# EFFECTS OF VITAMIN E AND HINDLIMB UNLOADING ON BIOMECHANICAL PROPERTIES OF BONE IN MATURE MALE RATS

By

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

# CHAPTER I

# RESEARCH PROBLEM

## Introduction to Topic

Long-duration space flight is a goal of space exploration that presents several physiological obstacles for the human body. Muscular atrophy, diminished cardiovascular function, and bone loss occur when astronauts are subjected to a microgravity state. The effect of near weightlessness on the human skeletal system is one of the most critical concerns in safely extending space missions (Smith et al. 1999). Therefore, mineral changes may be the most important limiting physiological factor for long-term human spaceflight (West 2000).

Disuse osteoporosis induced by bed rest is similar to flight-induced bone loss as indicated by negative calcium balance (Organov et al. 1991, LeBlanc et al. 1990, LeBlanc et al. 1987, Schneider et al. 1984, Donaldson et al. 1970). Both astronauts and bed-ridden individuals undergo a process of bone resorption exceeding bone formation when subjected to an unloaded state (Smith et al. 1998). Although the mechanisms are not likely to be the same, strategies developed to prevent disuse bone loss or to enhance the rate of recovery following space flight might have direct applicability to clinical medicine (LeBlanc and Schneider 1991).

Management of free radicals may achieve protection of the cells that make bone, osteoblasts, and the cells that resorb bone, osteoclasts. Bone metabolism is influenced by free radicals, which are toxic to osteoblasts (Moraeu et al. 1998) and activate osteoclasts (Garrett et al 1990, Suda 1991). Research has shown that vitamin E, an antioxidant,

affects bone metabolism by maintaining bone matrix trophism (Passeri and Provvedini 1983) and stimulating trabecular bone formation (Xu et al. 1995). Vitamin E, therefore, may be a countermeasure to a state of compromised bone quality that astronauts and bed-ridden individuals experience.

## Significance of the Problem

A decrease in weight-bearing forces caused by space flight, immobilization, or long-term bed rest contributes to diminished bone density and bone strength. Vitamin E may help maintain bone quality when weight-bearing forces are reduced and may also provide protection from free radical damage that could promote bone loss during space flight.

## Purpose of the Study

The purpose of this study is to examine the effects of vitamin E and mechanical unloading on bone biomechanics independently and interactively using an animal research model of weightlessness.

## Research Objectives

The following objectives were developed to examine the effects of three concentrations of vitamin E on properties of bone in mature hindlimb unloaded male rats.

1. To determine if vitamin E and hindlimb unloading affect mechanical strength of bone in 12-month old male rats.
2. To determine if vitamin E and hindlimb unloading affect bone mineral area, bone mineral content, and bone mineral density in 12-month old male rats.

3. To determine if vitamin E and hindlimb unloading alter biochemical markers of bone remodeling in 12-month old mature rats.

Hypotheses

The following null hypotheses were developed for this study.

1. Hindlimb unloading, vitamin E status, or their interaction will not significantly alter mechanical strength in 12-month old rats.
2. Hindlimb unloading, vitamin E status, or their interaction will not significantly alter bone mineral area, bone mineral content, or bone mineral density in 12-month old rats.
3. There will be no statistically significant effects of hindlimb unloading, vitamin E status, or their interaction on biochemical markers of bone remodeling (i.e. serum alkaline phosphatase activity and serum tartrate-resistant acid phosphatase activity) in 12-month old rats.

Limitations

The hindlimb unloading model provides some, but not all, of the physiological effects of microgravity. Only the hindlimbs are subjected to unloading while the forelimbs of the animal bear a normal weight and thus provide an internal control. With the animal being partially unloaded, this model provides site-specific skeletal effects yet does not provide a complete simulation of microgravity.

Research with immature animals provides insight on the effect of growing bone whereas mature rats can provide insight of microgravity on remodeling of bone. By studying 12-month old rats in a state of skeletal unloading, a greater understanding of

mechanical and biochemical changes in mature bone can be provided. Although the mature rat can be considered a model for human disuse osteoporosis, there are differences in rat bone when compared to human bone. Bone modeling is complete in mature human bone whereas bones of rats continue to grow gradually even after reaching a point of maturity. Results of rat studies therefore should not be directly extrapolated to humans.

# CHAPTER II

# REVIEW OF THE LITERATURE

## Bone Structure and Biology

Bone is a metabolically active tissue that is continually reforming. This dynamic structure owes its form, mass, and function to the specific cells within it (Smith 1993). Osteoclasts and osteoblasts are two specific bone cells that work synergistically to model bone during growth and to remodel bone during adult life. During development and growth, the skeleton is sculpted in order to achieve its shape and size by removal of bone from one site and by deposition at a different site (Manolagas 1998). This process is known as modeling. Once the skeleton has reached maturity, regeneration continues in the form of a periodic replacement of old bone with new bone at the same location and is known as remodeling (Manolagas 1998).

Bone remodeling is a process involving first resorption followed by formation. Remodeling is initiated by the osteoclast (Teitelbaum 1993). The physiological function of osteoclasts is to excavate small portions of the bone in preparation for bone formation by osteoblasts. Excavation is achieved by the osteoclast sealing off a selected area of bone and producing a very acidic environment within which enzymatic digestion of bone tissue occurs (Smith 1993). Osteoclasts excavate bone at a rate that increases the specific individual cavity about 20 μm in length and about 10 μm in width each day (Parfitt 1982). When the diameter reaches about 200 μm, resorption of the bone stops (Parfitt 1982).

Osteoblasts are activated to deposit new bone thus completing the remodeling process initiated by osteoclasts. These bone forming cells produce collagen and non-collagen

proteins besides mineralizing the organic bone matrix (Smith 1993). Osteoblasts lay down new bone at about 2 μm each day by narrowing the tunnels created by osteoclasts (Parfitt 1982). After laying down their protein-based matrix, known as osteoid, osteoblasts either bury themselves in the bony matrix and become osteocytes (Kroll 2000) or revert to an inactive cell form and line the bone surfaces as surface osteocytes or resting osteoblasts (Raisz and Kream 1983). The result is an area of newly remodeled bone. This synergistic cycle between osteoclasts and osteoblasts makes bone a dynamic structure rather than a static or inert one. Bone remodeling continuously renews the skeleton and is a process that repairs damaged bone and adapts the skeleton to changes in physical load (Christiansen 2001).

Bone is composed of two types of tissue: cortical and trabecular. Cortical tissue is the compact, or dense, structure of the bone that comprises 85% of total bone in the body whereas trabecular, also known as cancellous or spongy bone, comprises 15% (Mundy 1999). High concentrations of trabecular bone located within the human skeleton include femur, tibia, humerus, radius, vertebral bodies, and iliac crest (Goldstein 1987). The distinguishing morphological feature of trabecular bone is the level of porosity relative to cortical bone (Jacobs 2000). Trabecular material is more metabolically active, is remodeled more often than cortical bone, and therefore is considered younger than cortical bone on average (Rho et al. 1998). It is the trabecular component of bones that is most affected by an altered bone turnover due to prolonged bed rest, immobilization, or space flight (microgravity).

Biomechanics of Bone

The bones of the human skeleton serve a mechanical function besides providing protection for vital organs and a reservoir for calcium and other minerals (Jiang et al. 1999). They are the basis of posture (Rubin and Rubin 1999), endure functional load bearing, and provide rigid levers for muscles to pull against while remaining as light as possible to allow efficient locomotion (Turner 1998).

Mechanical stress corresponding to daily physical activity is one initiator of bone remodeling. Osteoclasts and osteoblasts are activated to cause bone resorption and formation when the bone is subjected to mechanical loading (Nomura and Takano-Yamamoto 2000). Bone remodeling continually reshapes the bone to adapt to changing mechanical environments. This mechanism, known as functional adaptation, matches bone mass and architecture to functional demand and ensures that bone is remodeled until prevailing strains match those genetically prescribed for that location (Lanyon 1987).

The mechanical properties of bones are governed by the same principles as those of man-made load-bearing structures with the exception that bone structure can adapt to changes in skeletal loading (Martin 1991). Age of the bone, anatomical location, and the kind of bone being tested (e.g., cortical or trabecular) can affect mechanical property outcomes (Turner and Burr 1993). Cortical bone mechanical properties are greatly influenced by the mineralization level, the porosity, and the organization of the solid matrix (Rho et al. 1998). Bone, regardless of its cortical or trabecular percentage, is subjected to the stress of tensile, compressive, and shear forces (Jiang et al. 1999). These forces invariably occur in combination, even under the simplest loading schemes (Turner and Burr 1993).

Several methods are available to test bone biomechanical properties *in vitro*. These tests provide quantitative assessment of mechanical behavior through the relationships between applied loads and the resulting deformations (Jiang et al. 1999). The three-point bending test is useful for measuring the mechanical properties of long bones from rodents and other small animals while the compression test is more suitable for testing vertebrae (Turner and Burr 1993). The three-point bending procedure involves a perpendicular force applied to the shaft of a long bone causing it to bend as the force is increased. Bending causes both compressive and tensile stresses to the bone tested (Turner and Burr 1993). In compression testing of vertebrae, loads are applied along the craniocaudal direction by two platens until fracture occurs (Akhter et al. 2001). This type of test best simulates *in vivo* loading conditions that the vertebral body is subjected to (Turner and Burr 1993). Both of these tests utilize a stress-strain curve, also known as load-displacement curve, that records the yield and ultimate load applied to the bone and allow for calculation of stiffness, yield stress, ultimate stress, and modulus.

Mechanical forces applied to the bone can be expressed in Newtons and reported as yield load and ultimate load. Bones possess an elastic property that allows some force to be applied without permanent damage (Turner and Burr 1993). However, the response to a load greater than the inherent elastic property will result in deformation and ultimately will fracture the bone. The yield point represents the instant at which deformation occurs due to excessive mechanical load. The force applied to bone to reach deformation is reported as the yield load and the highest force a bone can withstand before fracture is reported as the ultimate load.

