**Title:** Effects of skeletal unloading and vitamin E on bone in mature rats

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## Summary


- These data demonstrate that eleven-month-old rats are a valuable ground-based model for studying skeletal unloading. Twenty-eight days of hindlimb unloading (HLU) resulted in significant bone loss (femoral and vertebral bone mineral area, bone mineral content, and bone mineral density estimated by DXA and ash weights).

- Structural properties of bone were changed by HLU as indicated by decreased ultimate load and yield load in the HLU animals compared to the ambulatory (AMB) controls.

- Bone loss in unloaded animals resulted from both increased resorption and inhibition of bone formation. Histomorphometry data showed that trabecular surface expressed in relation to bone volume (BS/BV), labeled surface in relation to bone surface (LS/BS), trabecular thickness, and bone formation rate were all decreased in HLU animals compared to AMB.

- In addition to reducing total calcium in the femur and vertebra, HLU reduced total magnesium, iron, and zinc in both bones as well as copper in the vertebra.

- In HLU animals, high intakes of vitamin E had a positive effect on material properties of vertebrae measured by increased yield stress and by Young's modulus of elasticity.

- Based on histomorphometry data, trabecular surface in relation to total volume (BS/TV) as well as trabecular number (TbN) tended to increase with vitamin E intakes in HLU but not in AMB animals.

- Vitamin E dosage positively affected bone of unloaded animals while having no such effect in AMB controls.

## Introduction

Understanding the physiological changes associated with weightlessness is essential as NASA plans for future travel to Mars. Loss of bone is one of the critical challenges affecting crew members. Data collected on the Skylab missions revealed a significant correlation between bone loss and space flight duration (Smith et al., 1997). Leach et al. (1997) showed increased rate of bone resorption as well as decreased rate of bone formation was involved in bone loss occurring in astronauts on the Skylab missions.

Free radicals have been reported to have an effect on bone *in vivo* and *in vitro* by stimulating osteoclasts and increasing the rate of bone resorption (Garret et al., 1990). In spaceflight, the increased exposure to radiation enhances the possibility of oxidative damage to the bone. Antioxidant nutrients may have a protective effect on the skeletal tissue similar to that seen in other tissues (Ames et al., 1993). Due to the prohibitive expense and space limitations on mission flights, alternative methods of investigating microgravity-induced physiological changes are needed. Hindlimb unloading (HLU) produces a deficit in periosteal bone formation (Globus, et al., 1986), a decreased number of osteoblasts (Wronski & Morey, 1983) and has been utilized as an appropriate model for examining bones changes in simulated weightlessness (Wronski & Morey-Holton, 1987).

This study was designed to evaluate the potential of vitamin E for preventing bone loss associated with weightlessness in the HLU rat model. The long-term goal of this project is to identify an optimal dose of vitamin E to reduce the risk of bone loss and osteoporotic fractures under conditions of weightlessness such as those of astronauts and non-ambulatory or immobilized individuals.

The ***hypothesis*** of this project was that vitamin E, a dietary antioxidant, would dose-dependently reduce weightlessness-associated deterioration of bone, which has in part been linked to oxidative stress.

The specific aims of this project were:

***Specific Aim 1:*** To investigate whether vitamin E dose-dependently prevents loss of bone mineral density and deterioration of biomechanical properties in the HLU rat model of weightlessness.

***Specific Aim 2:*** To examine some of the underlying mechanisms by which vitamin E exerts bone protective effects using histomorphometric, biomechanical and biochemical analyses.

## Materials and Methods

Eight and one-half month-old rats were randomly assigned to one of three semi-purified diets containing 15, 75, or 500 IU dl-alpha tocopherol (low, recommended, or high), and ambulatory (AMB) or HLU treatments. Diets were fed for nine weeks prior to the start of the hindlimb unloading period. Subsequently rats were either HLU or AMB for four weeks. Eight days and one day prior to necropsy, rats were injected with calcein for dynamic bone histomorphometric analysis. Rats were scanned using the small animal software package available with the Hologic QDR4500 dual-energy x-ray absorptiometry (DXA) prior to unloading and at the end of the treatment period. Animals were anesthetized with ketamine/xylazine for scanning and then exsanguinated by cardiac puncture. Age at necropsy was approximately 11 months. Samples were aliquoted and appropriately stored for future analyses.

