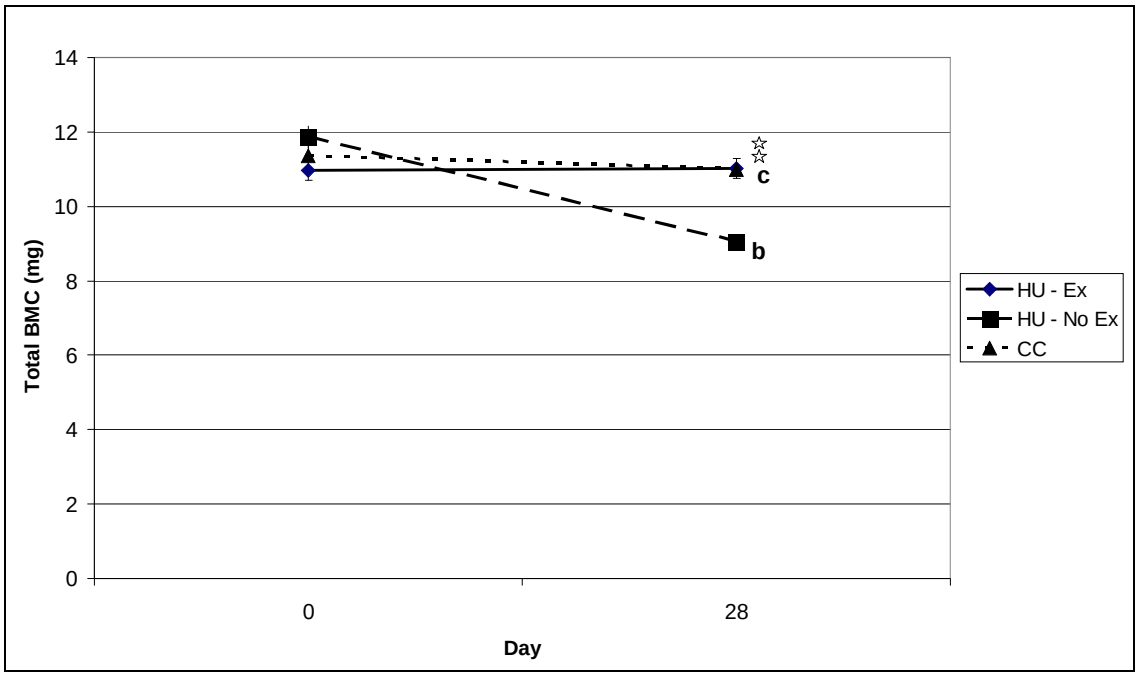

Figure 4.2: Total BMC of the tibial metaphysis during 28 days of unloading. The HU-Ex (left leg) was subjected to muscle contraction training. * P<0.05 vs.HU-No Ex, the untrained tibia of the HU rats. *b*, P<0.05 vs. 0 day values within the HU – No Ex group. *c*, P<0.05 vs. 0 day values within the CC group.

4.3.2 Cancellous BMC

The countermeasure does not impact changes in cancellous BMC when using a two way ANOVA (P = 0.486). Pairwise comparisons at 28 days do not reveal any differences among groups (Fig.4.3). The total BMC of the HU - EX group is 34.5%

larger than the HU - NO EX group (2.5 ± 0.2 mg vs. 1.8 ± 0.2 mg) but this difference is not statistically significantly (P = 0.151).

Within the treatment groups, post hoc comparisons between day 0 and day 28 reveal significant changes. Cancellous BMC in the HU – EX tibia decreases 24% from day 0 to day 28 (3.26 ± 0.2 mg vs. 2.5 ± 0.2 mg) and is statistically significant, (P = 0.019). The HU – No EX group decreases 44.8% from day 0 to day 28 (3.3 ± 0.2 mg vs. 1.8 ± 0.2 mg) and is statistically significant, (P < 0.001). The CC groups cancellous BMC does not change significantly. The counter measure does not abate bone loss as qualified by BMC due to disuse over a 28 day period.

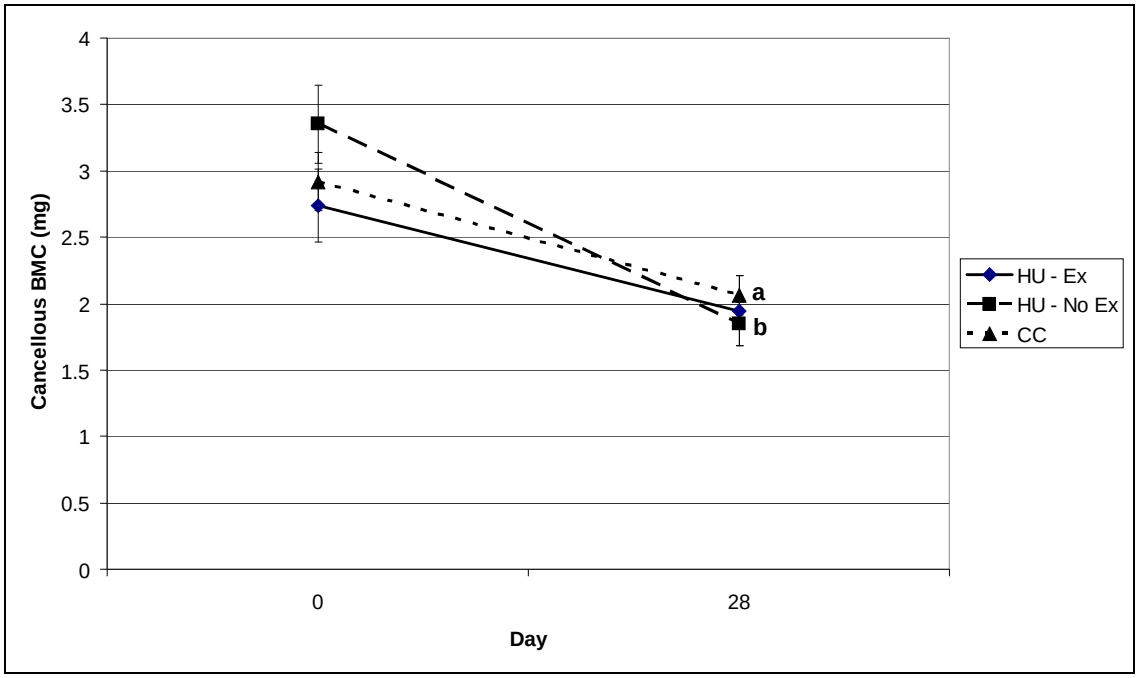

Figure 4.3: Cancellous BMC of the tibial metaphysis during 28 days of unloading. The HU-Ex left leg was subjected to muscle contraction training. There were no significant differences among group means at day 0 or day 28. *a*, P<0.05 vs. 0 day values within the HU - Ex group. *b*, P<0.05 vs. 0 day values within the HU – No Ex group.

4.3.3 Cortical BMC

Changes in cortical BMC were observed with administration of the countermeasure. Two way ANOVA analysis yields $P<0.001$ for differences due to the treatment. Post hoc pairwise analysis shows differences between specific groups (Fig.4.4). The cortical BMC of the HU - EX group is 22.9% larger than the HU - NO EX group at 28 days (8.1 ± 0.1 mg vs. 6.6 ± 0.1 mg), ($P<0.001$). There is not a statistically significant difference between the HU – EX and the CC groups at day 28. The cortical BMC of the CC group is 18.5% larger than the HU - NO EX group at 28 days (8.1 ± 0.1 mg vs. 6.6 ± 0.1 mg) and is statistically significantly, ($P<0.001$). The countermeasure effectively raises the BMC of the HU - EX group to the level of the CC group. The countermeasure abates losses due to 28 days of disuse. There are no statistical differences between treatment groups at day 0.

The cortical BMC significantly changes over the 28 day period. The HU – EX cortical BMC increases 8.6% from day 0 to day 28 (7.4 ± 0.1 mg vs. 8.1 ± 0.1 mg, $P < 0.001$), where as in HU – No EX, this parameter decreases 13.2% from day 0 to day 28 (7.6 ± 0.1 mg vs. 6.6 ± 0.1 mgt, $P < 0.001$). The CC group's cortical BMC increases 6.5% from day 0 to day 28 (7.4 ± 0.1 mg vs. 8.1 ± 0.1 mg, $P < 0.001$). The countermeasure abates losses to cortical BMC caused by 28 of disuse.

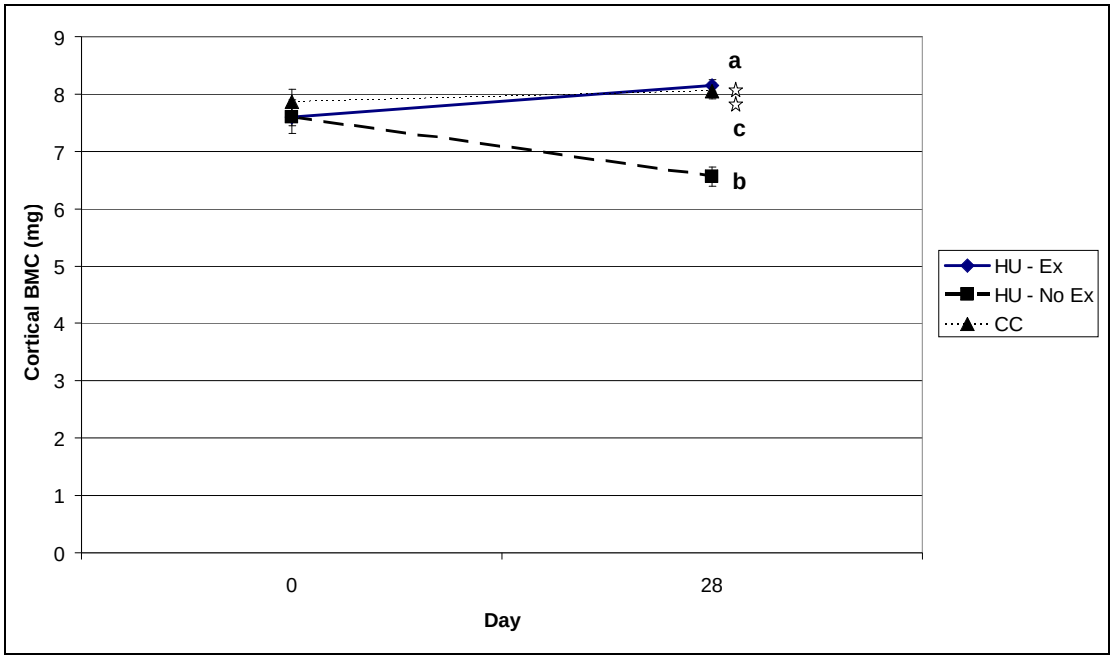

Figure 4.4: Cortical BMC of the tibial metaphysis during 28 days of unloading. The HU-Ex left leg was subjected to muscle contraction training. * $p<0.05$ when compared to HU - NO EX. *a*, $P<0.05$ vs. 0 day values within the HU - Ex group. *b*, $P<0.05$ vs. 0 day values within the HU – No Ex group. *c*, $P<0.05$ vs. 0 day values within the CC groups.