The extrinsic stiffness, or rigidity, of the bone is another biomechanical property demonstrated when force is applied past the yield point. Resistance to deformation under the applied load effectively describes stiffness as a structural property of bone (van der Meulen et al. 2001). Stiffness is defined as the slope of the linear portion of the load-displacement curve (Akhter et al. 2001) and indicates the rigidity of the bone as a whole. Bigger bones will naturally have greater rigidity than smaller bones (Jiang et al. 1999, Turner and Burr 1993) and therefore greater force would be required to fracture them. Stiffness, reported as Newtons per millimeter (N/mm), is categorized with yield load and ultimate load as a structural property of bone.

Material biomechanical properties of bone include yield stress, ultimate stress, and modulus of elasticity (Akhter et al. 2001). These properties reflect the material strength derived from the components of the bone rather than the bone as a whole. Both the yield stress and ultimate stress are reported in Newtons per millimeter squared ($N/mm^2$) and are calculated using the yield load and ultimate load, respectively.

Modulus of elasticity, also reported in the literature as Young's modulus, represents the intrinsic stiffness of the material (Akhter et al. 2001, Jiang et al. 1999, Turner and Burr 1993) and if often reported for compression testing of vertebrae. The modulus of bone tissue depends largely on the amount of mineral in it, the arrangement of crystal and collagen fibers, composition of collagen, and microfractures (Jiang et al. 1999). Calculation of the modulus of elasticity involves the cross-sectional area (mm2) and height (mm) of vertebral bodies and is reported as Newtons per millimeter squared ($N/mm^2$) (Akhter et al. 2001).

Bone Density

Bone mineral density (BMD) is an estimate of the amount of mineralized tissue contained within the periosteal envelope and is directly related to bone strength (Bonjour et al. 1994). Weight-bearing exercise increases the bone mineral density of skeletal sites that are impacted by activity (Bassey and Ramsdale 1995). While studying the effect of limb dominance and BMD, Chilibeck et al. (2000) reported that loading of the dominant arm correlated with a BMD difference between dominant and non-dominant arms in older women. Conversely, when the skeleton is unloaded due to prolonged bed rest, immobilization, or space flight, approximately 1-2% of BMD is lost at selected skeletal sites each month (Holick 1998, Whedon et al. 1974, LeBlanc et al. 1990, and Arnaud et al. 1991).

Although BMD is related to strength of the bone, it is not the sole determinant of the biomechanical performance of a bone. Density measurement neither provides information about the integrity of the trabecular architecture nor the mechanical properties of the trabeculae (van Lenthe et al. 2001). While studying the relationship between BMD and compressive strength in trabecular bone core samples of proximal femurs in severely osteoarthritic patients, Fazzalari and colleagues (1998) suggested that BMD could not be used to monitor changes in the mechanical properties of bone due to microdamage accumulation. Ideally, BMD should be used in combination with measurements of biomechanical properties such as mechanical testing, bone mineral area, and bone mineral content to determine overall bone quality.

Bone Strength

Bone strength is a reflection of both bone density and bone quality with reference to turnover, damage accumulation (e.g., microfractures), mineralization, and architecture (NIH Consensus Development Panel on Osteoporosis, Prevention, Diagnosis, and Therapy 2001). The internal structure of bone must be efficient so as to minimize skeletal weight while maintaining necessary strength (Hazelwood et al. 2001). Bone architecture refers to the three-dimensional arrangement of trabecular struts (Hans et al. 1997) and is another major contributor to bone strength besides BMD. The ability of the skeleton to bear load depends on sufficient bone mass suitably arranged in space (Mosley 2000). Microarchitectural deterioration of bone leads to decreased bone strength and is one characteristic of osteoporosis.

A common interpretation of bone strength is its resistance to fracture. The ability of bones to resist fracture depends on their mass, material properties, geometry, and tissue quality when subjected to loading (Forwood 2001). Fracture risk in vivo is commonly assessed by BMD measurement determined by dual energy X-ray absorptiomety (DEXA). Although DEXA is a noninvasive measurement of bone mass and structure, it can only predict the strength whereas direct biomechanical testing of bone in vitro can directly assess bone strength (Jiang et al. 1999). Tests involving compression, tensile, or shear stress ultimately determine the strength of bones, but are normally reserved for animal studies.

Bone Loss

Excessive bone resorption by osteoclasts without the coupled bone formation by osteoblasts results in a net loss of bone. Trabecular bone loss can occur after menopause (Type 1 osteoporosis) whereas both trabecular and cortical bone are affected by involutional osteoporosis (Type 2 osteoporosis) (Smith 1993). Prolonged bed rest, sedentary lifestyle, and space flight are also associated with bone loss (Vico et al. 2000) as are decreased estrogen and age. Both cortical and trabecular bone loss occur in weight-bearing bones when the skeleton is subjected to microgravity (Lafage-Proust et al. 1998).

Bone loss involves demineralization and is most often measured by DEXA, which can detect changes in bone mineral area and bone mineral content bone loss well before it becomes evident by conventional X-rays or by fracture (Kulak and Bilezikian 1999). Bone mineral content (BMC) in grams and bone mineral density (BMC divided by the estimated bone area) in $g/cm^2$ decrease when bone is lost.

Disuse Osteoporosis and Bone Quality

Mechanical force is important for maintaining skeletal bone mass (Peacock 1991) and immobility leads to increased bone loss (Schoutens et al. 1989). The reduction of graviational forces on the skeleton creates a unique type of osteoporosis with the severity dependent on the length of time that mechanical forces are absent or reduced (Doty and DiCarlo 1995). Immobilization via casting or paralysis, bed rest, or a microgravity environment all result in calcium loss and demineralization of bone (Whedon 1984). Bone quality, in terms of BMD and fracture risk, is directly affected by reduced mechanical loading and disuse osteoporosis can be the result. When weight is removed

from the skeleton, as with space travel or inactivity, bone mass is lost because bone resorption is greater than bone formation (Schneider and McDonald 1984).

Prolonged bed rest is a common human model used to study both disuse osteoporosis and weightless conditions (Bikle & Halloran 1999, Inoue et al. 2000, Scott et al. 1998, Zerwekh et al. 1998). Decreased mechanical loading of the skeleton occurs as it does in a microgravity state. Schneider and colleagues (1995) reported that gravitational forces exerted on the skeleton during bed rest are reduced by nearly 83%.

Bone loss occurs during bed rest and other simulated microgravity studies. LeBlanc and colleagues (1987) observed that six subjects lost 0.9% of their spinal bone mineral during a 5-week (35 day) bed rest study. Bed rest studies such as the 12-week (84 day) study conducted by Zerwekh and colleagues (1998), the 17-week (119 day) study conducted by LeBlanc et al. (1990), and the 120 day study conducted by Inoue et al. (2000) all resulted in significant bone loss due to long-term disuse of the skeleton. Each study reported bone loss but the specific alterations in bone metabolism resulting in bone loss are still unresolved. Zerwekh and colleagues reported that increased bone resorption occurred with a subtle decrease in bone formation. Inoue et al. (2000) reported that bone resorption increased in the early phase of their bed rest study whereas bone formation decreased in the late phase of bed rest. Studies such as these provide insight into the extent and rate of bone loss when disuse osteoporosis occurs.

Oxygen-Derived Free Radical (ODFR) Formation and Bone

Free radicals are common as transient intermediaries in chemical reactions, including body chemistry. Free radicals have certain beneficial roles but increased levels can

produce damage to tissues (Koul et al. 2000). When cells are exposed to oxidants, DNA damage frequently occurs by oxygen-derived species attacking DNA (Halliwell and Aruoma 1991). Reactive oxygen species (ROS) are responsible for tissue damaging effects such as lipid peroxidation (Stohs 1995). In biological fields, the major free radical species of interest have been those species associated with oxygen (Bergendi et al. 1999). Oxygen-derived free radicals (ODFR) and ROS are reported to have a negative impact on bone.

The formation of osteoclasts has been associated with ODFRs. Garrett et al. (1990) demonstrated that the generation of ODFRs in cultured bone was associated with the formation of new osteoclasts and enhanced bone resorption. Fukimiya et al. (1997) reported ODFRs to be stimulators of bone resorption in vitro and in vivo. Oxidative stress can then be considered as a promoter of bone resorption.

During bone resorption, osteoclasts release tartrate-resistant acid phosphatase (TRAP) into the circulation (Khosla and Kleerekoper 1999). Halleen and colleagues (1999) demonstrated that TRAP can generate ROS in vitro and also that these ROS can be targeted to destroy collagen and other proteins. Perhaps increased bone resorption, as in space flight, could increase ROS formation and possibly lead to tissue damage.

Radiation is considered a ROS-induced injury (Bauer and Bauer 1999), which could have negative health consequences. The radiolysis of chemical bonds and free radicals derived from oxygen accounts for the acute and chronic aspects of radiation injury (Sorenson 1992). Both galactic cosmic ray and solar cosmic ray radiation are present in outer space and pose a potential risk to humans during any duration of space flight (Spillantini et al. 2000).

Vitamin E and Bone Metabolism

Vitamin E (alpha-tocopherol), a fat-soluble vitamin and antioxidant, is considered the most potent lipid peroxyl scavenger (Traber and Packer 1995). This antioxidant prevents the propagation of free radical damage in biological membranes and may reduce free radical induced chromosomal damages through inhibition of free radical formation (Claycombe and Meydani 2001). Alpha-tocopherol is part of the body's defense system (Koul et al. 2000) in addition to superoxide dismutase, catalase, and glutathione peroxidase that protect tissue from damaging free radical effects (Bergendi et al. 1999). The influence of alpha-tocopherol on the status of other antioxidants and antioxidant enzymes may play a critical role in free radical-related diseases (Lii et al. 1998).