Serum was analyzed colorimetrically for markers of bone formation, alkaline phosphatase (ALP) activity, and bone resorption, tartrate resistant acid phosphatase (TRAP) activity, using the COBAS Fara II clinical analyzer (Roche, Inc., Montclair, NJ). Osteocalcin was analyzed using a rat specific immunoradiometric assay (IRMA) kit (Immutopics, San Clemente, CA). Ferric reducing ability of serum (FRA) and liver malondialdehyde were measured as indicators of antioxidant status and serum vitamin E was determined using high performance liquid chromatography (HPLC) and a C-18 column with methanol:water (95:5) as a mobile phase.

Mechanical strength of the femur was assessed via 3-point bending using an Instron Universal Testing Machine. The femur was placed on two rods spaced 20 mm apart and force was delivered to the mid-shaft by a crosshead moving at a constant speed of 1.0 mm/min. Compression analysis of the 4$^{th}$ lumbar vertebra was conducted by Dr. Mohammed P. Akhter. The fourth lumbar vertebral body (L4) was prepared with parallel flat ends and placed on a fixed lower pressure plate while an upper plate moved downwards at a rate of 3 mm/min causing compression in the anterior-posterior direction. Load displacement plots were analyzed to determine material properties. The average cross section for each vertebral body was used to calculate its ultimate stresses and Young's modulus.

Histomorphometric analysis of the femur was performed by Dr. Russell T. Turner as described elsewhere (Turner and Bell, 1986). Parameters evaluated were: bone volume/total volume (BV/TV); bone surface/total volume (BS/TV); bone surface/bone volume (BS/BV), labeled surface/bone surface (LS/BS); trabecular thickness (Tb.Th); trabecular separation (Tb.Sp); trabecular number (Tb.N); mineral apposition rate (MAR); bone-formation rate/bone surface (BFR/BS);

bone-formation rate/bone volume (BFR/BV); bone-formation rate/total volume (BFR/TV).

Bone mineral area, bone mineral content and bone mineral density of excised 5$^{th}$ lumbar vertebrae (L5) and of the left femur were estimated using the Hologic QDR4500 DXA with the small animal software package. Subsequently, femurs and 3$^{rd}$ lumbar vertebra were ashed according to a modification of the methods of Hill et al. (1986). After bones were fully ashed, the mineral residue was dissolved in concentrated double distilled nitric acid and diluted for analysis of calcium, magnesium, iron, copper, and zinc. Flame atomic absorption spectroscopy with an air-acetylene flame and deuterium background correction (Perkin Elmer Model 5100 PC, Norwalk, CT) was utilized at the most sensitive wavelength for each element.

Data analyses were performed using SAS version 8.0. The Generalized Linear Model (GLM) procedure was used with HLU vs. AMB and dietary vitamin E concentrations as main effects in a 2 x 3 factorial design. When a significant F statistic ($p < 0.05$) was noted from the analysis of variance, the least square means procedure was performed for separating means that were significantly ($p < 0.05$) different.

## Results

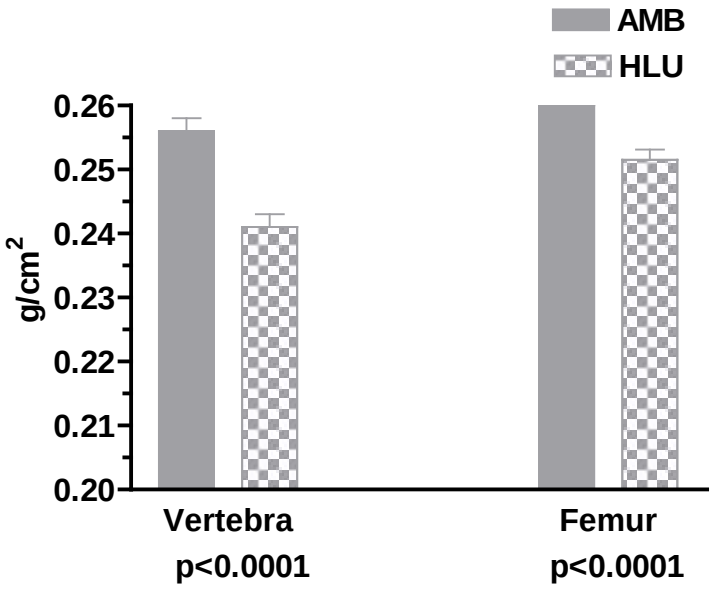
In this report, we have divided our findings into two parts: 1) comparisons of results between HLU and AMB conditions and 2) evaluation of dose-dependent effects of vitamin E on bone parameters of interest under both conditions.