4.4 Tibial Metaphyseal Area

4.4.1 Total Area

The total cross sectional area of the HU - NO EX group vs. the HU - EX is 3.1% less at 28 days (P = 0.355). The mean values of treatment groups are not significantly different at day 0 or day 28 (Figure 4.5). The area did change with respect to time (P<0.001). The total area for the HU – Ex group decreased 10% from day 0 to day 28 (18.5 ± 0.5 mm^2 vs. 16.6 ± 0.6 mm^2; P = 0.027). The CC group decreased 7.2% from day 0 to day 28 (18.9 ± 0.4 mm^2 vs. 17.5 ± 0 .5 mm^2; P =0.038). The HU – No Ex group decreased 10.5% from day 0 to day 28 (17.7 ± 0.5 mm^2 vs. 15.8 ± 0.6 mm^2; P = 0.026).

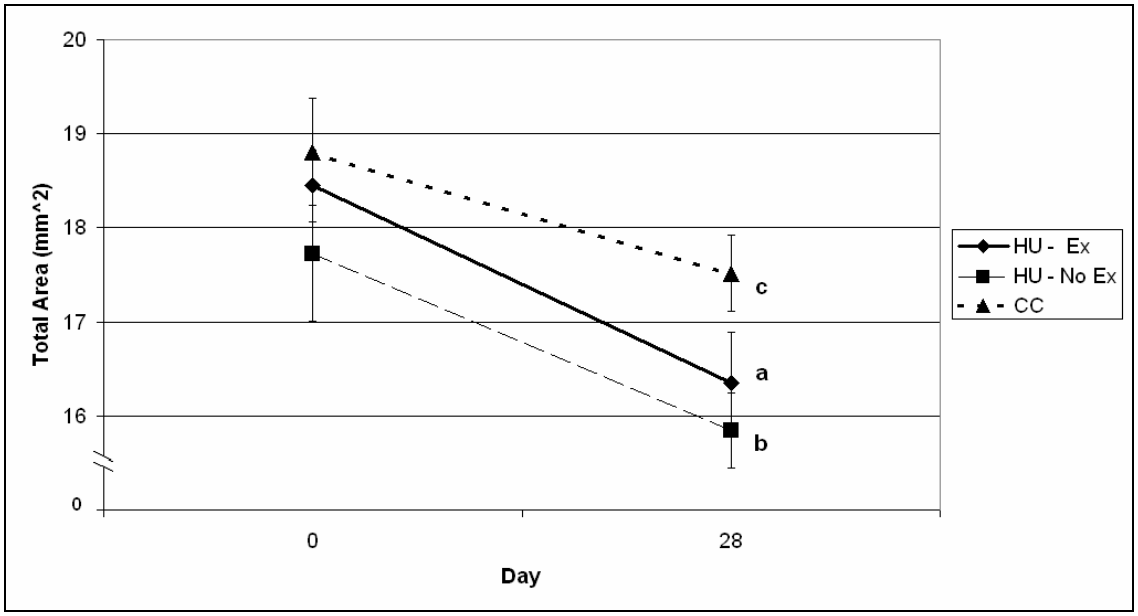

Figure 4.5: Total area of the tibial metaphysis during 28 days of unloading. The HU - Ex group's left leg was subjected to muscle contraction training. There were no significant differences among group means at day 0 or day 28. *a*, P<0.05 vs. 0 day values within the HU - Ex group. *b*, P<0.05 vs. 0 day values within the HU – No Ex group. *c*, P<0.05 vs. 0 day values within the CC groups.

4.4.2 Marrow Area

The group means for marrow area bounded by the endocortical perimeter in the tibial metaphysis are not statistically different at day 0 or at day 28. The countermeasure was unable to affect the marrow area lost due to disuse at 28 days (Fig.4.6). The HU – Ex's marrow area decreased 19.6% from day 0 to day 28 (10.69 ± 0.5 mm^2 vs. 8.6 ± 0.5 mm^2; P= 0.004). The HU – No Ex group's value decreases 8.8% but not significantly from day 0 to day 28 (9.8 ± 0.5 mm^2 vs. 8.9 ± 0.5 mm^2; P = 0.22). The CC group decreases 14.6% from day 0 to day 28 (10.9 ± 0.3 mm^2 vs. 9.3 ± 0.4 mm^2; P = 0.004. The marrow area is the area within the endocortical shell. The XCT-M analysis software defines a boundary between the cortical and cancellous bone based on attenuation thresholds set by the user to distinguish between higher density cortical bone and lower density cancellous bone. Changes in trabeculae architecture cause the endocortical boundary to vary, resulting in alterations in marrow area and cortical area.

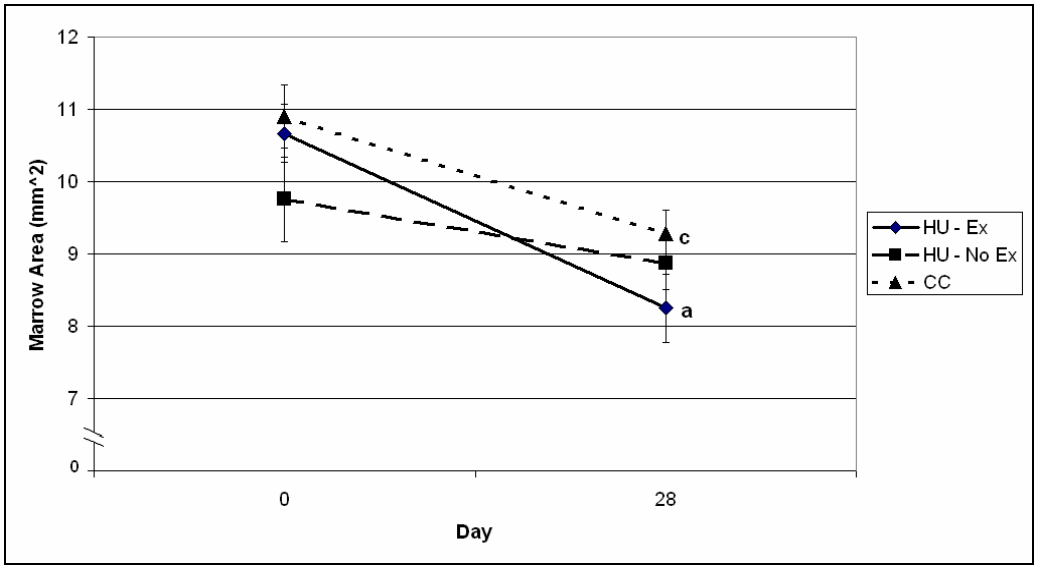

Figure 4.6: Marrow area of the tibial metaphysis during 28 days of unloading. The HU-Ex left leg was subjected to muscle contraction training. There were no significant differences among group means at day 0 or day 28. *a*, P<0.05 vs. 0 day values within the HU - Ex group. *c*, P<0.05 vs. 0 day values within the CC groups.

4.4.3 Cortical Area

There are no differences for cortical area among groups. The cortical area within treatment groups does not change over the 28 period. Furthermore, the countermeasure does not increase the cortical area (Fig.4.7).

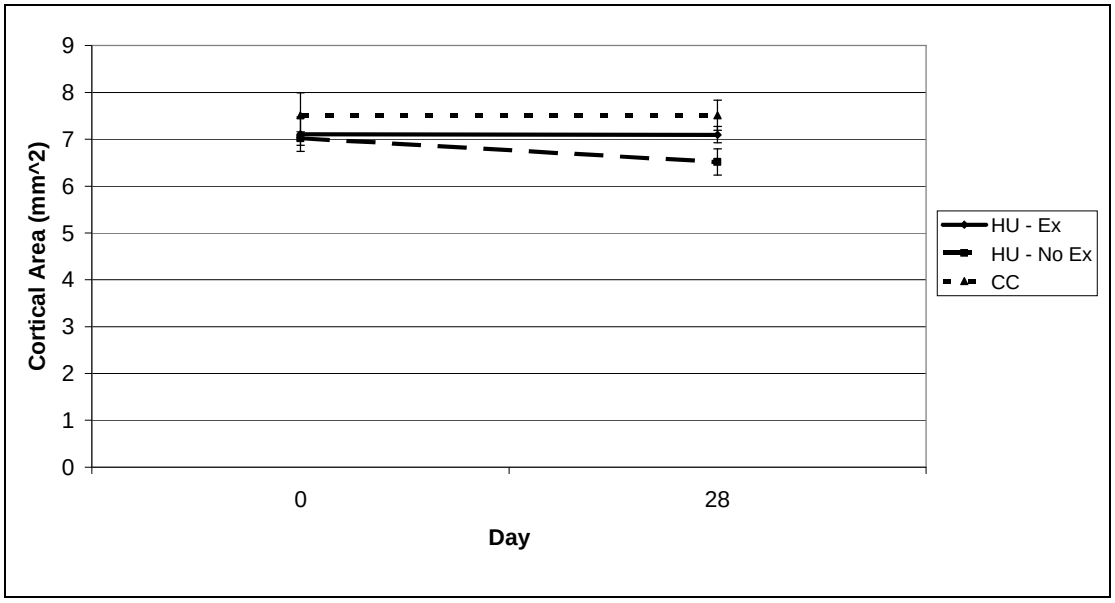

Figure 4.7: Cortical area of the tibial metaphysis during 28 days of unloading. The HU-Ex left leg was subjected to muscle contraction training electrically stimulated. There are no statistically significant relationships concerning the cortical BMC values.

4.5 Tibial Metaphyseal Bone Mineral Density (BMD)

4.5.1 Total BMD

See Figure 4.7 for total BMD data. After 28 days, the HU - NO EX leg has a total BMD 7.9% less than that in CC animals (586.2 ± 10.0 mg/mm^3 vs. 632.7 ± 6.4 mg/mm^3, respectively), consistent with previous studies [9]. The HU - EX received the countermeasure and after 28 days the total BMD is 11% greater than its contra-lateral

limb, HU - NO EX (658.7 ± 15.5 mg/mm^3 vs. 586.2 ± 10 mg/mm^3, respectively). The following figure shows the total BMD at day 0 and 28 (Fig.4.8).