Few studies exist that provide a relationship between alpha-tocopherol and bone. Ebina et al. (1991) reported vitamin E supplementation increased trabecular bone volume and decreased osteoclast number compared to non-supplemented rats. Xu and colleagues (1995) observed the effects of vitamin E on trabecular bone formation and epiphyseal cartilage and reported that normal bone growth and modeling were sustained by protection provided by vitamin E against cellular lipid peroxidation. Polyunsaturated fatty acids (PUFA) play an important role in cartilage and bone biology (Xu et al. 1995), and one function of alpha-tocopherol is to prevent PUFA oxidation (Valk and Hornstra 2000). Xu et al. (1995) also suggested that alpha-tocopherol may enhance bone mass by reduction in free radical concentrations that may be stimulating osteoclastic activity or inhibiting osteoblastic activity.

Assessment of Vitamin E Status in Rodents

The American Institute of Nutrition (AIN) published formulas for a semi-purified diet for experimental rodents in 1977 (AIN-76), 1980 (AIN-76A), and 1993 (AIN-93G and AIN-93M) that are currently in use. AIN-93M rodent diet was formulated to provide for adult maintenance while the AIN-93G provides for growth, pregnancy, or lactation (Reeves et al. 1993). The AIN-93M diet is lower in both protein and in fat content than the AIN-93G and follows the general guidelines used in the feeding of livestock, poultry, and companion animals where fewer nutrients such as protein and fat are utilized for maintenance (Reeves et al. 1993). The AIN-76A diet was utilized for all ages of rodents without discriminating for growth or maintenance phases.

The AIN-76A formula is still available but the AIN-93 diets may have a better balance of nutrients for rats. AIN-76A diet composition included 50 IU vitamin E and 50 g corn oil per kilogram of diet. The revised diets recommend an increased amount of vitamin E, i.e. 75 IU/kg diet, regardless of whether fat is 70g soy oil/kg diet (AIN-93G) or 40g soy oil/kg diet (AIN-93M). Workshop members revising AIN-76A concluded that if oil were increased from 50 to 70g/kg diet, the vitamin E content would also need to be increased to protect the increased PUFA content. The level of 75 IU/kg diet was selected for the AIN-93G, which contains 7% fat. Although the fat content of AIN-93M is only 4%, the decision was made to retain 75 IU/kg diet in the maintenance diet to alleviate possible age-associated biological and pathological changes caused by increased peroxidation and eicosanoid production as Meydani et al. suggested (1986; 1992). Thus vitamin E as currently recommended for the AIN-93M diet (75 IU for 40g soy oil) is approximately

twice as high in proportion to PUFA as dietary levels in older studies in which rodents were fed the AIN-76A diet.

Potential Risks of Space Flight to Bone

One of the most critical concerns in extending space missions safely is the effect of microgravity on bone tissue (Smith et al. 1999). Astronauts subjected to a state of weightlessness undergo bone alterations such as decreased bone mass, decreased bone mineral density, and decreased bone strength. Bone lost during spaceflight is primarily lost from those skeletal sites that are most loaded in our normal 1-G environment. Mack et al. (1971) and Vose et al. (1974) reported significant BMD decreases averaging 3.2% in the weight-bearing calcaneus of astronauts of the early Gemini and Apollo missions after approximately 10 days in space. Apparently, exposure to a microgravity environment even in a short space flight can induce significant bone loss.

Missions to Mars will last approximately two to three years (Buckey 1999, Grigoriev et al. 1998, and Lackner et al. 2000). This estimated travel period does not include time spent by astronauts on the planet in a decreased gravitational state. With data from Mir missions calculating bone loss of 0.35% to >1.0% per month (Buckey 1999), osteoporosis would most likely occur during long-term microgravity exposure. The physiological obstacle of bone loss would therefore restrict long-term space flight. Bone disorders must be prevented to allow a Mars mission or living on a lunar base (Miyamoto et al. 1998).

Another risk of space flight is radiation exposure to crewmembers. During interplanetary flights, they will be exposed to cosmic radiation with great risk to their health (Spillantini et al. 2000). Space radiation is a primary hazard for orbital flight

(Petrov et al. 1981) and a dose of cosmic radiation delivered in a few hours by a solar burst can easily exceed a dose accumulated in one year (Spillantini et al. 2000). Hazardous solar events are relatively rare (Reames 1999), yet radiation exposure is still present and is a health threat to astronauts.

Bone loss, overall, is a greater physiological dilemma for the astronaut compared to the potential radiation exposure. Because alterations in bone metabolism are expected, countermeasures such as artificial gravity, weight loading, and exercise are being sought to prevent bone loss. Three of the six astronauts on Skylab missions 3 and 4 suffered from bone loss although they did a considerable amount of exercise using treadmills, bicycle ergometers, and other equipment (Shigematus et al. 1997). Nutritional and pharmacological interventions, such as supplementation with alpha-tocopherol, may inhibit bone resorption and therefore minimize bone loss.

Space Flight, Bone, and Human Studies

Several studies have been conducted concerning astronauts and the physiological alterations they experience while in a microgravity state. Serum bone markers such as alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) are often measured in human space flight studies. Urinary bone markers such as deoxypyridinoline, pyridinium cross-links, N-telopeptide, and calcium excretion are used to measure bone resorption. DEXA measurements, used to assess bone mineral density (BMD), are also utilized in space flight studies.

ALP, secreted by osteoblasts, is an essential enzyme for mineralization of bone (Khosla and Kleerekoper 1999). In human space flight, serum ALP has been used as a

metabolic marker to assess bone formation, which decreases during space travel (Caillot-Augusseau et al. 1998, Miyamoto et al. 1998, Harris et al. 2000). Miyamoto and colleagues (1998) observed decreased ALP one week after space flight in two astronauts aboard the Space Shuttle: one for 8.9 days and a second one aboard for 14.7 days. Caillot-Augusseau et al. (1998) reported a 27% decrease in serum ALP for two cosmonauts orbiting for 180 days during the EUROMIR 95 space mission. Bone formation, also measured by serum ALP, showed a decreasing trend in one cosmonaut monitored aboard the EUROMIR 94 mission as observed by Collet et al. (1997).

Serum TRAP is the only osteoclast-specific product that has been evaluated to any extent as an indicator bone resorption (Khosla and Kleerekoper 1999). Miyamoto et al. (1998) reported a 10% increase in TRAP values in an astronaut following the 14.7-day Space Shuttle flight and also reported that TRAP "increased markedly (twice or more) from one week to 2 months after the flight" for the astronaut on an 8.9-day Space Shuttle mission.

Urinary markers of bone resorption and human space flight studies are often seen in the literature. Smith et al. (1998) reported that urinary markers of bone resorption indicated that astronauts aboard 28 day, 59 day, and 84 day Skylab missions, flown in 1973 and 1974, experienced elevated bone resorption as early as the first week. Seo et al. (1997) suggested that even a short period of exposure to microgravity could result in bone resorption after measuring a significant increase in urinary excretion of pyridinium cross-links of a Japanese astronaut aboard the 1992 Space Shuttle Endeavor.

DEXA measurements have been used to determine BMD in different skeletal locations for astronauts. Whole body and regional BMD were measured by DEXA assessment

before and after flight for cosmonauts aboard two MIR space station missions of 180 and 20 days. McCarthy and colleagues (2000) reported that DEXA measurements of the cosmonauts demonstrated significant variation between different sites with the greatest changes occurring in the proximal femur and the lumbar spine. Miyamoto et al. (1998) reported no significant change in BMD over the whole body in either 8.9- or 14.7-days for astronauts aboard the Space Shuttle, however they did observe a clear decrease of BMD in the weight-bearing midspine (L-2 to L-4). Organov et al. (1992) measured a decrease in BMD of up to 14% in the proximal femur of crewmembers of the sixth to the ninth MIR expeditions with durations of 132 to 176 days in space.

Space Flight, Bone, and Animal Studies

Various investigators have studied trabecular bone alterations and overall bone loss in rats flown in space. Most of these studies involve young, growing rats and the effects of space flight on bone modeling. Jee et al. (1983) observed significantly reduced mineralized tissue mass in the tibia of growing male rats flown for 18.5 days in orbit compared to ground-based control rats. Morey and Baylink (1978) examined tetracycline-labeled tibias of male rats that orbited for 19.5 days aboard the Soviet Cosmos 782 biological satellite and found that the periosteal bone formation rate was reduced by 47%. An approximate 50% inhibition of bone formation in the cancellous area of pelvic metaphyses and in thoracic vertebral bodies was reported for growing rats aboard a 17-day space flight (Zerath et al. 2000).

Mechanical testing of bone is also a common technique for studying the effects of microgravity on bone quality. Spengler and colleagues (1979) reported that a 19 day

space flight resulted in a significant decrease in mechanical strength of femurs in growing rats aboard the Soviet Cosmos 936.

Few studies, however, investigate mature rats and the effects of a space flight on the remodeling of the bone. Erben (1996) compared female rats aged 3 months to 12 months and observed remodeling as the main turnover activity of vertebral cancellous bones in all groups, yet the study was conducted in a normal gravitational environment. Vico and colleagues (1998) recommended that future researchers use adult rats in space flight to provide more bone remodeling area and also to minimize the variability involved with growing rats.

Comparison of Space Flight and Hindlimb Unloading

Weightlessness is the dominant and single most important factor that affects human health during space flight (White 1998). The effects of weightlessness (microgravity) on the human body include motion sickness, cephalic fluid shift, anorexia, reduced plasma volume, reduced red blood cell mass, increased kidney filtration rate, muscular atrophy, and bone loss (West 2000; White 1998). Cephalic fluid shift has been implicated as a cause of impaired cardiac output (Dunbar et al. 2000) and also of decreased organ weights (Witten et al. 1999).

Due to infrequent space missions, there is a limited number of actual microgravity studies in both human and animal research. In order to study selected tissues of animals in a weightless environment, a ground-based animal model was developed at the National Aeronautics and Space Agency (NASA)-Ames Research Center in the mid-1970s to simulate space flight (Morey-Holton and Globus 1998). This model, known as the tail-

suspension or the hindlimb unloading (HU) model, has been modified and improved over time.