### Effects of unloading in 11-month old male rats

This is the first study to examine the effects of skeletal unloading in 11-month old male rats. Significant effects of near weightlessness were demonstrated in these mature animals.

***DXA.*** Both 5th lumbar vertebra (L5) and femur bone mineral density (BMD) (Fig. 1) were significantly lowered in HLU animals compared to AMB controls, which is in agreement with ash weights (Figs. 2 & 3).

**Figure 1. Bone Mineral Density**




Bars represent mean ± SEM; n = 46 for AMB and 47 for HLU.

**Fig 2. Ash Weight - L5**

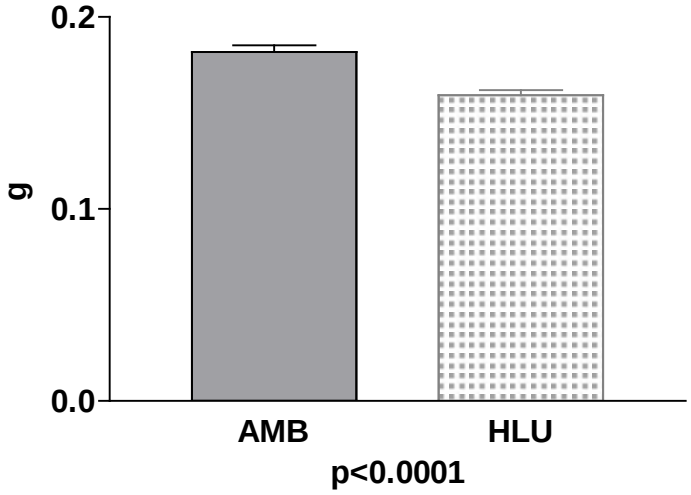
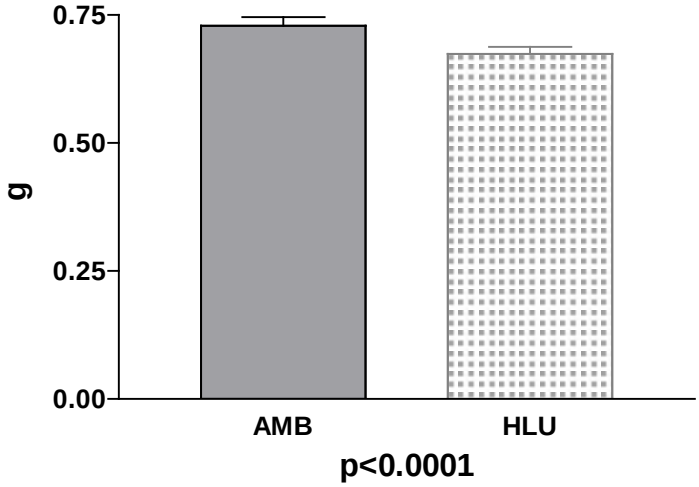
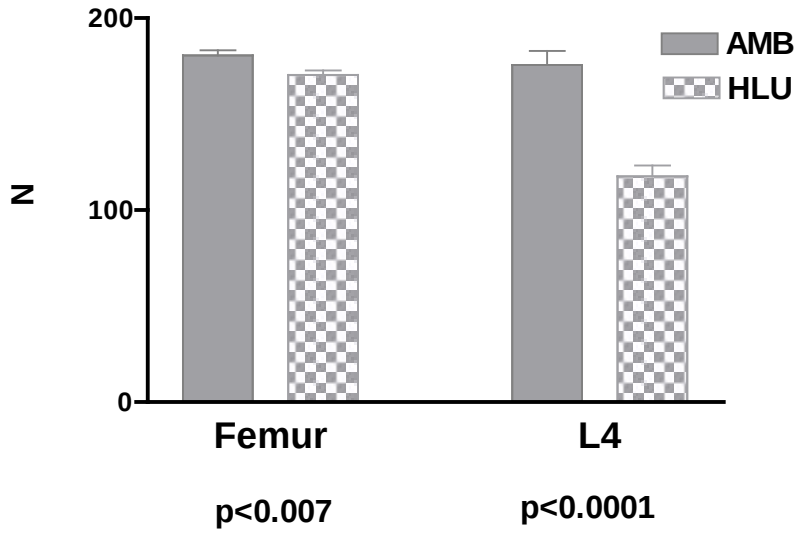



**Fig. 3. Ash Weight - Femur**




***Biomechanical Properties.*** The lower BMDs in the HLU group were accompanied by reduced structural properties of both the femur and L4 as indicated by reduced ultimate load (Fig. 4) and yield load.