A two way ANOVA determined the statistical significance between the groups. The mean values among the treatment groups at day zero values do not show statistical significance, indicating that all the groups started at the same level of total BMD. This confirms that the randomization of body weight and total BMD before day 0 was successful. At day 28 the mean values of the different treatment groups were significantly different (P = 0.002). Post hoc pairwise comparisons reveals that the HU - EX and CC groups were not different (P=0.11) and the HU - EX and HU – No EX groups were statistically different for total BMD after 28 days (P<0.001). The CC and HU – No EX groups were also statistically different (P=0.001). Exercise training during disuse prevents losses to total BMD due to disuse. The total BMD of the HU – EX and CC are statistically the same and the total BMD of the disuse limb (HU – No EX) is less then CC at day 28.

Differences occur between day 0 and 28 in the different treatment groups. The HU-EX group's total BMD increases 7.8% (607.2 ± 9.2 mg/cm^3 vs. 653.7± 9.7 mg/cm^3) over 28 days (P=0.001). By contrast, the HU- No EX group's total BMD changes only over 28 days 3.9% (609.6 ± 9.2 mg/cm^3 vs. 586.2 ± 10.3 mg/cm^3) which is not statistically significant (P=0.094). The CC group's total BMD marginally increases over the 28 day period by 3.9% (607.8 ± 6.3 mg/cm^{3}vs. 632.7 ± 8.7 mg/cm^3; P=0.025).

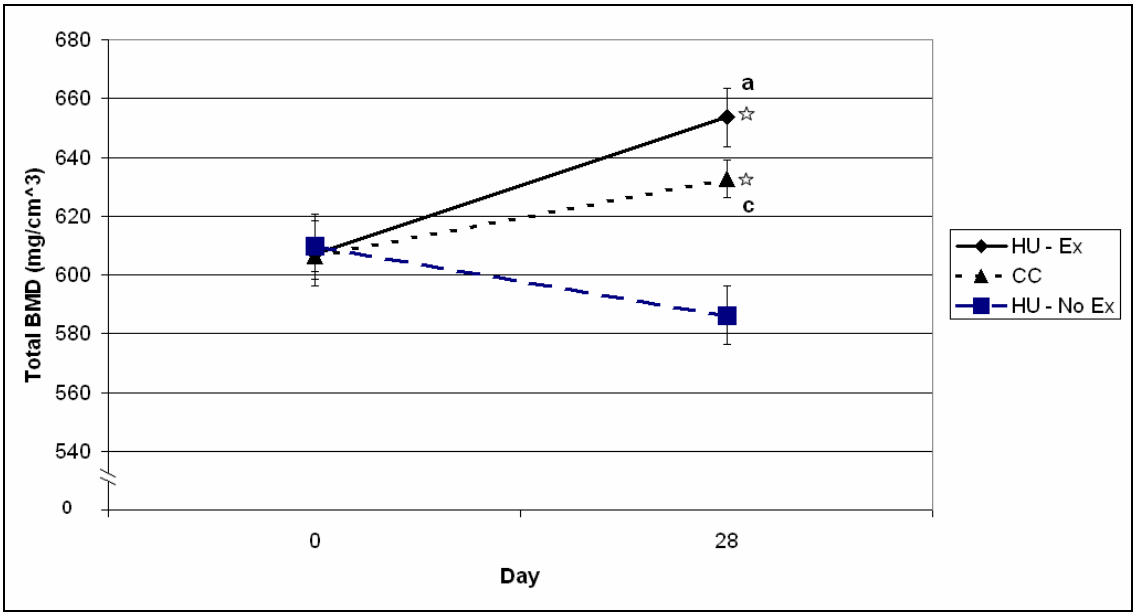

Figure 4.8: Total BMD of the tibial metaphysis during 28 days of unloading. The HU-Ex left leg was subjected to muscle contraction training. * $p<0.05$ when compared to HU-No Ex at day 28. *a*, $P<0.05$ vs. 0 day values within the HU - Ex group. *c*, $P<0.05$ vs. 0 day values within the CC groups.

4.5.2 Cancellous BMD

The following figure illustrates there was not a statistically detectable effect of stimulated muscle training on cancellous BMD in the HU rats. The HU - EX group exhibited a loss in cancellous BMD that were similar to that in the CC group. The HU - EX group had a greater cancellous BMD than the HU - NO EX group at day 28 (213.9 ± 10.42 mg/mm^{3}vs. 189.4 ± 10.2 mg/mm^3, respectively). The countermeasure was unable to raise the cancellous BMD in the tibial metaphysis above that BMD observed in the HU - NO EX group (Fig.4.9).

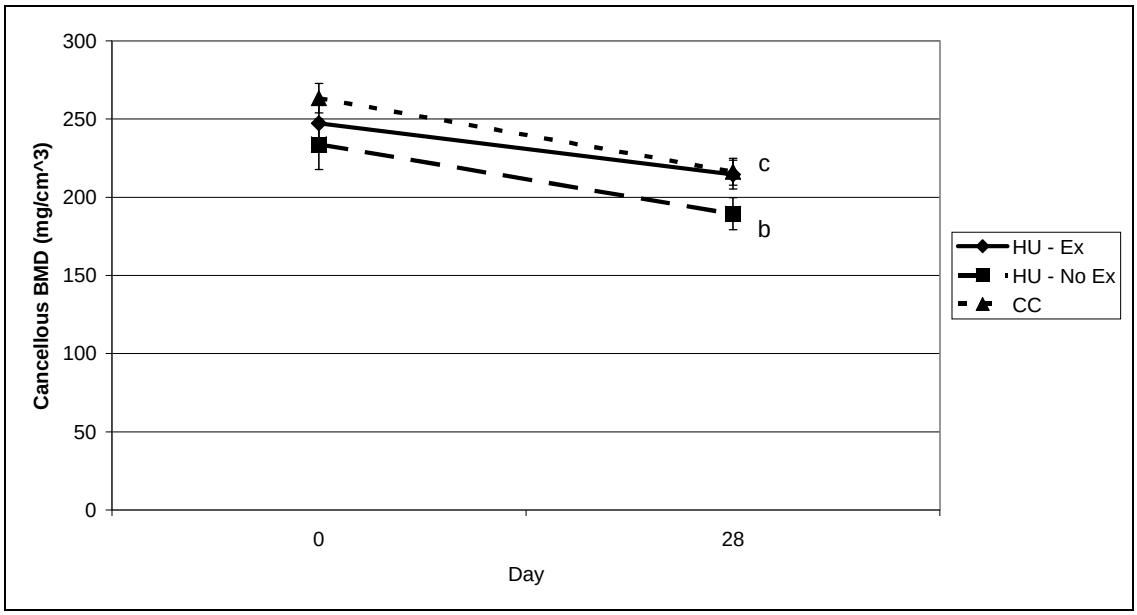

Figure 4.9: Cancellous BMD of the tibial metaphysis during 28 days of unloading. The HU - EX rat's groups left leg was subjected to muscle contraction training. There were no significant differences among group means at day 0 or day 28. *b*, P<0.05 vs. 0 day values within the HU – No Ex group. *c*, P<0.05 vs. 0 day values within the CC groups.

The countermeasure increases the cancellous BMD to the level of its pair feed cage control equivalent; the affect is not statistically different significant between HU - No Ex, HU – Ex, or CC. A two-way ANOVA reveals a statistically time effect ($P<0.001$). The HU- Ex group's cancellous BMD decreased over 28 days 13.3% but was not significant (247.4 ± 11.9 mg/mm^{3}vs. 214.5 ± 12.6 mg/mm^3; P=0.062). The HU- No Ex group's cancellous BMD decreased over 28 days 19.0% (233.9 ± 11.9 mg/mm^3 vs. 189.4 ± 13.3 mg/mm^3; P = 0.016). The CC group's cancellous BMD decreased over 28 days 17.0% (260.1 ± 8.2 mg/mm^3 vs. 216.4 ± 11.4 mg/mm^3; P <0.001).

4.5.3 Cortical BMD

Attenuating the losses of cortical BMD in the tibial metaphysis is attributed to the application of the countermeasure. Cortical BMD in HU - Ex group was 5.7% greater than that of the HU - No Ex group at day 28(1141.28 ± 10.95 mg/mm^3 vs. 1079.79 ± 25.28 mg/mm^3, respectively) but the difference did not achieve statistically significance (P = 0.093). The following figure illustrates the effect of muscle training on a HU animal's cortical BMD (Fig.4.10).

The control group's cortical BMD increases 3.8% from day zero to 28 but not statistical (1065.0 ± 13.0 mg/mm^3 vs. 1107.0 ± 18 mg/mm^3; P = 0.066). The HU – Ex group's cortical BMD increases 5.5% from day zero to 28 (1078.0 ± 18.9 mg/mm^{3}vs. 1141.0 ± 19.9 mg/mm^3; P = 0.025). The HU – No Ex group's cortical BMD does not statistically change from day zero to 28 (1077.0 ± 18.9 mg/mm^3 vs. 1080 ± 21.1 mg/mm^3; P = 0.91).

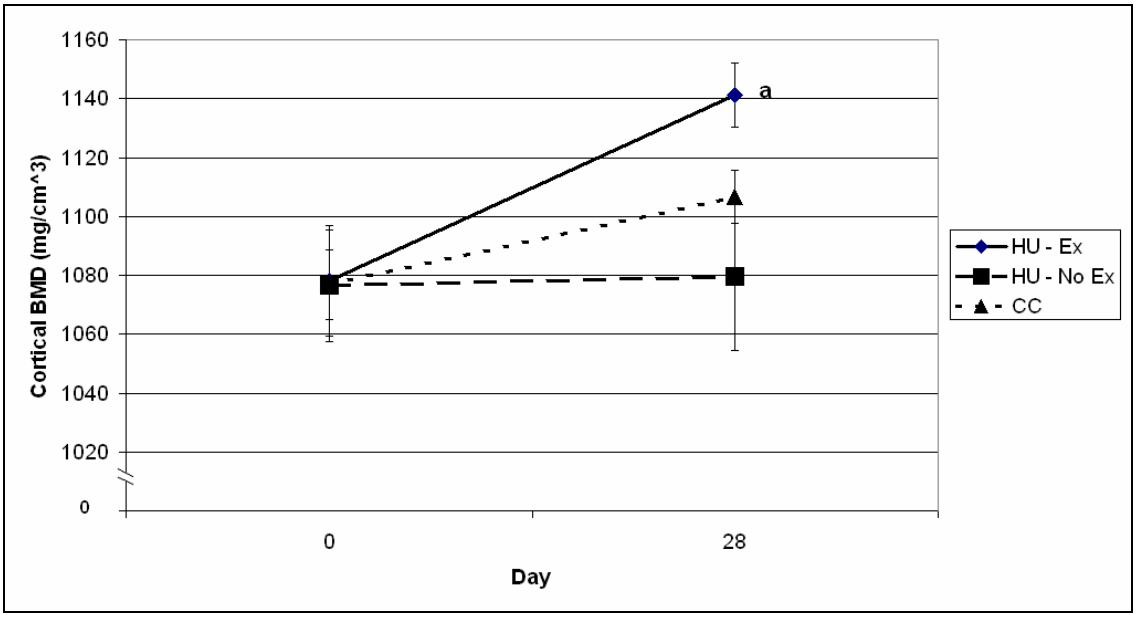

Figure 4.10: Cortical BMD of the tibial metaphysis during 28 days of unloading. The HU - Ex group's left leg was subjected to muscle contraction training. There were no significant differences among group means at day 0 or day 28. *a*, $P<0.05$ vs. 0 day values within the HU - Ex group.