The Animal Care and Use Committee at National Aeronautics and Space Administration-Ames Research Center has approved protocols for the model currently in use. The criteria established for HU models include the following: (1) The system should not stress the animals; (2) the model should unload limbs without paralysis or restriction of motion and with the ensuing ability to reload and recover from the response to unloading; (3) the pattern of muscle atrophy should be similar to that which occurs during space flight; (4) the model should produce a cephalic fluid shift similar to space flight; and (5) the physiological responses of the experimental animals to unloading should be similar to space flight (Morey-Holton and Globus 1998).

HU is accomplished by suspending the rodent by the tail at a 30° angle without the hindlimbs touching the bottom of the cage and with minimal touching of the sides of the cage. The goal is to prevent mechanical resistance to the hindlimbs and to prevent pushing off from the side of the cage, which could provide a loading force. Any resistance provided by the hindlimbs contacting the sides of the cage could interfere with potential bone loss. Much of the animal's weight is on the forelimbs yet the apparatus should be adjusted so that the angle of suspension ensures that loading is at a normal level (DeHority et al. 1999). This angle also provides a cephalic fluid shift as outlined in the listed protocols. The animal can reach of both food and water and normal eating habits should be expected.

This model attempts to mimic the physiological changes in a weightless environment. Some stress would occur whether the animal was aboard an authentic space flight or

whether weightlessness was simulated with the HU protocol. Minimizing stress is a goal, and a confirmation that stress was minimized would be for mature HU rats to maintain weight throughout the experiment (Morey-Holton and Globus 1998). Another indication of low stress would be the adjustment to suspension indicated by eating, drinking, and normal grooming activities (LeBlanc et al. 1985).

Restriction of motion is not an issue for the HU rat if the apparatus is built to provide a 360° range of motion. Traction tape applied to the tail provides a loop to suspend the animal from a hook attached to a pulley system. The 30° angle of suspension is important to provide the correct weight distribution on the forelimbs without overloading them. This system allows free movement about the cage while maintaining the 30° head tilt, presumably producing a fluid shift similar to that observed in astronauts during space flight (LeBlanc et al. 1985).

The purpose of the HU model is to simulate the diminished gravitational loading on bone and to study those consequences. Wronski and Morey (1982) were among the first to demonstrate the skeletal alterations induced by simulated weightlessness by use of an orthopedic taped harness bonded to the shaved backs of rats by silicone rubber. Their study reported a depressed rate of longitudinal bone growth in the proximal tibia and humerus of 10 male rats suspended for a two-week period in comparison to a group of 10 male control rats. A significant reduction in mineralized tissue in the proximal tibia of suspended rats at all regions of the metaphysis except for the area immediately beneath the growth plate was also observed. Both the tibia and humerus of HU animals had a significant decrease in the number of osteoblasts while the number of osteoclasts significantly increased.

LeBlanc and colleagues (1985) modified the tail HU model by drilling through the second caudal vertebra and inserting a stainless steel ring. The purpose of the study was to extend the duration of suspension in order to determine if the physiological changes were progressive. A second purpose was to accurately measure the degree, rate, and relationship of bone and muscle atrophy for comparison with changes using conventional immobilization methods. A total of 27 animals, aged 24-29 weeks at end of each experiment, were suspended for various lengths of time such as 14, 30, 60, or 90 days. Results included a significant decrease in total calcium content in femurs of the 60-day rats, and a significant decrease in bone mineral content in the distal region of the femur in 30-day rats. After 90 days, the bone mineral deficit in the shaft of the femur was similar in magnitude to the distal region. LeBlanc et al. (1985) also reported that the cortical bone of the femoral shaft looked almost osteoporotic at 90 days of suspension. There was no significant difference in bone length between control and suspended animals for any of the groups. The pattern of bone loss with suspension was reported to be similar to immobilization-induced atrophy, but the amount of bone mineral loss was considerably less.

Globus and colleagues (1986) further modified the HU model using orthopedic tape wrapped around the base of the tail with tape applied to opposite sides of the tail and a clip in the middle. Groups of 6 week old rats were suspended for 2, 5, 7, 10, 12, or 15 days. The weight and calcium content of HU tibiae and L-1 vertebrae of those suspended for 7 days were less than control animals. In the 10-day HU animals, L-1 vertebrae and tibial metaphyseal weights decreased to 75% and 82% respectively when compared to controls. For 15-day HU animals, L-1 vertebrae metaphyseal weights were 81% of

controls, tibial metaphyseal weights were 78% of controls, and calcium content decreased to 71% for L-1 and 67% of controls for tibia. Although both matrix formation and mineralization returned toward control levels between the 7th and 15th day of unloading, there was evidence that hypomineralization had occurred.

In order to examine the appropriateness of HU as a model for diminished mechanical loading in adults and to determine the influence of age on bone responsiveness to skeletal unloading, DeHority and colleagues (1999) studied the effects of HU in 6-month old, adult male rats. Animals were suspended for either 1, 3, or 5 weeks. Mineralizing surfaces (MS), mineral apposition rate (MAR), and bone formation rate on the periosteal surface decreased by as much as 80% during unloading. DeHority et al. (1999) reported that the decreases in MS and MAR were consistent with the observed decrease in osteoblast number and also suggested a decrease in bone-forming activity per cell. Osteoclast surface did not change significantly suggesting that the decrease in bone mass was primarily a consequence of decreased bone formation. Overall, DeHority and co-workers stated that the HU of adult rats was a useful model for simulating the loss of gravitational loading in adult humans during space flight. This study also demonstrated that the effects of reduced load on the bone are prolonged and have a greater relative effect on mature bone formation of adults than on bone of growing rats.

Additional research is still needed in the area of prevention of bone loss from mature bone. Although six months of age may be considered mature for a rat, further studies are needed utilizing animals older than six months. Reducing the rate of bone loss, or actually preventing it, are goals many are attempting to achieve. Ground-based models, such as

the HU model, provide a way to understand bone loss without the limitations in size of sample or frequency of flights associated with actual space missions.

# CHAPTER III

# MATERIAL AND METHODS

## Experimental Design

The research design used for this study was a two by three (2 x 3) factorial design. Main effects included ambulatory versus hindlimb unloading (HU) and three levels of vitamin E. The vitamin E levels utilized were 15 mg/kg, 75 mg/kg, and 500 mg/kg of diet to represent low, recommended, and high intake respectively.

## Animals and Diet

Ninety-seven male Sprague-Dawley rats (Harlan Teklad, Indianapolis, IN) were acquired in three separate groups (n=25, n=35, n=36) at age 8.5 months. A limited number of metabolic cages with the HU apparatus were available therefore three animal replications were needed. Two animals died during the research study. One animal could not tolerate hindlimb unloading and was terminated before research could be completed and a second animal died under anesthesia during a bone scan.

Animals were housed in the Oklahoma State University Laboratory Animal Resources (LAR) facility with free access to deionized water. A twelve-hour light/dark cycle was utilized in a temperature-controlled setting. Weekly body weights were recorded and animals were monitored daily. Rats were initially maintained in individual cages during the weeks prior to HU and then were transferred to individual specialized metabolic cages for the HU intervention.

Rats were fed a standard commercial pelleted animal diet during acclimation to the facility. Then all animals were fed AIN-93M powdered diet containing tocopherol-stripped soybean oil prepared by Harlan Teklad with no added vitamin E for 10 days.

Animals were randomized to the six treatment groups. The vitamin E levels were fed for a period of 8 weeks prior to suspension. Immediately before suspension, an intraperitoneal injection of xylazine (2.5 mg/kg body weight) and ketamine (50 mg/kg body weight) was administered to all animals to facilitate DEXA scanning and tail blood collection. Full body DEXA scans were performed utilizing a Hologic QDR 4500A Fan Beam X-ray Bone Densitometer (Waltham, MA) and the small animal software package designed for high resolution scans.

HU was accomplished utilizing the model developed by Wronski and Morey-Holton (1979). The HU model currently approved by the NASA-Ames Animal Care and Use Committee (Morey-Holton and Globus 1998) incorporates orthopedic traction tape applied to the tail. To form a more adhesive surface for the tape, tails were swabbed with isopropyl alcohol and a coat of tincture of benzoin was applied. Traction tape was then immediately placed approximately one half inch from the base of the tail and laid lengthwise down one side. A loop for suspension was created by leaving one inch of tape extended away from the end of the tail before placing the tape down the opposite side. The traction tape was further secured by wrapped cotton gauze and tape.

All animals were then placed individually into metabolic cages for a period of 4 weeks and fed their assigned vitamin E diets. Control rats in all three replications were pair-fed based on the intake of the suspension group during the first replication. Those in the HU metabolic cages were positioned with a suspension mechanism so that the

hindlimbs were not allowed to bear weight. Rats were able to move freely about by use of their forelimbs. Tension of the apparatus was adjusted to provide an approximate 30° angle to place weight similar to a normal weight-bearing only on the forelimbs (Hargens et al. 1984). The head-down position generated a cephalic fluid shift similar to that occurring during space flight (Morey 1979).

Necropsy

At the end of the 4-week suspension period, both HU and control animals were injected with xylazine (5 mg/kg body weight) and ketamine (100 mg/kg body weight) before necropsy. Each animal's final weight was noted and a full body DEXA scan was performed before exsanguination from the abdominal aorta. Blood samples were collected and chilled for approximately 30 minutes before centrifugation at 3000 x g for 20 minutes at 4°C. Aliquots of the separated serum were then frozen at -20°C for further analysis.

Vertebral columns, humeri, femora, tibiae, calvaria, livers, intestines, gastrocnemii, and solei were collected for analysis. Both the vertebral column and the left femur of each animal were carefully cleaned of excess tissue without removing the periosteum. Vertebral columns were then stored at -20°C to be separated later to obtain individual lumbar vertebrae. Left femurs were stored in phosphate buffered saline (PBS) at -20°C for mechanical testing at a later date. The serum, left femur, and 4$^{th}$ lumbar (L4) vertebrae were the only tissues analyzed specifically for this thesis.