**Figure 4. Ultimate Load**




Furthermore, the material properties of L4, ultimate stress (Fig. 5), yield stress and Young's modulus of elasticity (Fig. 6) were significantly decreased by suspension.

**Figure 5. Material Properties of L4**

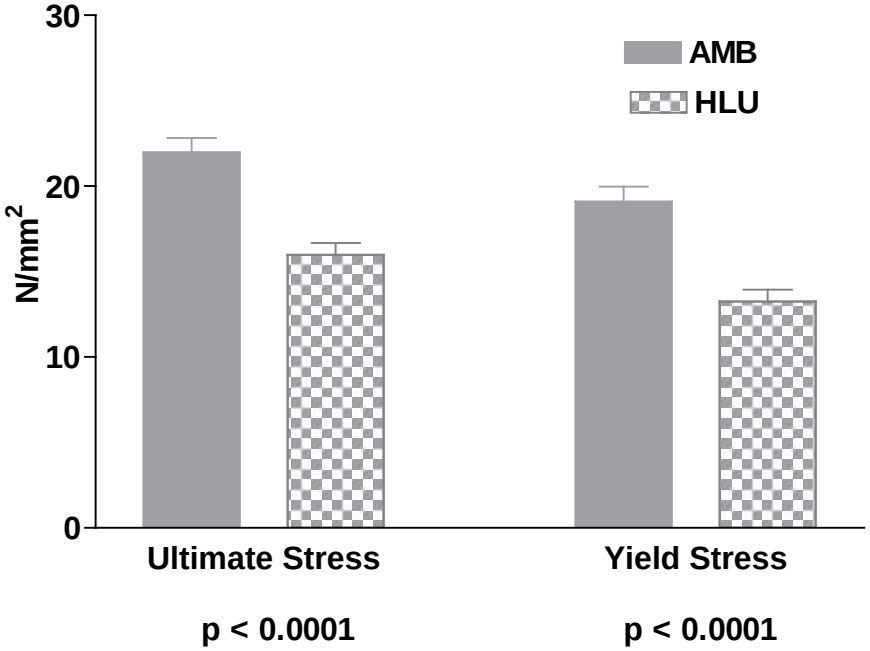
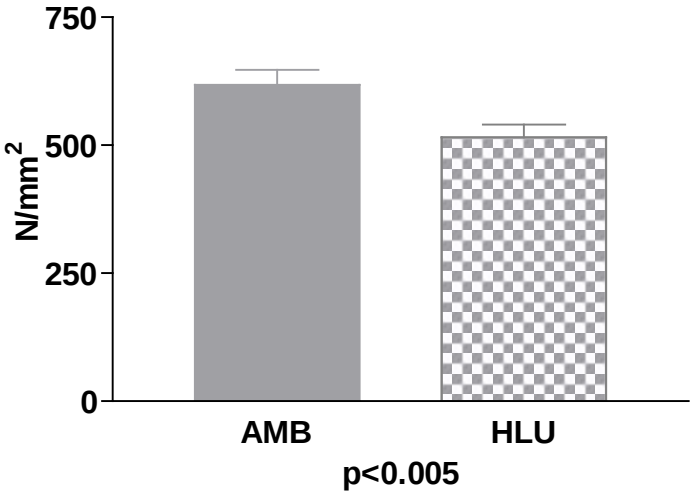


**Figure 6. Young's Modulus of Elasticity - L4**




***Histomorphometry.*** The histomorphometric data indicated that HLU decreased the rate of bone formation as assessed by lower rate of bone formation (BFR/BV) (Fig. 7)and increased the rate of bone resorption as assessed by LS/BS (Fig. 8) and trabecular thickness (Fig. 9).