4.6 Mechanical Properties

RPC testing was used to determine the differences in mechanical properties of metaphyseal bone. The countermeasure was found to attenuate of the detrimental effects on the bone integrity of hindlimb unloading at 28 days. Statistical differences for the elastic modulus were found using a One way ANOVA, (P = 0.012). The elastic modulus of the HU - No Ex tibia was 81% lower than that of the HU – Ex group (3.17±1.33 MPa vs. 17.12± 5.81MPa, respectively; P<0.05) and 78.6% lower than the pair feed CC group (3.17±1.33 MPa vs. 14.81± 4.07 MPa, respectively; P<0.05), see Figure 4.11. The CC and HU – Ex group's values are not statistically different.

Statistical differences among the 3 group's ultimate stress values were found using a One way ANOVA, (P = 0.02). The ultimate stress for HU - No Ex tibia was 68% lower than the HU - Ex group (0.17± 0.05 MPa vs. 0.53± 0.14 MPa, respectively; P<0.05). The HU - No Ex group was 53% lower than the pair feed CC group but not statistically significant (0.17 ± 0.05 MPa vs. 0.36± 0.11 MPa, respectively). The HU-Ex and the CC group's values are not statistical different. Figure 4.12 illustrates the ultimate stress of the groups.

Expressing the elastic modulus and ultimate stress as a percentage of CC values allows one to answer: does electrical muscle stimulation attenuate the decreases in the cancellous bone material properties due to hind limb unloading at 28 days? Figure 4.13 shows an increase in elastic modulus from CC of 15.62 ± 39% in the HU - Ex and a decrease of 78.6 ± 8.98% in the HU - No Ex group; these changes are significantly different (P=0.035). The countermeasure dramatically reduces the losses in the elastic modulus due to hind limb unloading. The ultimate stress values are significantly different (P=0.032), and documents that the countermeasure halts decreases in ultimate stress due to hind limb unloading. The countermeasure results in an ultimate stress value that is 48.07 ± 40.01 % higher in the HU - Ex group, where as the HU - No Ex group value is 52.83 ± 13.69 % lower.

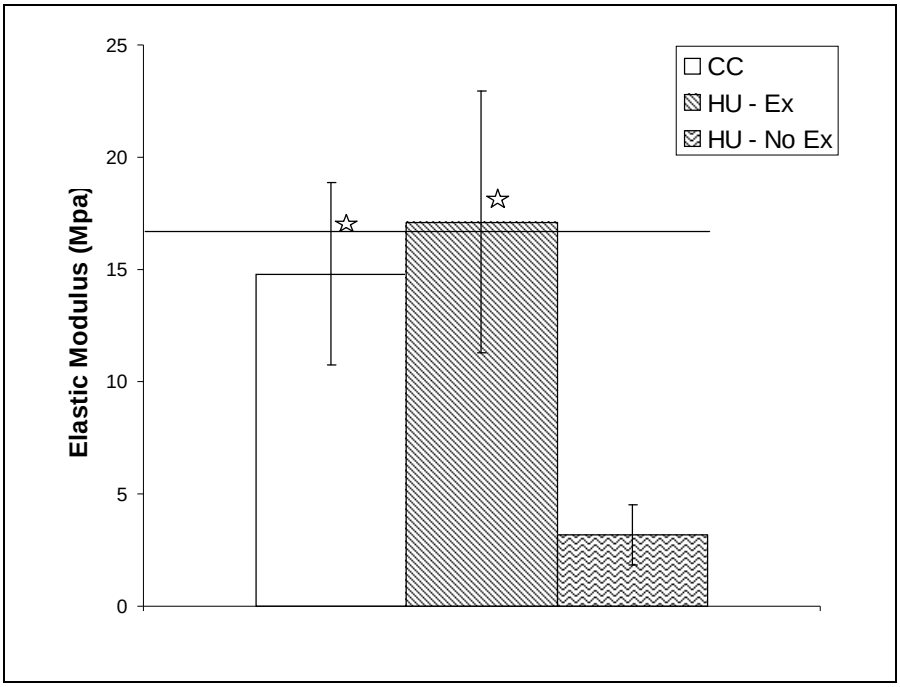

Figure 4.11: The elastic modulus of the tibial metaphysis after 28 days of unloading. The HU - Ex group's left leg was subjected to muscle contraction training electrically stimulated. * $p<0.05$ when compared to HU - No Ex. Line represents day zero baseline group's elastic modulus.

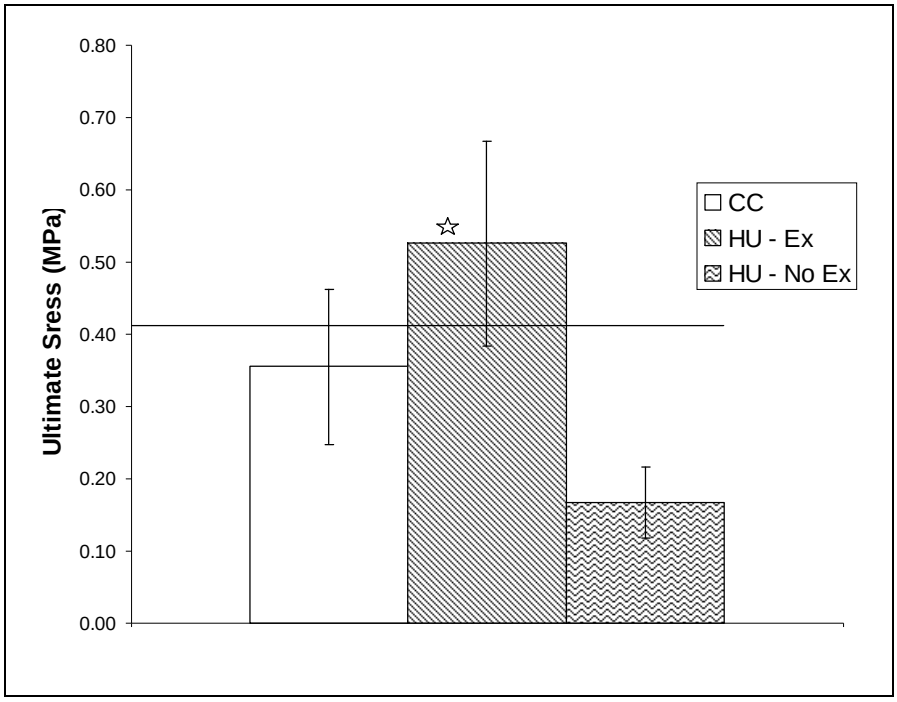

Figure 4.12: The ultimate stress of the tibial metaphysis after 28 days of unloading. The HU - Ex group's left leg was subjected to muscle contraction training. * $p<0.05$ when compared to HU - No Ex. Line represents day zero baseline group's ultimate stress.

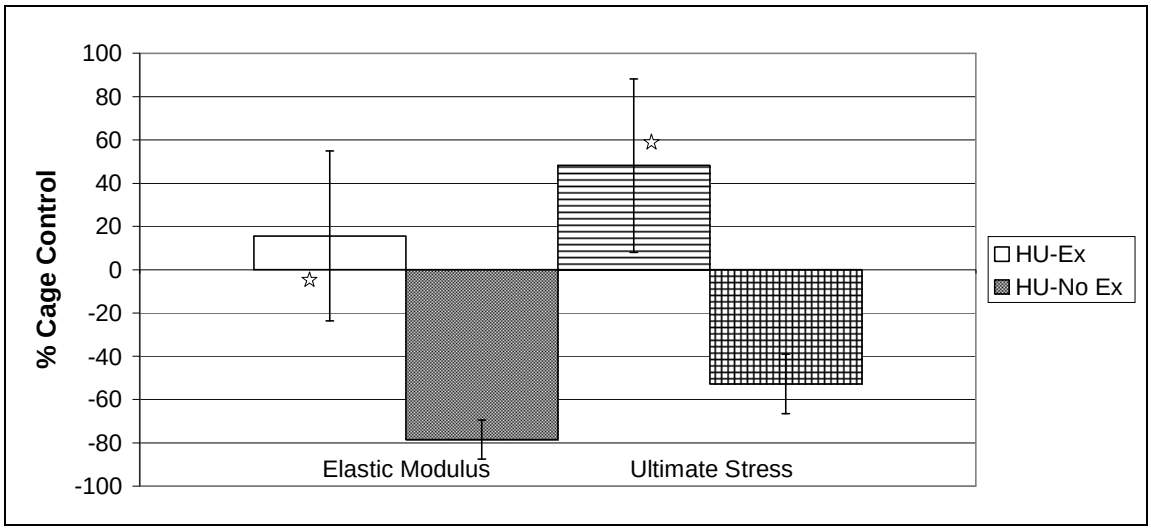

Figure 4.13: The changes in intrinsic properties expressed relative to CC values. * $p<0.05$ when compared to HU - NO EX..

4.7 Summary

See Table 4.1 for a summary of all this studies data and statistical significance.

Table 4.1: Complete summary of the results obtained at days 0 and 28.