Biochemical Analysis

Aliquots of serum attained after necropsy were thawed and analyzed for serum alkaline phosphatase (ALP) and tartrate resistant acid phosphatase (TRAP) activity to assess bone metabolism. Ferric reducing ability (FRAP) activity was measured according to the method of Benzie and Strain (1996) to assess total antioxidant capacity. ALP, TRAP, and FRAP analyses were performed on a Cobas-Fara II Clinical Analyzer (Roche, Montclair, NJ). Commercially available reagents were used for ALP and TRAP (Roche Diagnostic Systems, Indianapolis, IN) while appropriate reagents for FRAP were prepared in our laboratory.

Initial blood samples were collected from the tail and final samples were collected from the abdominal artery to provide serum to be analyzed for alpha-tocopherol concentration by high-performance liquid chromatography (HPLC). The combined methods of Bieri et al. (1979) and Ortega et al. (1998) were modified to determine the vitamin E values. The internal standard was prepared by dissolving alpha-tocopherol acetate (Sigma, T-3376) in ethanol. Standards of 25,50, and 100 μg/ml were prepared from alpha-tocopherol (Sigma, T-3251) dissolved in a 95% ethanol solution. The internal standard (200 μl) was combined with 200 μl of each sample and vortexed vigorously. Serum samples were centrifuged at 4000 x g for 10 minutes at 4°C after addition of 1000 μl hexane. The hexane layer containing alpha-tocopherol extracted from the sample was carefully removed and placed in a new microcentrifuge tube. Samples were evaporated by nitrogen followed by addition of 25 μl diethyl ether and 75 μl methanol prior to injection. Duplicate samples (10 μl each) were injected into a HPLC unit with a reverse phase C-18 column (Supelco, Bellfonte, PA). HPLC conditions consisted of a mobile

phase of methanol:water (95:5) at a flow rate of 2.0 ml/min with a UV detector wavelength set at 290 nm (Water Tunable Absorbance Detector, Milford, MA). The sample analysis run time was set for 16 minutes with an alpha-tocopherol retention time of 9 minutes and alpha-tocopherol acetate retention time of 13 minutes.

Densitometric Measurements

Left femurs and 5$^{th}$ lumbar (L-5) vertebrae were removed from -20°C storage and allowed to thaw at room temperature before DEXA scans were performed. Each sample was placed in a container with deionized water approximately 2 cm deep to simulate a soft tissue environment. DEXA scans were completed and bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD) were recorded. Femurs were then returned to their respective storage containers with PBS solution at room temperature before mechanical strength testing commenced.

Mechanical Testing

Bone strength was determined by a three-point bending test utilizing an Instron Universal Testing Machine (Model TM-S) at room temperature. Femoral length and external diameter of the midshaft were recorded prior to mechanical testing. External shaft diameters were measured perpendicular to the point of load and parallel to the point of load. Femurs were positioned so that the posterior surface rested on two rods (4 mm in diameter) spaced 20 mm apart. Force (load) was applied at the rate of 1.0 mm/min to the anterior surface of the femur until bone failure occurred. Internal diameters perpendicular

to the point of load and parallel to the point of load were immediately measured after each femur was tested (fractured).

A load-displacement curve was recorded during the test to provide measurement of structural strength variables such as yield load, ultimate load, and stiffness. Yield load is defined as the level of force at which the bone begins to experience permanent structural damage. The maximum load the bone can sustain defines the ultimate load. Both yield load and ultimate load were measured in Newtons (N). Stiffness (elastic modulus) was determined by calculating the slope of the linear portion of the curve.

Material strength variables of yield stress and ultimate stress were calculated via the load-displacement curve. Yield stress is defined as tensile stress applied to the femur that results in the bending of the bone. Ultimate stress is the point where the bone no longer bends and fracture begins to occur before the bone actually breaks. Yield stress and ultimate stress were both measured in units of Newtons per millimeter squared ($N/mm^2$).

The $4^{th}$ lumbar vertebrae (L-4) samples were shipped to Creighton University Biomechanics Laboratory (Omaha, NE) for compression testing. Each L-4 vertebra was tested at room temperature with loads applied along the craniocaudal axis. Compression was applied at 3.0 mm/min deformation rate until fracture. Yield load, ultimate load, stiffness, yield stress, and ultimate stress were determined.

Data Analysis

Data were analyzed as a 2x3 factorial design utilizing SAS version 8.1 (SAS Institute, Cary, NC). Means and standard error of the mean for each treatment group were calculated. The general linear model (GLM) procedure in SAS was used to analyze data

and the least squares means procedure was used for means separation tests. Differences were considered significant at $p<0.05$.

# CHAPTER IV

## RESULTS AND DISCUSSION

Final body weight of hindlimb unloaded (HU) animals was significantly lower ($p<0.0001$) than ambulatory animals after four weeks of suspension (Table 1). Unloaded animals weighed 6.7% less than ambulatory when weighed at necropsy. Initial weight loss in HU animals is usually due to a decrease in appetite after attachment of the HU apparatus and was compensated in this study by pair feeding ambulatory animals. Although a goal is to minimize stress of HU animals, stress may still have been a factor contributing to weight loss. Loss of muscle weight may also contribute to loss of body weight as noted by LeBlanc and colleagues (1985) in 6-7 month old rats. They observed that muscle weight loss due to HU was maximized within 14-30 days of suspension. Dehority et al. (1999) observed a 3.2% decrease in body weight in 6 month old male rats after 5 weeks of HU. Garber and colleagues (2000) noted a 10% weight loss by day six in HU 6-month old female rats with weight stabilizing below the weight of ambulatory animals at the end of 2 weeks.

HU animals had significantly lower ($p<0.02$) liver weights than ambulatory animals (Table 2). Dietary vitamin E or interactions of HU and vitamin E did not produce significant differences in liver weight. Because liver weights were proportional to body weights, no significant difference existed due to hindlimb unloading, vitamin E, or their interaction when liver was expressed as a percentage of body weight.

Spleen weights were not significantly affected by vitamin E, HU, or the interaction of the two (Table 3) indicating that spleens were unaffected by the weight loss that occurred

Table 1. Final body weight at necropsy from ambulatory (Amb) and hindlimb unloaded (HU) rats fed 15, 75, or 500 IU vitamin E/kg of diet.[1]

| | N | Initial Body Weight (g) | N | Final Body Weight (g) | Change in Body Weight (g) |
|---|---|---|---|---|---|
| Amb, E-15 | 15 | 492±6 | 15 | 496±6 | 4±7 |
| Amb, E-75 | 15 | 494±8 | 15 | 499±8 | 5±7 |
| Amb, E-500 | 16 | 483±10 | 16 | 494±9 | 11±7 |
| HU, E-15 | 17 | 484±9 | 16 | 463±8 | -18±9 |
| HU, E-75 | 16 | 479±9 | 15 | 461±9 | 16±11 |
| HU, E-500 | 16 | 502±12 | 14 | 472±12 | -22±10 |
| | | | | | |
| E-15 | 32 | 488±6 | 31 | 479±6 | -8±6 |
| E-75 | 31 | 486±6 | 30 | 480±7 | -6±7 |
| E-500 | 32 | 493±8 | 30 | 484±8 | -5±7 |
| | | | | | |
| Amb | 46 | 490±5 | 46 | 496±5[a] | 7±4[a] |
| HU | 49 | 488±6 | 45 | 465±5[b] | -19±6[b] |
| | | | | | |
| Source of Variation | | *p*-values | | *p*-values | *p*-values |
| HU | | 0.82 | | <0.0001 | <0.0001 |
| E Diet | | 0.80 | | 0.92 | 0.99 |
| HU*E Diet | | 0.16 | | 0.67 | 0.57 |

[1]Values represent means ± SEM. Values that do not share a superscript for the same parameter are significantly different ($p<0.05$)

Table 2. Liver weight at necropsy from ambulatory (Amb) and hindlimb unloaded (HU) rats fed 15, 75, or 500 IU vitamin E/kg of diet.[1]

| | N | Liver Weight (g) | N | Liver Weight as % of Body Weight |
|---|---|---|---|---|
| Amb, E-15 | 15 | 15.93±0.28 | 15 | 3.21±0.06 |
| Amb, E-75 | 15 | 16.13±0.49 | 15 | 3.22±0.06 |
| Amb, E-500 | 16 | 16.10±0.35 | 16 | 3.26±0.05 |
| HU, E-15 | 17 | 14.92±0.42 | 16 | 3.21±0.07 |
| HU, E-75 | 16 | 14.77±0.38 | 15 | 3.18±0.07 |
| HU, E-500 | 16 | 15.86±0.74 | 14 | 3.29±0.13 |
| E-15 | 32 | 15.40±0.27 | 31 | 3.21±0.05 |
| E-75 | 31 | 15.43±0.32 | 30 | 3.20±0.05 |
| E-500 | 32 | 15.98±0.40 | 30 | 3.28±0.07 |
| Amb | 46 | 16.06±0.22[a] | 46 | 3.23±0.03 |
| HU | 49 | 15.18±0.31[b] | 45 | 3.23±0.05 |
| Source of Variation | | *p*-values | | *p*-values |
| HLU | | <0.02 | | 0.94 |
| E Diet | | 0.35 | | 0.46 |
| HU*E Diet | | 0.46 | | 0.87 |

[1]Values represent means ± SEM. Values that do not share a superscript for the same parameter are significantly different ($p<0.05$)

Table 3. Spleen weight at necropsy from ambulatory (Amb) and hindlimb unloaded (HU) rats fed 15, 75, or 500 IU vitamin E/kg of diet.[1]

| | N | Spleen Weight (g) | N | Spleen Weight as % of Body Weight |
|---|---|---|---|---|
| Amb, E-15 | 15 | 0.77±0.02 | 15 | 0.155±0.004 |
| Amb, E-75 | 14 | 0.78±0.02 | 14 | 0.158±0.004 |
| Amb, E-500 | 15 | 0.81±0.03 | 15 | 0.164±0.007 |
| HU, E-15 | 16 | 0.83±0.03 | 15 | 0.179±0.006 |
| HU, E-75 | 16 | 0.80±0.02 | 15 | 0.175±0.006 |
| HU, E-500 | 14 | 0.82±0.02 | 14 | 0.175±0.005 |
| E-15 | 31 | 0.80±0.02 | 30 | 0.167±0.004 |
| E-75 | 30 | 0.80±0.02 | 29 | 0.167±0.004 |
| E-500 | 29 | 0.81±0.20 | 29 | 0.169±0.004 |
| Amb | 44 | 0.79±0.02 | 44 | 0.159±0.003[b] |
| HU | 46 | 0.82±0.01 | 44 | 0.176±0.003[a] |
| Source of Variation | | *p*-values | | *p*-values |
| HU | | 0.16 | | <0.0004 |
| E Diet | | 0.75 | | 0.84 |
| HU*E Diet | | 0.61 | | 0.50 |

[1]Values represent means ± SEM. Values that do not share a superscript for the same parameter are significantly different ($p<0.05$)

in the HU animals. Spleen weight as percentage of body weight however was significantly higher ($p<0.0004$) in the HU group due to the maintenance of the spleen weight despite weight loss associated with unloading. Witten et al. (1999) reported significant decreases in liver and spleen weights as a percentage of body weight of rats subjected to a 45° head-down tilt for 24 hours. Although Witten and colleagues observed an organ weight change in only one day with a larger degree of tilt, it is also probable that a change in the liver and spleen would occur with a 30° tilt utilized for a longer time period. The change in spleen weight as a percentage of body weight did decrease in this study however the liver did not have decrease as a percentage of body weight.