**Fig. 7. Bone Formation Rate/ Bone Volume**

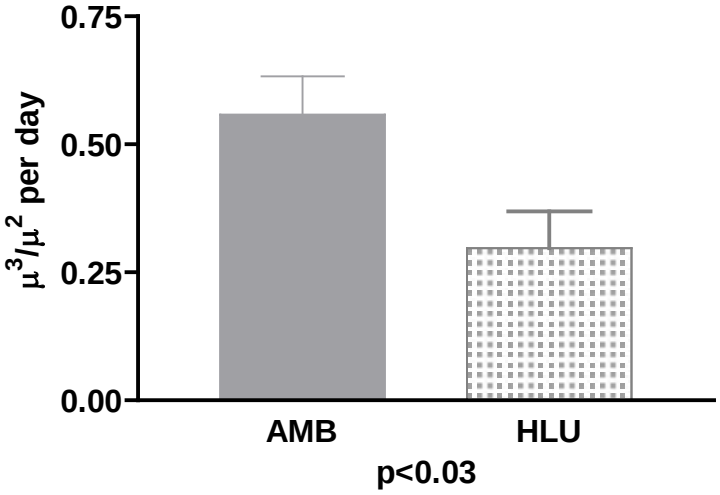


**Fig. 8. Labeled Surface/Bone Surface**

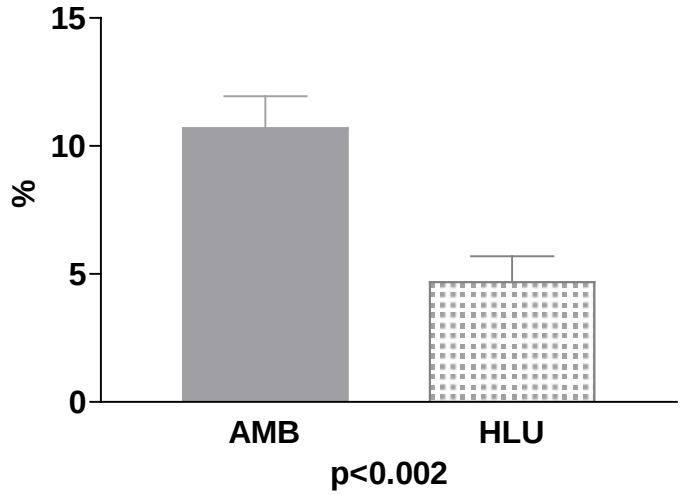
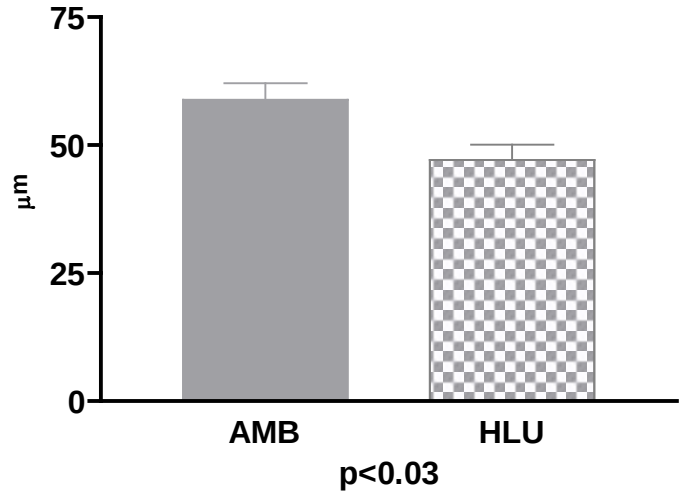
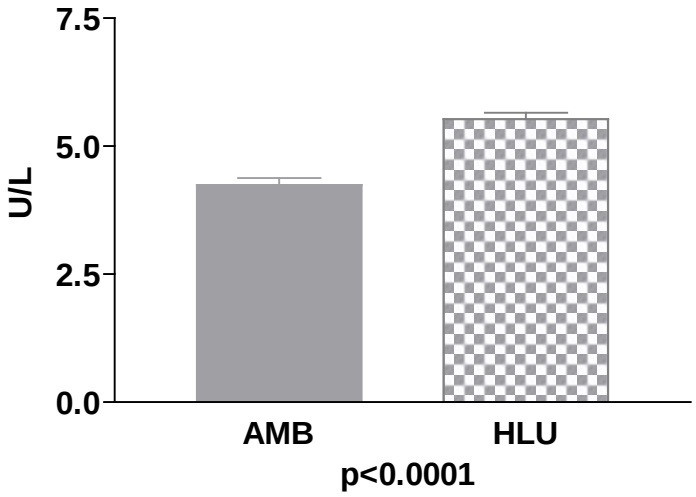



**Figure 9. Trabecular Thickness**




***Serum markers of bone metabolism.*** Serum markers indicated an increase (*p*<0.001) in tartrate-resistant acid phosphatase (TRAP) activity (Fig. 10), a marker of bone resorption, but no change in either marker of bone formation alkaline phosphatase (ALP) activity or osteocalcin (OC).

**Figure 10. Serum TRAP**




The findings of this study confirm that unloading exerts similar deleterious effects on bone of older animals to those reported by other investigators using younger growing rats. However, alterations in bone metabolism resulting in bone loss in mature rats appear to more closely mimic the metabolic bone changes occurring in the astronauts in space.