	Day	BL	CC	HU - EX	HU - NO EX
Total BMD (mg/cm^3)	0	609.24 ± 7.73	606.52 ± 5.34	607.22 ± 9.47	609.59 ± 11.09[c]
	28		632.67 ± 6.40[a,b]	658.66 ± 15.4[a,b]	586.17 ± 9.95
Cancellous BMD (mg/cm^3)	0	216.36 ± 8.63	263.35 ± 9.51	247.42 ± 13.77	233.93 ± 16.13
	28		256.52 ± 20.92[b]	214.50 ± 9.21	189.45 ± 10.20[b]
Cortical BMD (mg/cm^3)	0	1052.11± 20.92	1076.89 ± 21.16	1078.24 ± 18.71	1076.52 ± 18.96
	28		1106.64 ± 14.67	1141.28 ± 10.95[b]	1079.79 ± 25.28
Total BMC (mg)	0	10.96± 0.30	11.37±0.27	10.98± 0.27	11.87± 0.29
	28		10.99± 0.20	11.02± 0.26[c]	9.03 ± 0.19[b,c]
Cancellous BMC (mg)	0	2.74± 0.29	2.92± 00.22	2.74± 0.25	3.35± 0.29
	28		2.06± 0.15	1.95± 0.21[b]	1.85 ± 0.16[b,c]
Cortical BMC (mg)	0	7.59± 0.25	7.86± 0.17	7.60± 0.15	7.60 ± 0.08
	28		8.06± 0.08[,b]	8.15±0.10[,b]	6.56 ± 0.13[b,c]
Total Area (mm^2)	0	18.93 ± 0.40	18.81 ± 0.57	18.45 ± 0.39	17.73 ± 0.72
	28		17.51 ± 0.40[b]	16.34 ± 0.54[b]	15.84 ± 0.39[b]
Marrow Area (mm^2)	0	10.98 ± 0.39	10.90 ± 0.44	10.66 ± 0.40	9.76 ± 0.58
	28		9.27 ± 0.33[b]	8.24 ± 0.47[b]	8.86 ± 0.36
Cortical Area (mm^2)	0	7.60 ± 0.40	7.51 ± 0.48	7.11 ± 0.11	7.02 ± 0.14
	28		7.51 ± 0.32	7.09 ± 0.06	6.51 ± 0.27
Elastic Modulus (MPa)	0	17.31 ± 0.42			
	28		14.81 ± 4.07[a]	17.12 ± 5.81[a]	3.17 ± 1.33[c]
Ultimate Stress (MPa)	0	0.419± 0.087			
	28		0.357± 0.107	0.526± 0.14[a]	0.167± 0.049
Body Weight	0	417.5 ± 9.24	431.09 ± 11.80	418.7 ± 8.53	418.7 ± 8.53
	28		452.09 ± 8.96[a]	383 ± 7.32[b,c]	383 ± 7.32[c]

a, statistical significance when comparing to HU - NO Ex.

b, statistical significance when compared to day 0.

c, statistical significance when comparing to CC.

5. DISCUSSION

5.1 Purpose and General Findings

The study's aim was to investigate the utility of high intensity resistance type training as a countermeasure to hindlimb suspension. The tibial metaphysis in a disuse environment is observed to have a decrease in mechanical properties, density, and mineral content. An effective countermeasure has matching levels of mechanical properties, bone density and bone mineral content between the HU + Ex and the age match pair fed control. Based on the data presented, the countermeasure appears to attenuate losses in intrinsic mechanical properties due to disuse. The elastic modulus and ultimate strength of the exercised limb are higher than the non-exercised limb. The total BMC and total BMD of the exercised limb are also higher than the non-exercised limb.

5.2 Procedural Caveats

5.2.1 Hindlimb Suspension

Hindlimb unloading via tail suspension provides a ground based model for space flight. The skeletal changes in the suspended animals are similar to changes seen in rats exposed to space flight using histomorphometric analyses of bone cell activity [27]. However, differences in bone response vary between tail suspension and space flight. "Osteoclasts and active resorption surfaces increase 2-fold within 7 days of tail suspension, but was not affected by micro-gravity." [15] There are dissimilarities in osteoblast activity between the tail suspension and space flight. Greater declines of cancellous osteoid formation sites are seen in space flight as opposed to HU (55% less vs 22% less) [15]. During space flight bone formation decreases, while hindlimb suspension causes smaller decreases in formation and increases in bone resorption. The model is not an exact analog for space flight but the bone exhibits a similar behavior.

The hindlimb suspension method creates animal welfare issues. If the harness is too wide it will impede blood flow creating sores on the tail. Eventually, the rat's tail can become necrotic. If tail necrosis occurs the animal is sacrificed immediately, in an effort to humanely treat animals.

The suspension sling is attached with marine glue. Occasionally the natural exfoliation of the skin allows the sling to separate from the rat's tail. This allows the rat to ambulate freely, loading its bones. Thus the animal is not in a pure disuse environment. If sling separation commonly occurs the disuse model is not realistic and will not adequately replicate microgravity.

5.2.2 Simulated Resistive Training via Electrical Muscle Stimulation

During the muscle stimulation protocol the foot attaches to the servo motor using a plastic foot mold and tape. The fore foot is securely fixed to the pedal but the heel can still separate from the footplate. It is important to maintain consistent contact between the foot and the footplate to ensure that the force of the contraction is effectively transmitted to the footplate.

The knee is clamped to keep the femur and the tibia at 90 degrees. During a contraction, the torque produced occasionally (less than 5 times) overcomes the restraining force of the clamp, allowing the knee to move with the exercise. This occurred vary rarely during the initial training sessions before the HU animals lost muscle. If this was observed, the clamp strength was supplemented by the operator.

Stimulated muscle contractions are not the same as voluntary exercise, because the animal is anesthetized during the exercise protocol. The unconscious animal does have a generally active sympathetic nervous system. The sympathetic nervous system's outflow affects the distribution of blood flow during exercise. In a rat model, the animal is sedated during electrically lowering the sympathetic activation in the skeletal muscles more than in the kidneys. This leads to less vasoconstriction in the skeletal muscles, as opposed to the kidneys. It is suggested that "neural mechanisms mediated by central command contributes to the blood flow distribution by evoking differential sympathetic

outflow during exercise" [28]. To summarize, the anesthetized rat's sympathetic nervous system (SNS) is generally inactive and thus unable to increase blood flow to its muscles by way of the SNS. The decrease in blood flow to skeletal muscles due to SNS mediation is a physiological difference when comparing electrical muscle stimulation to voluntary exercise.

Stimulated muscle contractions provide some significant advantages: the ability to systematically control the velocity of the movement, angle of rotation, and level of exercise intensity. This advantage is not available with any current voluntary exercise paradigm for rodents. The direct control of muscle torque, movement and duration of contraction allows investigators to precisely control the level and type of exercise.

5.2.3 Issues Related to the pQCT Method

5.2.3.1 Discussion of Decreases Observed in Longitudinal Area Data

The total cross sectional area of all three groups decreased over the 28 day study. The 7% decrease in total area from day zero in the CC group's tibia is not consistent with previous studies, which usually indicate no decrease in this parameter over 28 days [7, 9]. In this study, the HU group's total area of the tibial metaphysis at 28 days is less than the control group while not statistically significant; this is consistent with previous studies [9]. In a previous 2 week study the total area of the cage control increased slightly while the HU group decreases [29]. The unexpected change in this metaphyseal bone site's longitudinal total area in weight bearing animals raises questions concerning the pQCT methodology.

5.2.3.2 Systematic Distal Positioning Error

The decrease in area may be due to positioning error when scanning the animal. Day 28 scans may be more distal than the day zero scan. The basic shape of the proximal tibia is such that the total cross-sectional area decreases with the distance (distally) from the tibial plateau. To support this observation, the rate of total area

decrease within groups is similar (Fig 4.5), thus leading one to believe that scans taken at day 28 were performed at more distal sites than on day zero. However, this inconsistency is unlikely because all scans were not performed at the same time. The experiment was preformed in 2 staggered cohorts. Any positioning errors would not be isolated to only day 28 and 56. A consistent error with 4 weeks of spacing is unlikely.

It is possible that the distal positioning error is due to changes in longitudinal growth. Consistent positioning of the scout view coupled with slight longitudinally growth would yield a different area, illustrated in Figure 5.1.

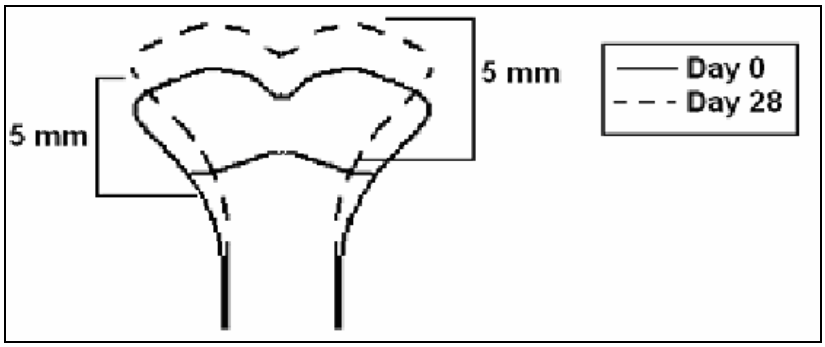

Figure 5.1: Longitudinal growths vs. area.

This scenario suggests that it is possible for total area to appear as if it is decreasing over time. The change in area may be an artifact of longitudinal growth and positioning. A comparison of day 0 and day 28 bone lengths and geometries would yield insight into this concern, but these data are not readily available.

The cage control total BMC does not change over time (see Fig 4.2). Previous observations show that the aging Sprague-Dawley rat's cancellous BMC and cancellous BMD is constant between 3 and 6 months when using pQCT to determine these properties [30]. Distal positioning errors would cause the total BMC to decrease, if the BMD is constant and the defined bone area decreases. The data in this study show the total BMC is constant for the cage control group. The cage control group has a higher BMD in a smaller total area at 28 days as opposed to day 0.

The BMD is least affected by positioning error because the total BMD is the total BMC normalized by the area and a constant thickness. Therefore one can surmise that the calculation of BMD abates the affects of positioning error; the BMD is an intrinsic property. The use of BMD to quantify tissue level bone properties is more transparent than the use of BMC because BMD is normalized by area.

5.2.3.3 Definition of the Cortical and Marrow Area

The contrast between unmineralized tissue and bone mineral is large enough to obtain the total area values easily. The contrast between cortical and cancellous bone is not as clear. Different threshold values within the pQCT instrument, coupled with peeling algorithms, systematically sort mineralized tissue into cancellous and cortical compartments. The identification of layers within the total specimen depends upon the density of the cancellous and cortical bone. The layers include cortical, sub-cortical, cancellous, and sub-cancellous. The cancellous and cortical layers are reported, while the sub-cortical and sub-cancellous layers are not. The exclusion of the sub layers may decrease the precision of layer separation.