Bone resorption increased ($p<0.0001$) in the HU treatment group as measured by serum tartrate resistant acid phosphatase (TRAP) activity (Table 4). Neither vitamin E nor the interaction of vitamin E and HU produced a significant difference in serum TRAP values. Bone resorption was expected to increase in the unloaded limbs and lumbar vertebrae when utilizing the HU model. Wronski and Morey (1982) reported an increase in osteoclast number for ten male rats after utilizing one of the first HU models, however, HU does not appear to cause consistent changes in osteoclast number or in bone resorption (Morey-Holton and Globus 1998). Changes were not found in osteoclast numbers when body weights were not significantly different between HU and ambulatory animals (Machwate et al. 1995; Machwate et al. 1994; Wronski and Morey-Holton 1987). Increased osteoclast activity however has been observed in HU studies where there is a weight loss reported for HU animals (Vico et al. 1991; Wronski and Morey-Holton 1987). The increased TRAP in this experiment may reflect an increase in number of osteoclasts as reported in other studies where there was significant weight loss.

Table 4. Biochemical markers of bone metabolism and total antioxidant capacity in ambulatory (Amb) and hindlimb unloaded (HU) rats fed 15, 75, or 500 IU vitamin E/kg of diet.[1, 2]

| | N | Serum Alkaline Phosphatase (ALP) ($\mu$kat/L) | N | Serum Tartrate-Resistant Acid Phosphatase (TRAP) (U/L) | N | Serum Ferric-Reducing Ability (FRAP) ($\mu$mol/L) |
|---|---|---|---|---|---|---|
| Amb, E-15 | 15 | 1.65±0.13 | 15 | 4.43±0.29 | 15 | 553±35 |
| Amb, E-75 | 15 | 1.57±0.08 | 14 | 4.27±0.24 | 15 | 575±20 |
| Amb, E-500 | 16 | 1.85±0.17 | 16 | 4.04±0.21 | 16 | 659±35 |
| HU, E-15 | 17 | 1.60±0.12 | 17 | 5.73±0.17 | 17 | 533±26 |
| HU, E-75 | 16 | 1.54±0.13 | 16 | 5.47±0.23 | 16 | 578±20 |
| HU, E-500 | 16 | 1.82±0.14 | 16 | 5.38±0.23 | 16 | 727±47 |
| E-15 | 31 | 1.62±0.09 | 32 | 5.12±0.20 | 31 | 542±21[b] |
| E-75 | 30 | 1.56±0.08 | 30 | 4.91±0.20 | 30 | 576±14[b] |
| E-500 | 30 | 1.83±0.11 | 32 | 4.71±0.19 | 30 | 693±30[a] |
| Amb | 46 | 1.69±0.08 | 45 | 4.24±0.14[b] | 46 | 597±19 |
| HU | 45 | 1.65±0.08 | 49 | 5.53±0.12[a] | 47 | 611±22 |
| Source of Variation | | *p*-values | | *p*-values | | *p*-values |
| HLU | | 0.73 | | <0.0001 | | 0.50 |
| E Diet | | 0.10 | | 0.23 | | <0.0001 |
| HU*E Diet | | 0.99 | | 0.97 | | 0.35 |

[1]Values represent means ± SEM. Values that do not share a superscript for the same parameter are significantly different ($p<0.05$)
[2]Marker of bone formation (i.e. serum alkaline phosphatase), marker of bone resorption (i.e. serum tartrate-resistant acid phosphatase), and total antioxidant capacity (i.e. serum ferric-reducing ability) are reported.

Bone formation, as indicated by serum alkaline phosphatase activity (ALP), was not significantly affected by vitamin E, HU, or the interaction of the two (Table 4). This indicates that bone formation in these mature rats was maintained during the 28 days of HU yet was not increased to match the increased resorption that occurred as indicated by TRAP.

Serum ferric reducing ability (FRAP) was significantly different ($p<0.0001$) within vitamin E groups as measured from serum collected during necropsy (Table 4). Animals consuming the 15 mg E/kg (low intake) and 75 mg E/kg (recommended intake) diets had significantly lower FRAP values compared to those animals consuming the 500 mg E/kg of diet (high intake). Rats consuming the high E diet had a FRAP level 27.9% greater than rats consuming low E diet and 20.3% greater than the recommended E diet. The serum antioxidant potential was clearly affected by the high intake level of vitamin E. Serum FRAP levels though were not affected by HU or the interaction of HU and vitamin E.

Dietary vitamin E (alpha-tocopherol) concentrations had a dose-dependent effect on serum alpha-tocopherol concentrations. The initial serum was collected after 8 weeks of diet treatment and before hindlimb unloading (HU) occurred. Final serum was collected at necropsy. Initiation and final serum for each animal were compared and a significant difference ($p<0.0001$) in alpha-tocopherol concentration existed among all three vitamin E groups at initiation of HU as well as at necropsy (Table 5). Animals consuming the recommended E intake had serum alpha-tocopherol concentrations 84% greater at initiation and 58% greater at necropsy than animals consuming the low E intake. Those consuming the high E level had serum alpha-tocopherol concentrations 188% greater at

Table 5. Vitamin E concentrations of serum collected prior to hindlimb unloading and at necropsy from ambulatory (Amb) and hindlimb unloaded (HU) rats fed 15, 75, or 500 IU vitamin E/kg of diet and measured by high performance liquid chromatography (HPLC).[1]

| | N | Initial (μg/ml) | N | Final (μg/ml) |
|---|---|---|---|---|
| Amb, E-15 | 6 | 10.43±2.43 | 15 | 15.45±1.19 |
| Amb, E-75 | 14 | 20.94±2.19 | 13 | 25.38±2.53 |
| Amb, E-500 | 15 | 29.78±2.20 | 15 | 32.17±2.20 |
| HU, E-15 | 11 | 11.40±1.24 | 17 | 16.70±1.10 |
| HU, E-75 | 14 | 19.85±2.08 | 15 | 25.49±1.77 |
| HU, E-500 | 14 | 34.20±3.40 | 14 | 37.25±2.94 |
| | | | | |
| E-15 | 17 | 11.06±1.14$^{c}$ | 32 | 16.12±0.80$^{c}$ |
| E-75 | 28 | 20.40±1.49$^{b}$ | 28 | 25.44±1.48$^{b}$ |
| E-500 | 29 | 31.91±2.00$^{a}$ | 29 | 34.62±1.85$^{a}$ |
| | | | | |
| Amb | 35 | 22.93±1.78 | 43 | 24.28±1.57 |
| HU | 39 | 22.62±2.08 | 46 | 25.82±1.67 |
| | | | | |
| Source of Variation | | *p*-values | | *p*-values |
| HU | | 0.50 | | 0.19 |
| E Diet | | <0.0001 | | <0.0001 |
| HU*E Diet | | 0.49 | | 0.44 |

[1]Values represent means ± SEM. Values with different superscripts for the same parameter are significantly different ($p<0.05$)

initiation and 145% greater at necropsy when compared to those consuming the low E intake. Differences between serum alpha-tocopherol concentrations decreased over time, however, the serum concentration of each vitamin E treatment group actually increased during the 28 days. Final serum alpha-tocopherol concentrations of low, recommended, and high E diet increased by 46%, 25%, and 8% respectively when compared to the same diet at initiation. The percentage change for the high E diet, 8%, is much lower than the other percentage increases and may reflect a plateau in the group fed 500 mg E/kg of diet.

Other research groups have also reported that animals of different ages and species fed diets with different vitamin E concentrations developed significantly different serum alpha-tocopherol concentrations. Lii et al. (1998) reported an increase in serum alpha-tocopherol concentrations in weanling male rats fed 0, 100, or 1500 ppm vitamin E for 8 weeks and observed a 78% increase between the moderate level and the high level. Xu and colleagues (1995) reported a significant increase in alpha-tocopherol concentrations of young chicks fed three times the requirement of vitamin E (90 IU/kg) for 14 days compared to chicks fed the ambulatory level of 30 IU/kg. Although two different species were studied, the alpha-tocopherol concentrations were increased significantly by increased vitamin E in the diet.