**The effects of vitamin E on bone parameters under hindlimb unloading and ambulatory conditions**

The dietary treatments were effective in creating differences in serum vitamin E concentrations even though treatments were not begun until the rats were eight and one-half months of age (Fig. 11). The dietary treatments produced significant differences in ferric reducing ability of serum (Fig. 12) and strong trends for decreased malondialdehyde (Fig. 13), a product of lipid peroxidation, in liver.

**Figure 11. Serum Vitamin E**

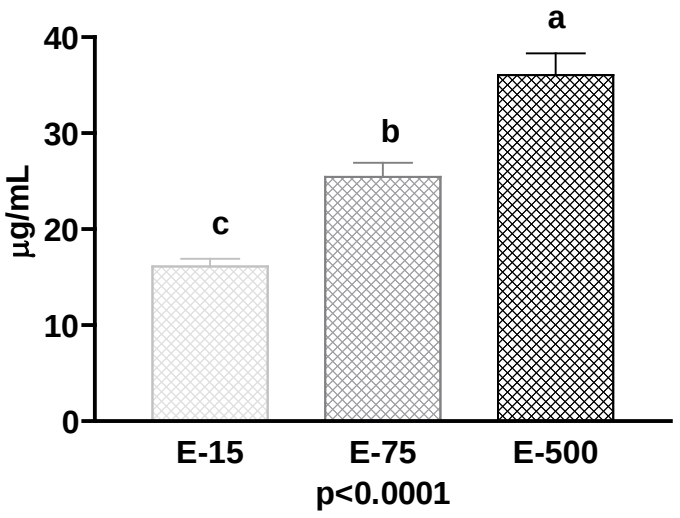



**Fig. 12. Serum Ferric Reducing Ability**

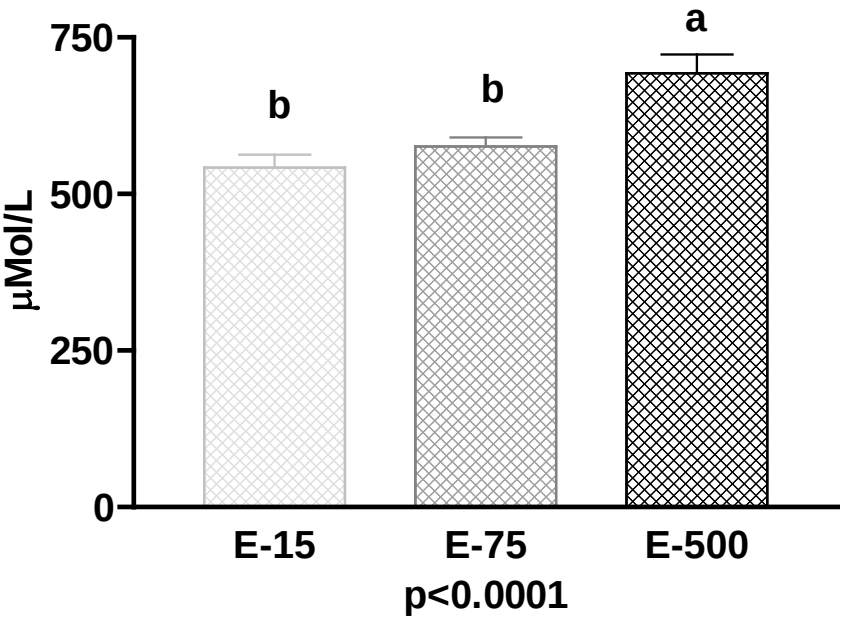
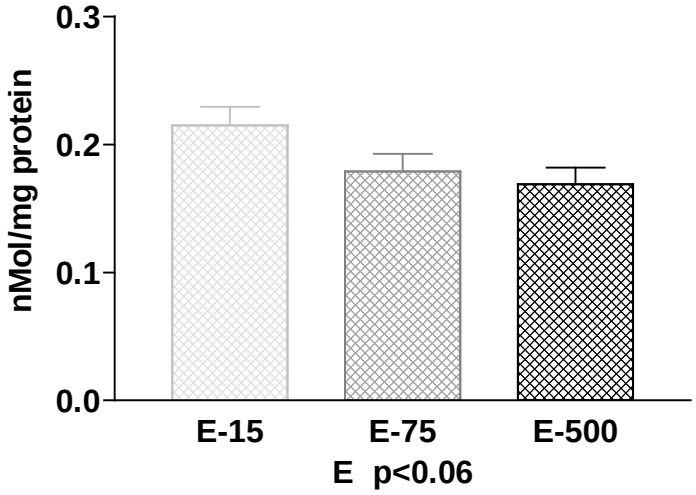


**Figure 13. Liver Malondialdehyde**




***Biomechanical Properties***. In HLU animals, high intakes of vitamin E had a positive effect on material properties of vertebrae measured by increased yield stress (Fig. 14) and by Young's modulus of elasticity (Fig. 15), but not in AMB controls.