Problems arise when disuse alters the cortical and cancellous border. Disuse is known to stimulate increased osteoclastic activity along the endocortical border, creating large resorption cavities and creating new trabeculae out of what used to be cortical bone [(13)]. The analysis may sort a portion of cortical bone as cancellous bone, if excessive resorption has occurred at the endocortical surface. Furthermore the program creates sub regions, which do not clearly belong in either cancellous or cortical layers.

5.3 Avoiding the Problems with Decreases in Longitudinal Area

The significant decrease over time in total area even in the cage control group over the course of only 28 days was unexpected. However, the purpose of the study is to investigate the intrinsic bone properties (total BMD, elastic modulus, and ultimate stress) between treatment groups at the end of the study. At day 28, the total area of CC was larger than HU-Ex and HU-Ex was larger than HU – No Ex, while not statistically

different. The longitudinal total area datum does not provide the expected relationship, comparisons between treatment groups at day 28 are the most important and remain valid and revealing.

The control rat's total BMC would appear to decrease over time if there were distal positioning errors. There is a smaller cross section in a more distal sample. The mineral content of the CC group should not change over time. Since the BMC is constant while the area decreases, the percentage of mineralized tissue must increase to maintain a constant BMC. Distal positioning variances may be responsible for total area anomalies; however, the consistency of the cage control's longitudinal total BMC creates ambiguities with this explanation.

The study is primarily interested in the density and intrinsic mechanical properties of the proximal tibial metaphysis between treatment groups. The density is normalized over the volume of bone and this decreases the effect of positioning errors. The mechanical properties investigate the tissue level material properties. The total area is an extrinsic property which is not of primary interest in this study.

5.4 Changes in the Material Properties

5.4.1 Change in Intrinsic Mechanical Properties

Elastic Modulus of the HU - Ex group was significantly higher than the HU - No Ex group. As compared to the cage control group values the HU - Ex group's elastic modulus was not significantly different, or equal (see Fig 4.11). The countermeasure negates the detrimental effect of hindlimb suspension on the elastic modulus to the same level as its age matched pair fed control.

Ultimate Stress of the HU - Ex group was significantly higher than the HU - No Ex group. The cage control group values compared to the HU - Ex group's ultimate stress was not significantly different, or equal (see Fig 4.12). The resistive muscle training counteracts the detrimental effect of hindlimb suspension on the ultimate stress. The ultimate stress at 28 days is effectively equal to its age matched pair fed control.

We are not aware of another study which investigates the effect of resistive exercise during hindlimb suspension, on the elastic modulus and ultimate stress of cancellous bone in the proximal tibia. Previous studies have investigated the effect of resistive exercise on total BMD in the tibial metaphysis [36, 34] but have not addressed the mechanical properties of the cancellous bone in the proximal tibia.

5.4.2 The Relationship between BMD and Mechanical Properties

This section investigates the site-specific-on-axis modulus to density relationship of the proximal tibial metaphysis. There is not a clear linear relationship between elastic modulus and BMD or the ultimate stress and BMD. A previous investigation used a power law regression to relate the elastic modulus and BMD. The relationship between BMD and elastic modulus indicates that small changes in total BMD can lead to large changes in mechanical properties [31]. Machined cylinders of cancellous bone provide material properties independent from the cortical bone.

When the cancellous BMD exceeds 250 mg/cm^3 in the machined cylinders of cancellous bone from the human proximal tibia the elastic modulus begins to rise rapidly. For instance, when the cancellous BMD is raised from 200 mg/cm^3 to 250 mg/cm^3 the elastic modulus increases from 500 MPa to 750 MPa. A change in cancellous BMD from 250 mg/cm^3 to 300 mg/cm^3 increases elastic modulus from 750 MPa to 1750 MPa [31]. As the cancellous BMD increases and passes a critical threshold the mechanical properties begin to change rapidly. While a modulus to density relationship may provide some degree of predictive insight, it does not explain changes in bone architecture responsible for changes in the modulus [31].

The cancellous BMD vs. elastic modulus and the total BMD vs. elastic modulus create a generally similar relationship in the rat proximal tibia in the current study (Fig 5.2 and 5.3). The cancellous BMD is expected to have a more direct relationship because RPC testing directly loads the cancellous bone only.

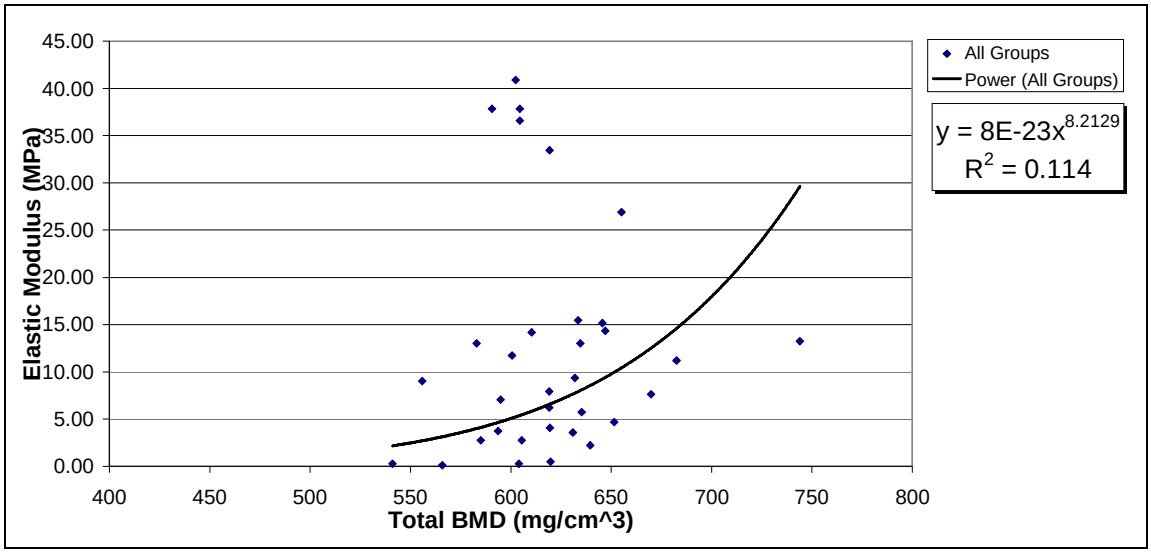

Figure 5.2: Total BMD vs. elastic modulus. A power law relationship indicates that small changes in total BMD have larger impacts on elastic modulus.

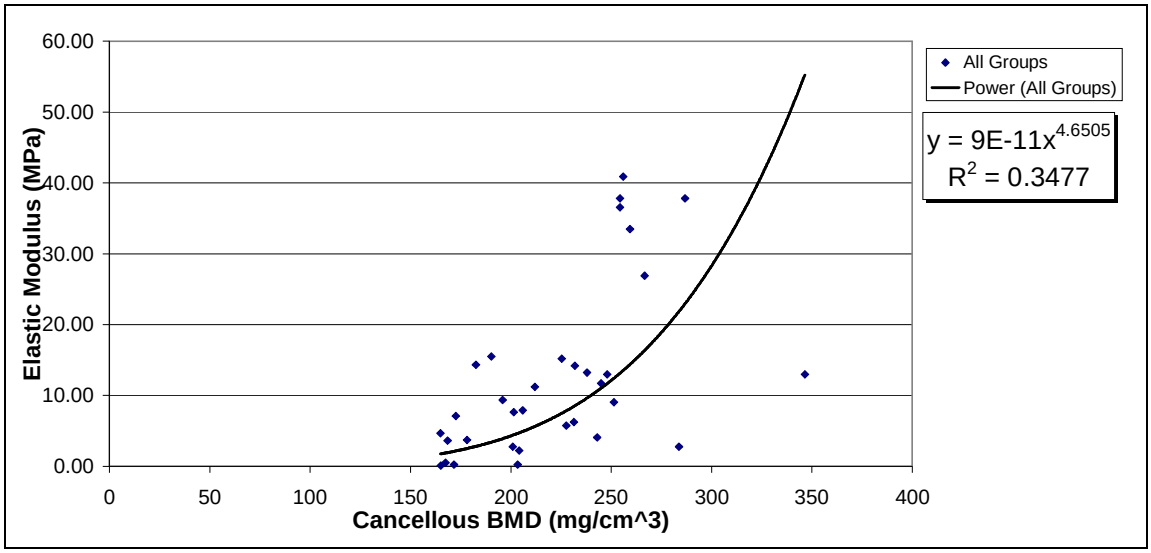

Figure 5.3: Cancellous BMD vs. elastic modulus. A power law relationship indicates that small changes in total BMD have larger impacts on elastic modulus.

A relationship between the total BMD and modulus is desired because of the statistical differences exhibited by the group's total BMD values. The relationship between total density and elastic modulus is not clear because the cortical bone is denser than cancellous bone. When cortical bone and cancellous bone data are combined the effect of cancellous bone is not as prominent.

The low R^2 value associated with the regression line between the elastic modulus and total BMD (Fig 5.2) when compared to the cancellous BMD (Fig 5.3) indicates that the total BMD is not suitable choice. It is interesting to note, that the elastic modulus begins to increase swiftly after the cancellous density exceeds 250 mg/cm^3 but the values are highly variable. The variability of the data points is due to the quality of the cancellous bone in the samples. The samples were not machined cancellous bone. An interaction between the cortical and cancellous bone during compression will affect the elastic modulus.

Ultimate stress in the proximal tibia exhibits a similar relationship with total and cancellous BMD (Fig 5.4 and 5.5). Small changes in cancellous BMD cause large changes in ultimate stress. The total BMD does not exhibit as strong of a relationship with the ultimate stress as it does with the cancellous BMD. The low R^2 value associated with the regression line indicates that this is not an effective model.