As expected, 28 days of HU had a significant effect on femur bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD) (Table 6). A reduction in femur BMA ($p<0.001$) occurred in HU animals indicating that bone geometry was altered due to unloading. BMC was also reduced ($p<0.0001$) in the femur and was most likely a result of increased resorption as indicated by the increase in TRAP activity. A significant reduction ($p<0.001$) in BMD also occurred. BMA decreased by 3.0%, BMC

Table 6. Femur BMA, BMC, and BMD measured by dual energy X-ray absorptiometry (DEXA) in ambulatory (Amb) and hindlimb unloaded (HU) rats fed 15, 75, or 500 IU vitamin E/kg of diet.[1]

| | N | Femur BMA ($cm^2$) | Femur BMC (g) | Femur BMD ($g/cm^2$) |
|---|---|---|---|---|
| Amb, E-15 | 15 | 2.5231±0.0309 | 0.6594±0.0105 | 0.2613±0.0019 |
| Amb, E-75 | 15 | 2.5846±0.0404 | 0.6860±0.0145 | 0.2651±0.0019 |
| Amb, E-500 | 16 | 2.5112±0.0355 | 0.6890±0.0161 | 0.2579±0.0032 |
| HU, E-15 | 15 | 2.4990±0.0270 | 0.6322±0.0095 | 0.2529±0.0024 |
| HU, E-75 | 16 | 2.4246±0.0287 | 0.6125±0.0106 | 0.2526±0.0029 |
| HU, E-500 | 16 | 2.4738±0.0392 | 0.6177±0.0161 | 0.2492±0.0031 |
| E-15 | 30 | 2.5110±0.0203 | 0.6458±0.0074 | 0.2571±0.0017 |
| E-75 | 31 | 2.5020±0.0282 | 0.6481±0.0110 | 0.2586±0.0021 |
| E-500 | 32 | 2.4925±0.0262 | 0.6333±0.0116 | 0.2536±0.0023 |
| Amb | 46 | 2.5390±0.0208[a] | 0.6645±0.0082[a] | 0.2614±0.0015[a] |
| HU | 47 | 2.4651±0.0188[b] | 0.6206±0.0072[b] | 0.2515±0.0016[b] |
| Source of Variation | | *p*-values | *p*-values | *p*-values |
| HU | | <0.001 | <0.0001 | <0.001 |
| E Diet | | 0.86 | 0.45 | 0.13 |
| HU*E Diet | | 0.10 | 0.16 | 0.68 |

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$)
[2]Bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD) are reported.

decreased 7.1%, and BMD decreased 3.9% by the end of our study. Vitamin E diet alone did not have a significant effect on BMA, BMC, or BMD of the femur nor did the interaction of HU and vitamin E.

A reduction in BMC or both BMA and BMC together can result in a reduction of BMD values. Other researchers also reported decreased BMD in weightlessness conditions. Lafage-Proust and colleagues (1998) reported a detectable bone loss in the femoral metaphysis/epiphysis of 2-month old male space-flown rats after a 14-day mission. Utilizing a HU model, Mosekilde et al. (2000) observed a significant decrease of 4.4% in the tibeal BMD of 4-month old female rats unloaded for 28 days.

BMA ($p<0.003$), BMC ($p<0.0001$), and BMD ($p<0.0001$) were also significantly decreased in the L-5 vertebrae of HU rats (Table 7). The BMA in lumbar vertebrae of unloaded animals decreased 4.7% while BMC decreased 11.7% and BMD decreased 6.2%. The larger reduction in BMC of the L-5 vertebrae compared to the femur suggests a higher metabolic activity due to the larger amount of trabecular bone present in vertebrae.

HU significantly decreased ($p<0.05$) femur length of animals compared to those in the ambulatory group (Table 8). A 1.4% decrease in length occurred in unloaded animals after 28 days. Garber and co-workers (2000) reported a significant decrease in bone length in 6-month old rats unloaded for 14 days (1.8% decrease) and for 28 days (2.4% decrease) when compared to ambulatory animals. However, other research studies have not observed any change in bone length involving rats in unloaded conditions. Lafage-Proust et al. (1998) did not detect a change in bone length between 2-month old rats flown in space for 14 days and ambulatory rats. No significant changes in bone length were

Table 7. Fifth lumbar (L-5) BMA, BMC, and BMD measured by dual energy X-ray absorptiometry (DEXA) in ambulatory (Amb) and hindlimb unloaded (HU) rats fed 15, 75, or 500 IU vitamin E/kg of diet.[1]

| | N | L-5 BMA ($cm^2$) | L-5 BMC (g) | L-5 BMD ($g/cm^2$) |
|---|---|---|---|---|
| Amb, E-15 | 15 | 0.6975±0.0111 | 0.1758±0.0037 | 0.2520±0.0038 |
| Amb, E-75 | 15 | 0.7197±0.0134 | 0.1895±0.0055 | 0.2628±0.0037 |
| Amb, E-500 | 15 | 0.7029±0.0182 | 0.1784±0.0071 | 0.2527±0.0047 |
| HU, E-15 | 16 | 0.6770±0.0108 | 0.1642±0.0036 | 0.2424±0.0031 |
| HU, E-75 | 16 | 0.6625±0.0066 | 0.1592±0.0028 | 0.2402±0.0033 |
| HU, E-500 | 15 | 0.6861±0.0141 | 0.1643±0.0036 | 0.2397±0.0038 |
| | | | | |
| E-15 | 31 | 0.6869±0.0078 | 0.1697±0.0028 | 0.2470±0.0025 |
| E-75 | 31 | 0.6902±0.0089 | 0.1738±0.0041 | 0.2511±0.0031 |
| E-500 | 30 | 0.6945±0.0114 | 0.1713±0.0041 | 0.2462±0.0032 |
| | | | | |
| Amb | 45 | 0.7067±0.0083[a] | 0.1812±0.0033[a] | 0.2558±0.0024[a] |
| HU | 47 | 0.6750±0.0062[b] | 0.1625±0.0019[b] | 0.2408±0.0019[b] |
| | | | | |
| Source of Variation | | *p*-values | *p*-values | *p*-values |
| HU | | <0.003 | <0.0001 | <0.0001 |
| E Diet | | 0.89 | 0.54 | 0.20 |
| HU*E Diet | | 0.23 | 0.07 | 0.17 |

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$)
[2]Bone mineral area (BMA), bone mineral content (BMC), and bone mineral density (BMD) are reported.

Table 8. Femur length at necropsy from ambulatory (Amb) and hindlimb unloaded (HU) rats fed 15, 75, or 500 IU vitamin E/kg of diet.[1]

| | N | Femur Length (mm) |
|---|---|---|
| Amb, E-15 | 15 | 42.4±0.2 |
| Amb, E-75 | 15 | 42.4±0.5 |
| Amb, E-500 | 16 | 42.3±0.2 |
| HU, E-15 | 16 | 41.9±0.3 |
| HU, E-75 | 16 | 41.5±0.3 |
| HU, E-500 | 16 | 42.0±0.4 |
| | | |
| E-15 | 31 | 41.2±0.2 |
| E-75 | 31 | 41.9±0.3 |
| E-500 | 32 | 42.1±0.2 |
| | | |
| Amb | 44 | 42.4±0.2[a] |
| HU | 46 | 41.8±0.2[b] |
| | | |
| Source of Variation | | *p*-values |
| HU | | <0.05 |
| E Diet | | 0.81 |
| HU*E Diet | | 0.75 |

[1]Values represent means ± SEM. Values that do not share a superscript for the same parameter are significantly different ($p<0.05$).

observed by LeBlanc and colleagues (1985) in bones of 6-7 month old rats suspended for periods of 14, 30, 60, or 90 days. Likewise, hindlimb immobilization of the right leg of 4-month old females in a study conducted by Mosekilde and colleagues (2000) did not significantly change femoral length when compared to the ambulatory left leg. It is not entirely clear why bone length in HU animals was altered in this study given that rates of bone growth are slower in 12-month old rats than in younger animals.

Femurs of HU animals did not accommodate increased loads during mechanical strength testing as well as did femurs of ambulatory animals. Ultimate load of the femora was significantly lower ($p<0.006$) in HU animals than in ambulatory animals (Table 9) indicating that less force was needed to break the bone. Femur BMD and femur ultimate load were positively correlated (r=.73, $p<0.0001$). Yield load, stiffness, yield stress, and ultimate stress were not significantly affected by HU, vitamin E, or their interaction. The decrease in ultimate load observed during three-point bending of the femur may reflect porosity or a decrease in mineralization in the HU animals. Ultimate load was likewise significantly decreased in 6-month old rats unloaded for 14 days and 28 days (Garber et al. 2000).

Compression testing of the 4$^{th}$ lumbar vertebrae (L-4) provided significantly different results in both structural and material variables of HU animals compared to ambulatory animals. Yield load, ultimate load, and stiffness (structural variables) as well as the yield stress, ultimate stress, and modulus (material variables) were all lower ($p<0.005$) in L-4 vertebrae of the HU group (Table 10). Because of the high trabecular content of vertebrae, the decline of both structural and material strength in HU animals is not surprising. A dramatic decrease of 55.9% in yield load, 49.1% in ultimate load, and

Table 9. Mechanical properties of load, stiffness, and stress in femurs of ambulatory (Amb) and hindlimb unloaded (HU) rats fed 15, 75, or 500 IU vitamin E/kg of diet.[1]

| | Structural Property | Structural Property | Structural Property | Material Property | Material Property |
|---|---|---|---|---|---|
| | Yield Load (N) | Ultimate Load (N) | Stiffness (N/mm) | Yield Stress ($N/mm^2$) | Ultimate Stress ($N/mm^2$) |
| Amb, E-15 | 149.8±6.5 | 179.3±4.0 | 499.6±14.6 | 117.9±5.7 | 157.0±6.6 |
| Amb, E-75 | 159.9±9.4 | 189.1±4.6 | 519.3±17.7 | 123.0±5.9 | 166.6±6.8 |
| Amb, E-500 | 148.6±7.3 | 173.4±4.9 | 484.9±21.7 | 120.8±5.9 | 159.4±6.9 |
| HU, E-15 | 140.6±6.3 | 170.3±3.8 | 477.9±14.0 | 117.2±5.7 | 160.2±7.1 |
| HU, E-75 | 144.4±7.2 | 170.1±4.1 | 476.8±14.2 | 117.3±5.9 | 160.0±6.4 |
| HU, E-500 | 144.1±7.0 | 170.7±4.8 | 478.8±19.0 | 112.9±5.7 | 153.0±6.4 |
| E-15 | 145.4±4.5 | 175.0±2.9 | 489.1±10.2 | 117.5±4.0 | 158.6±4.9 |
| E-75 | 151.9±5.9 | 179.0±3.5 | 498.0±11.8 | 120.1±4.2 | 163.3±4.7 |
| E-500 | 146.4±5.0 | 172.0±3.4 | 481.7±14.1 | 116.9±4.1 | 156.2±4.7 |
| Amb | 152.5±4.4 | 180.6±2.7[a] | 501.6±10.4 | 120.6±3.4 | 161.0±3.9 |
| HU | 143.1±3.9 | 170.4±2.4[b] | 477.8±9.1 | 115.8±3.3 | 157.7±3.8 |
| Source of Variation | *p*-values | *p*-values | *p*-values | *p*-values | *p*-values |
| HU | 0.11 | <0.006 | 0.10 | 0.31 | 0.55 |
| E Diet | 0.61 | 0.23 | 0.64 | 0.84 | 0.55 |
| HU*E Diet | 0.75 | 0.18 | 0.56 | 0.81 | 0.71 |

[1]Values represent means ± SEM (n for treatment groups = 13-16). Values with different subscripts for the same parameter are significantly different ($p<0.05$).