**Figure 14. Yield Stress - L4**

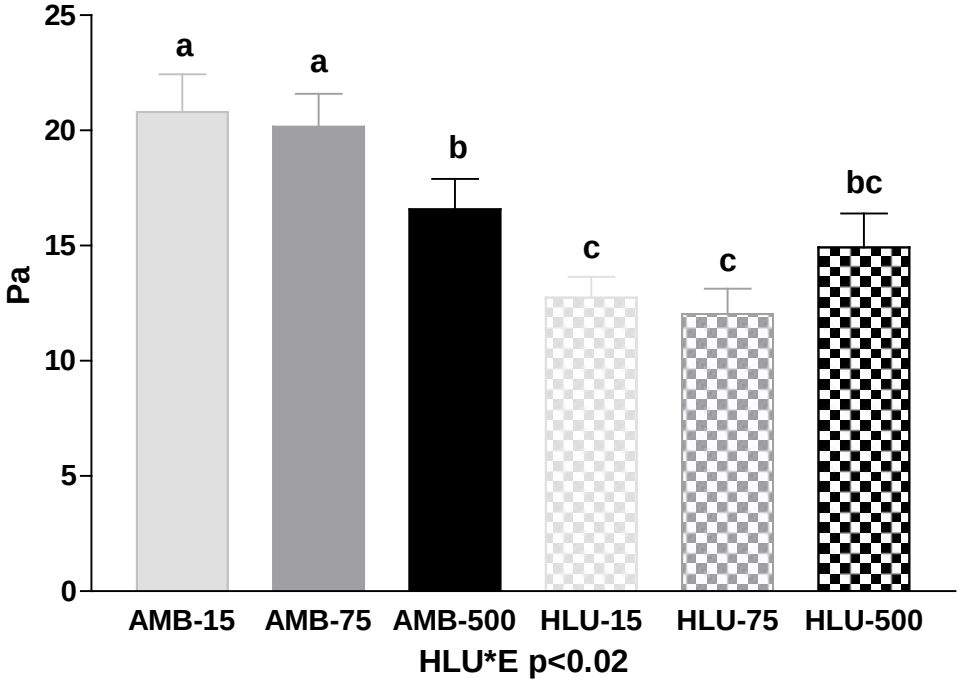
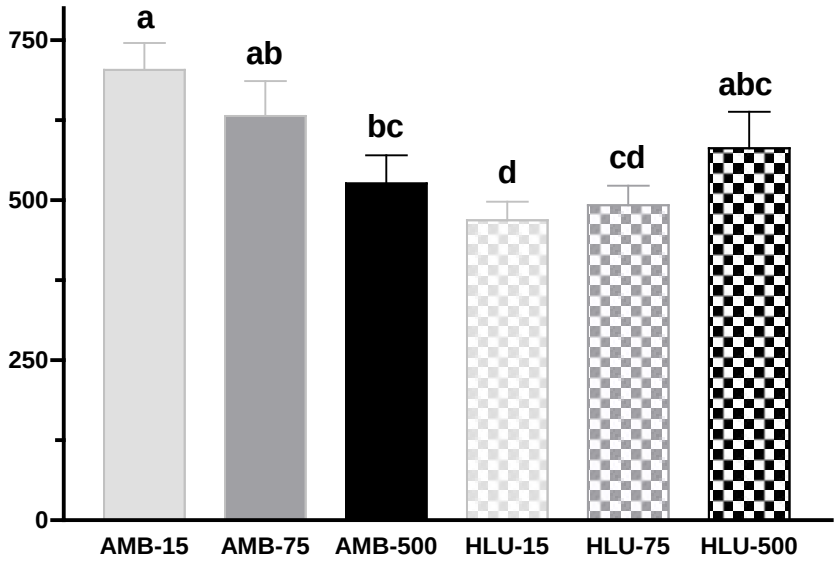


**Figure 15. Young's Modulus Of Elasticity-L4**




**HLU * E p<0.006**

***Histomorphometry.*** The histomorphometry data indicated that higher doses of vitamin E exerted positive effects on bone volume BV/TV (Fig. 16), BS/TV (Fig. 17) and Tb.N (Fig. 18) in HLU animals, but contrary to our expectations, the same doses of vitamin E had negative effects on these histomorphometric parameters in AMB controls.