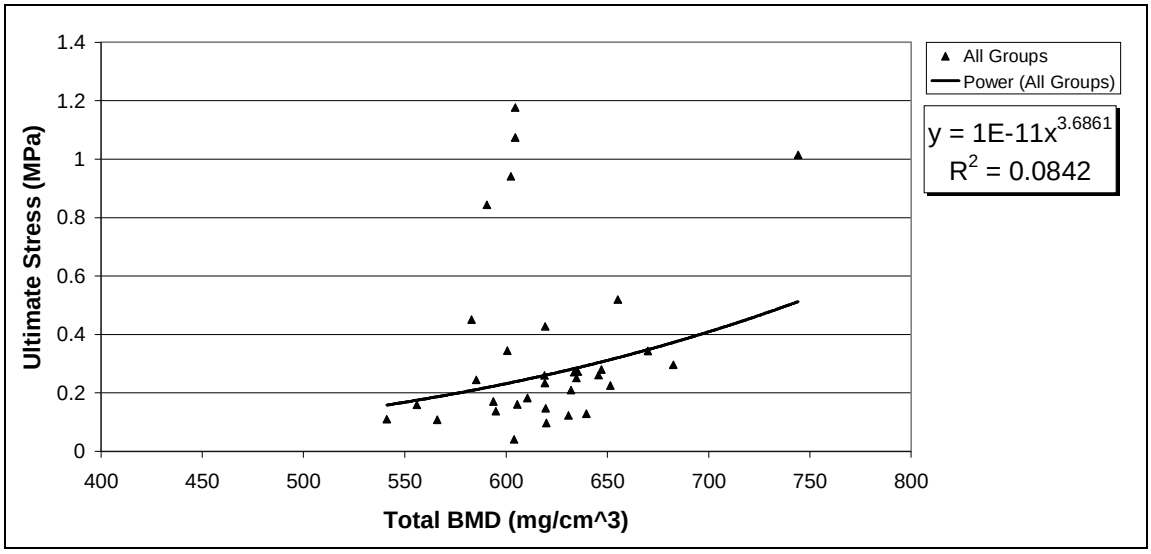

Figure 5.4: Total BMD vs. ultimate stress. Using a power law relationship to indicate that small changes in total BMD have larger impacts on ultimate stress.

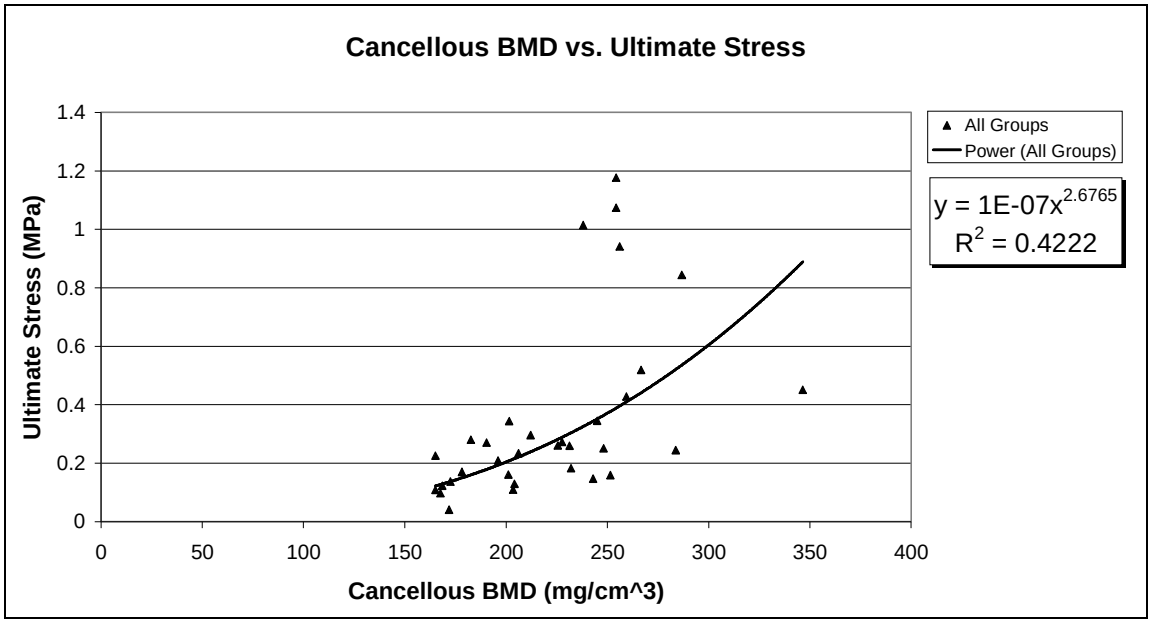

Figure 5.5: Cancellous BMD vs. ultimate stress. Using a power law relationship to indicate the small changes in total BMD have larger impacts on ultimate stress.

There are limitations to the predictive power of a modulus-to-density relationship and ultimate stress to density relationship. The modulus-to-density relationship has limitations in its predictive power. The cancellous bone density is not the only factor mediating the material properties. Variations in the trabecular architecture are dominant forces in determining the tissue level properties of cancellous bone and should be included in the development of a predictive model [31]. Trabecular architecture information is not available with pQCT. This indicates that models using only BMD to predict tissue level properties are not comprehensively characterizing the bone.

5.5 Comparison of Previous HU + Exercise Models

This section discusses different methods of exercise interventions used during disuse. Differences include whether the loading pattern is physiological, if the applied loads are of a physiological magnitude, and what material properties the studies quantify. The following studies will help illustrate the unique niche that the current study fills. The exercises vary whether the loading pattern are physiological, load magnitudes are larger than a rat will exert voluntarily, or if the animal's muscles create the loads. Not all exercise protocols afford the investigator the ability to precisely control the intensity of the contractions, nor do they report intrinsic mechanical properties and bone mineral density of cancellous bone. Other existing studies do not provide the design advantages and unique outcome measures found in this study.

5.5.1 Electrical Muscle Stimulation of the Free Hanging Limb during HU

Forty-five male Windsor rats (9 weeks old) received resistive muscle contractions for 10 days [32]. The groups in the study were CC, HU, HU-exercise at 50 Hz, and HU-exercise at 100 Hz. The exercise protocol dictates the application of surface electrodes to the hind quarter of the adult HU animal at 1, 50, and 100 Hz. The protocol consisted of contraction durations of 1 second, followed by 4 seconds of rest and continued for 4 hours (2880 contractions total). The animals rest for 6 hours following

an exercised. The animals exercise twice a day for 10 days. The legs hang freely during the exercise. The animals were anesthetized during the procedure [32].

There was not a statistical change in tibial BMD, measured by dual energy X-ray absorptiometry (DEXA), between any HU + exercise group and the HU group. The BMD was not significantly different between any groups. The BMC of the 50 Hz exercise group was significant greater when compared to the suspended non-exercised group. HU+exercise 50 Hz was significantly grater than its non-exercised contra-lateral control. The changes in BMC support the notion that stimulation of the muscles creates loads which can abate detrimental effects due to hindlimb unloading [32].

This study did not investigate the mechanical properties associated with the combination of HU and exercise. Furthermore, its exercise protocol does not measure the forces applied to the tibia during electrical muscle stimulation. The protocol is very aggressive in the number of cycles the animal receives, but does not quantify the magnitude of each contraction. The loading magnitude has been shown to be a factor in determining the osteogenic effect of a countermeasure [33]. The absence of change in the BMD may be due to the length of study or the exercise protocol. The length of the study was too short because the resorptive phase of remodeling is estimated to last 10 days in cancellous bone. This resorptive phase is followed by bone formation that takes approximately 3 months [13]. Therefore, to observe changes one should choose a study length that will allow the bone to enter the formation stage. The exercise protocol does not insert long enough rest periods between contractions. Evidence suggests that applying a given number of loads multiple times a day is more osteogenic than applying the same number of loads in a single daily session [5].

5.5.2 Voluntary Flywheel Exercise of HU Rats

Male, Sprague–Dawley rats (n = 14; 6-month old) were used for this study [34]. Resistance exercise training using flywheel technology attenuated skeletal muscle mass losses during 4 weeks of adult rat hindlimb suspension. Before the actual experiment began, all rats in the study were trained to perform a squat-like exercise in an upright

box in response to a light stimulus. The hindlimb unloaded group performs the squat like exercise in a horizontal box while maintaining suspension. The exercise consisted of 25 repetitions over the rat's maximum range of movement, performed 3 days a week for 4 weeks for a total of 11 sessions. [34]

After 4 weeks total BMD at the distal femur, using DEXA, decreased 7.7% from day zero in the hindlimb unloaded rat. The cage control rats decrease in total BMD was not statistically different from HU + exercise group at the distal femur. This indicates that the administration of the exercise negates the detrimental effects of disuse on BMD [34].

The flywheel exercise protocol is a voluntary exercise. The effect of the active sympathetic nervous system may have an effect on bone health. The effect on total BMD in long bone metaphysis is similar to that observed after electrical muscle stimulation. Both methods attenuate bone loss from a cancellous site during hindlimb suspension. However, the flywheel exercise study does not address the changes to cancellous bone's mechanical properties. Nor does it allow investigators to manipulate the level of exercise intensity. The exercise ultimately depends on animal motivation and voluntary precipitation.

5.5.3 Limb Immobilization, Mechanical Loading, and Calcium Restriction

This study's objective was to investigate the effect of mechanical loading on the bone properties over a six week period of disuse concurrent with feeding of calcium deficient diet to 28 female Sprague-Dawley retired breeder rats, aged 5 months [35]. The groups were randomly assigned to cage control, immobilized, and immobilized + loading. The immobilized and immobilized + loading rats received low calcium diet (0.1% calcium), whereas the cage control group received standard rat chow (1.85 % calcium). If calcium deficiency is coupled with the effects of disuse, the rate of bone loss is accelerated because of the resultant increase in serum parathyroid hormone (PTH). Chronically elevated serum PTH can contribute to increased rates of bone resorption and loss of bone mass [35].

The right hindlimb was immobilized by taping it to the body. The rats were able to ambulate normally on three legs. The immobilized + exercise group received an external loading treatment from a four-point loading device. A cartoon describing the positions of the applied loads is given in Fig. 5.6.

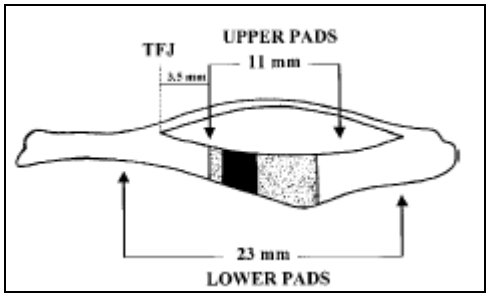

Figure 5.6: Locations of the applied loads during 4 point bending. Loading extends 3.5 mm from the tibiofibular junction (TFJ) to 14.5 from the TFJ. The lateral surfaces experience tension while the medial surface experiences compression [35].

The loads were applied 3 days a week for 5 weeks, starting a week after the initiation of immobilization using loads of 32 N at 2 Hz for 36 cycles. At no time during the experiment did the rat bear weight on the immobilized limb. Three point bending determine the mechanical properties of the tibia [35].