Table 10. Mechanical properties measured by compression test of fourth lumbar (L-4) vertebral body in ambulatory (Amb) and hindlimb unloaded (HU) rats fed 15, 75, or 500 IU vitamin E/kg of diet.[1, 2]

| Property: | N | Structural Yield Load (N) | Structural Ultimate Load (N) | Structural Stiffness (N/mm) | Material Yield Stress ($N/mm^2$) | Material Ultimate Stress ($N/mm^2$) | Material Modulus ($N/mm^2$) |
|---|---|---|---|---|---|---|---|
| Amb, E-15 | 11 | 152.2±14.3 | 168.0±13.8 | 826.0±50.9 | $20.8±1.6^{a}$ | 23.1±1.6 | $703.9±41.9^{a}$ |
| Amb, E-75 | 11 | 160.5±13.0 | 184.4±14.9 | 891.2±69.1 | $20.2±1.4^{ab}$ | 23.1±1.6 | $631.9±54.4^{ab}$ |
| Amb, E-500 | 12 | 145.0±11.8 | 174.2±10.5 | 758.9±52.5 | $16.6±1.3^{bc}$ | 19.9±1.0 | $526.3±43.8^{bc}$ |
| HU, E-15 | 11 | 96.3±8.2 | 121.9±8.4 | 616.3±40.9 | $12.7±0.9^{d}$ | 16.2±1.1 | $468.7±29.0^{c}$ |
| HU, E-75 | 12 | 91.0±9.8 | 110.3±10.1 | 591.2±47.7 | $12.0±1.1^{d}$ | 14.7±1.1 | $492.2±30.6^{c}$ |
| HU, E-500 | 12 | 105.7±10.3 | 121.1±10.5 | 680.0±55.0 | $14.9±1.5^{cd}$ | 17.0±1.4 | $581.2±57.0^{ac}$ |
| E-15 | 22 | 124.2±10.1 | 144.9±9.4 | 721.2±39.2 | 16.8±1.3 | 19.6±1.2 | 586.3±35.7 |
| E-75 | 23 | 124.2±10.8 | 145.8±11.7 | 734.7±51.5 | 15.9±1.2 | 18.8±1.3 | 559.0±33.3 |
| E-500 | 24 | 125.4±8.7 | 147.7±9.1 | 719.4±38.1 | 15.8±1.0 | 18.4±0.9 | 553.8±35.6 |
| Amb | 34 | $152.3±7.3^{x}$ | $175.5±7.4^{x}$ | $823.4±33.7^{x}$ | $19.1±0.9^{x}$ | $22.0±0.8^{x}$ | $617.9±29.2^{x}$ |
| HU | 35 | $97.7±5.4^{y}$ | $117.6±5.6^{y}$ | $629.6±28.0^{y}$ | $13.2±0.7^{y}$ | $16.0±0.7^{y}$ | $515.4±24.7^{y}$ |
| Source of Variation | | | | | | | |
| HU | | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.0001 | <0.005 |
| E Diet | | 0.99 | 0.97 | 0.90 | 0.74 | 0.66 | 0.75 |
| HU*E Diet | | 0.41 | 0.46 | 0.12 | <0.03 | 0.10 | 0.0056 |

[1]Values represent means ± SEM. Values with different subscripts for the same parameter are significantly different ($p<0.05$).
[2]Mechanical property N=Newtons

30.8% in stiffness indicated that unloaded L-4 vertebrae were structurally damaged at a much lower force and were ultimately fractured before vertebrae from control animals. Material properties also displayed dramatic decreases of 44.7% in yield stress, 37.5% decrease in ultimate stress, and 19.9% in modulus. No significant difference existed between vitamin E treatment groups indicating that vitamin E alone did not protect vertebral strength.

A significant difference in yield stress of L-4 vertebrae existed within the ambulatory groups but did not exist within the HU groups of vitamin E. The Ambulatory E-15 (Amb Low E) group tolerated a 25.3% greater yield stress than the Ambulatory E-500 (Amb High E) group. Interestingly the yield stress values in ambulatory rats decreased as the vitamin E level increased as if a high level of vitamin E actually weakened the bone. HU groups, however, did not display the same pattern of less sustained stress with more dietary vitamin E. No significant difference occurred among HU dietary groups in regards to yield stress of L-4 vertebrae.

The modulus of L-4 vertebrae was significantly lower ($p<0.005$) in HU animals. The interaction between dietary vitamin E and HU was nearly significant ($p=0.0056$). A 19.9% decrease in this material property was observed in unloaded animals when compared to ambulatory animals. Modulus values were highest in the ambulatory E-15 group and 50.2% greater than the lowest values in the HU E-15 group when compared. The modulus values decreased within the ambulatory groups as the vitamin E level increased but this pattern was not repeated within the HU groups. No significant difference existed among dietary groups that were HU.

All L-4 vertebrae biomechanical properties were significantly decreased in HU animals whereas the only property significantly affected by HU in femora was the ultimate load. Vertebrae in this experiment had greater alterations in biomechanical properties than femora did during the 28 days of HU.

# CHAPTER V

## SUMMARY AND CONCLUSION

### Summary

The purpose of this research was to examine the effects of vitamin E, hindlimb unloading, or their interaction on biomechanical properties of bone in mature male rats. Mechanical strength of femora and vertebrae, bone mineral area, bone mineral content, bone mineral density, selected biochemical markers of bone formation and resorption, total antioxidant capacity, and serum alpha-tocopherol concentrations were determined.

### Results of Hypotheses Testing

The following hypotheses were developed for this study:

1. Hindlimb unloading, vitamin E status, or their interaction will not significantly alter mechanical strength in 12-month old rats.

This hypothesis was rejected due to the significant decrease in the ultimate load of femora in hindlimb-unloaded animals and the significant decrease in all structural and material properties of the L-4 vertebrae with unloading. An interaction between vitamin E and hindlimb-unloading was also observed in the yield stress of L-4 vertebrae.

2. Hindlimb unloading, vitamin E status, or their interaction will not significantly alter bone mineral area, bone mineral content, or bone mineral density in 12-month old rats.

Hypothesis #2 was rejected based on dual-energy X-ray absorptiometry (DEXA) scans that indicated a significant reduction in bone mineral area, bone mineral content, and bone mineral density of both femora and L-4 vertebrae in hindlimb-unloaded animals. DEXA scans of animals did not display any significant difference due to Vitamin E nor due to the interaction of vitamin E and hindlimb unloading.

3. There will be no statistically significant effects of hindlimb unloading, vitamin E status, or their interaction on biochemical markers of bone remodeling (i.e. serum alkaline phosphatase activity and serum tartrate-resistant acid phosphatase activity) in 12-month old rats.

This hypothesis was rejected due to the significant increase ($p<0.0001$) of tartrate-resistant acid phosphatase activity (TRAP), a marker of bone resorption, in unloaded animals. Bone formation did not increase to compensate for the resorption that occurred during the 28 days of hindlimb unloading as indicated by similar levels of serum alkaline phosphatase (ALP) activity between loaded and unloaded animals. Vitamin E alone did not significantly affect biochemical markers nor did an interaction of vitamin E and hindlimb unloading of mature male rats.

## Conclusion and Suggestions

Although the serum alpha-tocopherol concentration and the total antioxidant capacity were increased by dietary supplementation, vitamin E did not significantly affect any of our measurements of biomechanical strength in either femora or L-4 vertebrae of skeletally unloaded animals. Regardless of bone mineral density measurements, fracturing the bone is the ultimate test of bone strength and no

significant differences in the three point bending of the femur or the compression test of the fourth lumbar vertebra (L-4) were demonstrated by vitamin E within this time period.

Hindlimb unloading (HU) resulted in decreased strength in both the femur and the L-4 vertebrae. The only interaction between vitamin E dietary treatments and hindlimb unloading was observed in the yield stress and the modulus of the L-4 vertebrae. The larger the dosage of vitamin E in ambulatory animals, the less yield stress the L-4 could sustain before deformation would occur. Equivalent dietary vitamin E provided to the hindlimb-unloaded animals did not produce the same pattern in L-4 vertebrae for either yield stress or modulus. Perhaps a longer feeding period may have produced the same trend in the femur but could not be detected due to the smaller amount of trabecular bone in the femur compared to the vertebrae.

Bone loss that occurred during our study is attributed to increased bone resorption without a coupled increase in bone formation. Studies involving hindlimb unloading and young growing rats have demonstrated a reduction in bone formation, followed by resumption, with a net effect of diminished longitudinal bone growth. Mature male rats in one study of unloading demonstrated a dramatic decrease in bone formation when compared to young growing rats. The present study did not provide the same results in mature male rats and therefore substantiates the need for further research. Mature rats have not been widely used in studies of weightlessness or in bone metabolism research and should be considered when seeking an understanding of bone loss and long-term bed rest, immobilization, or space flight.

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