After 6 weeks the tibia's total BMC and BMD at midshaft in the immobilized group was significantly lower (10 and 12 %, respectively) than in the cage control group. The total BMC and BMD at the tibial midshaft in the immobilized + exercise group were significantly greater than the immobilization group values and equal to the cage control's values.

The intrinsic properties (elastic modulus and ultimate stress) at the tibial midshaft did not show a change with immobilization. The extrinsic properties were different among groups. The ultimate load of the immobilized group was 19 % lower than cage control, but the ultimate load in the immobilized + exercise group was greater than that of the immobilized group and not different from the cage control group. The stiffness of

the immobilized limb was 20% lower than cage control. The immobilized + exercise limb stiffness was not statistically different from the cage control or the immobilized limb group. The cross sectional moment of inertia (CSMI) of the immobilized and immobilized + exercise groups was 20% and 8% lower, respectively, compared to cage control values [35].

The smaller CSMI accounted for changes to the extrinsic properties. The intrinsic properties did not change, implying that the tissue level material properties were not affected by immobilization or external loading. The application of a loading protocol effectively attenuated the losses seen due to immobilization and calcium deficiency. However, the loading protocol used is not physiological, since the load is not applied by the muscle but externally. An advantage the investigators had was the able to control the intensity of the loading. The animals are sedated which is similar to stimulated muscle contractions, but as stated previously, this could be a disadvantage because the sympathetic nervous system is not active. It is important to note that this study's region of interest was the mid-diaphysis of the tibia, which consists of cortical bone. The current study focuses on the proximal metaphysis of the tibia, which is a site of mixed cancellous and cortical bone.

5.6 Conclusion

The density, area, BMC and intrinsic properties of the tibial metaphysis decrease during hind limb suspension. The changes in the proximal tibia total density (a decrease of 3.9% in total BMD from day 0 in group HU - No Ex) are consistent with previous work [7]. The addition of a countermeasure negated the detrimental affects of hind limb unloading on the total BMD and the intrinsic mechanical properties of cancellous bone in the proximal tibia.

Changes to BMD and BMC without observable changes in cross sectional area create a material with greater tissue level mechanical characteristics. The application of the countermeasure attenuated losses due to disuse. The intrinsic properties provide a transparent picture of the materials behavior. The application of the countermeasure

abated material changes caused by disuse. As the bone is deformed during exercise, the body does not remodel the bone to adapt to disuse but maintains its structure to match that of its pair-fed cage control. The countermeasure's physiological loading pattern and loading level is rigorous enough to avoid losses due to disuse.

6. SUMMARY OF FUTURE WORK

6.1 Current Problems

6.1.1 Absence of HU Control Group

The addition of an HU control group with no training intervention would account for any possible systemic affects of the countermeasure. It is not expected that there is a measurable systemic affect but the current study design cannot prove this. This study's non-stimulated HU limb (HU - NO EX) had a decrease in total BMD of 3.7% decrease with respect to baseline. This decrease is equal to that of previous studies, with no training or other countermeasure applied during HU [(9)].

6.1.2 Countermeasure May Cause Muscle Damage

Muscles were harvested at necropsy and should be analyzed for muscle damage. For animal welfare concerns, one should determine if eccentric contractions at 120% of peak isometric torque cause muscle damage. Optimizing the level of eccentric contractions to a level that doesn't damage muscle will help create a more physiological loading pattern. The determination of the lowest contraction intensity which causes muscle damage will determine an upper bound for the countermeasure intensity. It should be noted, however, that mild muscle damage is routinely observed after resistance training or unaccustomed exercise and is an important stage in events leading to muscle hypertrophy.

6.2 Vary Intensity of Countermeasure

The intensity of eccentric contractions was based on a percentage of the peak isometric torque. The countermeasure should be optimized to the greatest osteogenic affect. A future study should contain an experiment which systematically varies the intensity and the duration of total eccentric contraction. The contraction intensity

should range between 100% of peak isometric torque up to the level found to not damage the muscle.

6.3 Strain Level to Projected BMD Relationship

Strain levels have been measured using uni-axial strain gages. The use of tri-axial strain gages may characterize strain more fully. Exploring the relationship relating strain levels and changes in BMD over a longitudinal study would create a way of predicting the osteogenic potential of a countermeasure. A predictive relationship at a particular site will help determine the osteogenic potential of other loading methods.

For instance, the flywheel and muscle stimulator exercise protocols are to be compared during upcoming. During the operation of the flywheel the *in vivo* strain gage is implanted medial proximal diaphysis surface of the bone. During the exercise the strain is recorded. The use of the site specific predictive relationship between strain levels and changes in BMD over a longitudinal study will aid in the choice of the best exercise regime.

6.4 Drug and Exercise Experiments

The next phase is a series of experiments that varies levels of bisphosphonates and countermeasure. It is desirable to quantify the effect of bisphosphonates and exercise level attenuation on the detrimental affects of hind limb unloading. The combination of two countermeasures may more fully attenuate bone loss due to disuse.

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APPENDIX A

ELECTRICAL MUSCLE STIMULATION DATA

Table A-1: Voltage (V) for peak isometric torque at 175 Hz

	1	2	3	4	5	6	7	8	9	10	11	12
1504	4	4	5	3	3	4	3	4	4	6	3	4
1506	4	3	4	4	4	5	4	4	4	6	8	5
1507	5	5	4	5	5	4	3	4	4	7	6	5
1519	3		3	3	3	3	4	3	8	3	3	3
1520	3		5	3	3	4	3	3	4	3	3	3
1522	3		3	4	3	4	3	4	5	3	3	3
1523	4		3	3	4	3	5	4	4	4	3	3
31530	4		5	5	3	3	3	3	6	3	3	4

Table A-2: Peak isometric (N/mm) torque at 175 Hz

	1	2	3	4	5	6	7	8	9	10	11	12
1504	0.24	0.24	0.22	0.25	0.21	0.23	0.21	0.21	0.24	0.23	0.25	0.25
1506	0.20	0.32	0.27	0.22	0.23	0.24	0.24	0.21	0.27	0.28	0.24	0.23
1507	0.22	0.20	0.18	0.19	0.26	0.19	0.16	0.18	0.16	0.18	0.15	0.15
1519	0.25		0.24	0.25	0.26	0.23	0.27	0.23	0.19	0.23	0.24	0.25
1520	0.21		0.18	0.19	0.19	0.19	0.19	0.18	0.16	0.18	0.18	0.19
1522	0.25		0.24	0.21	0.22	0.24	0.20	0.22	0.22	0.21	0.21	0.22
1523	0.26		0.23	0.29	0.21	0.22	0.25	0.24	0.24	0.24	0.27	0.27
1530	0.23		0.21	0.21	0.20	0.20	0.21	0.26	0.20	0.22	0.22	0.22

Table A-3: Frequency need for each 120% peak isometric torque

	1	2	3	4	5	6	7	8	9	10	11	12
1504	60	60	60	60	55	60		60	48	45	50	47
1506	60	60	60	55	60	55		60	50	45	47	45
1507	50	45	50	60	55	50		58	52		50	30
1519	60		60	58	47	50	50	50	52	55	55	50
1520	50		60	45	47	55	47	55	55	55	47	50
1522	58		55	45	47	55	55	55	55	55	55	50
1523	55		50	45	45	47	55	52	50	50	55	53
1530	30		45	45	40	47	50	50	50	55	52	52

APPENDIX B

RPC MEASURE DATA

Table B-1: Platen size for left leg specimens

LEFT			
AM #	MAX	70%	PLATEN
1500	2.3	1.61	1.6
1502	2.9	2.03	2.05
1503	2.75	1.925	1.95
1504	3	2.1	2.1
1506	2.75	1.925	1.95
1507	2.75	1.925	1.95
1509	2.5	1.75	1.75
1510	3	2.1	2.1
1511	3	2.1	2.1
1512	2.75	1.925	1.95
1513	2.5	1.75	1.75
1514	2.5	1.75	1.75
1517	2.75	1.925	1.95
1519	2.9	2.03	2.05
1520	2.3	1.61	1.6
1522	2.9	2.03	2.05
1523	2.75	1.925	1.95
1524	3	2.1	2.1
1525	2.75	1.925	1.95
1526	2.5	1.75	1.75
1527	3	2.1	2.1
1529	2.9	2.03	2.05
1530	2.2	1.54	1.55
1531	3	2.1	2.1
1532	2.6	1.82	1.8
1534	2.75	1.925	1.95
1535	2.6	1.82	1.8
1536	2.75	1.925	1.95

Table B-2: Platen size for right leg specimens

RIGHT			
AM #	MAX	70%	PLATEN
1504	2.75	1.925	0.195
1506	2.3	1.61	1.6
1507	2.3	1.61	1.6
1519	2.3	1.61	1.6
1520	2	1.4	1.45
1522	2.1	1.47	1.45
1523	2.1	1.47	1.45
1530	2	1.4	1.45

Table B-3: Thicknesses for right leg specimens

(NE: No Epiphysis)

RIGHT		
AM #	Epi	Specimen
1504	4.52	2.03
1506	NE	2.03
1507	NE	2.06
1519	NE	2.03
1520	5.21	2.03
1522	NE	2.03
1523	NE	2.16
1530	5.08	2.03

Table B-4: Thicknesses for left leg specimens

(NE: No Epiphysis, and NR: Not Recorded)

LEFT		
AM #	Epi	Specimen
1500	4.11	2.34
1502	4.45	2.11
1503	4.57	2.13
1504	4.37	2.06
1506	4.65	2.03
1507	4.42	2.03
1509	4.88	2.03
1510	NE	2.06
1511	NE	2.03
1512	NE	2.03
1513	NE	1.91
1514	4.45	2.11
1517	5.00	2.03
1519	NE	1.98
1520	5.11	2.06
1522	4.50	2.03
1523	4.65	2.08
1524	4.95	2.06
1525	NE	2.03
1526	4.32	2.03
1527	4.24	2.03
1529	NE	2.06
1530	NR	NR
1531	NE	1.91
1532	NE	2.03
1534	4.62	2.03
1535	4.95	2.03
1536	4.22	2.03

VITA

Justin Dow Alcorn received his Bachelor of Science degree in Mechanical Engineering from The University of Texas at Austin in 2003. He entered the Mechanical Engineering program at Texas A&M University in January 2004, and he received his Master of Science degree in May 2006. His research interests include bone quality and tissue mechanics. He won the HSEMB Louis C. Sheppard Award for Best Poster.

Publications:

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Contact via:

Justin Dow Alcorn c/o Dr. Harry Hogan
206 Engineering/Physics Building
3123 TAMU
College Station, TX 77843

alcornjd@neo.tamu.edu