**Figure16. Bone Volume/Total Volume**

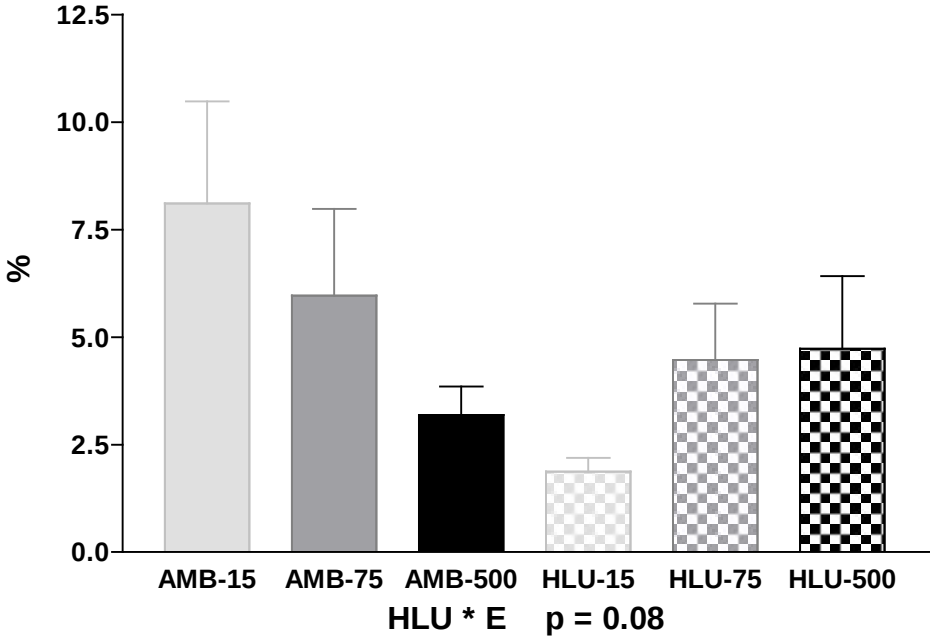



**Figure 17. Bone Surface/Total Volume**

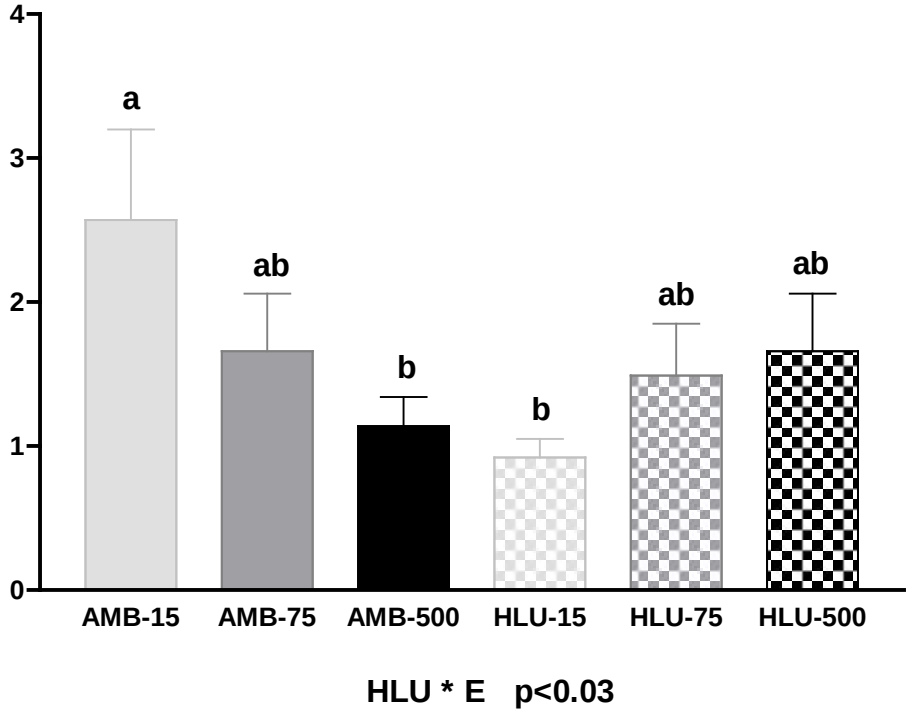
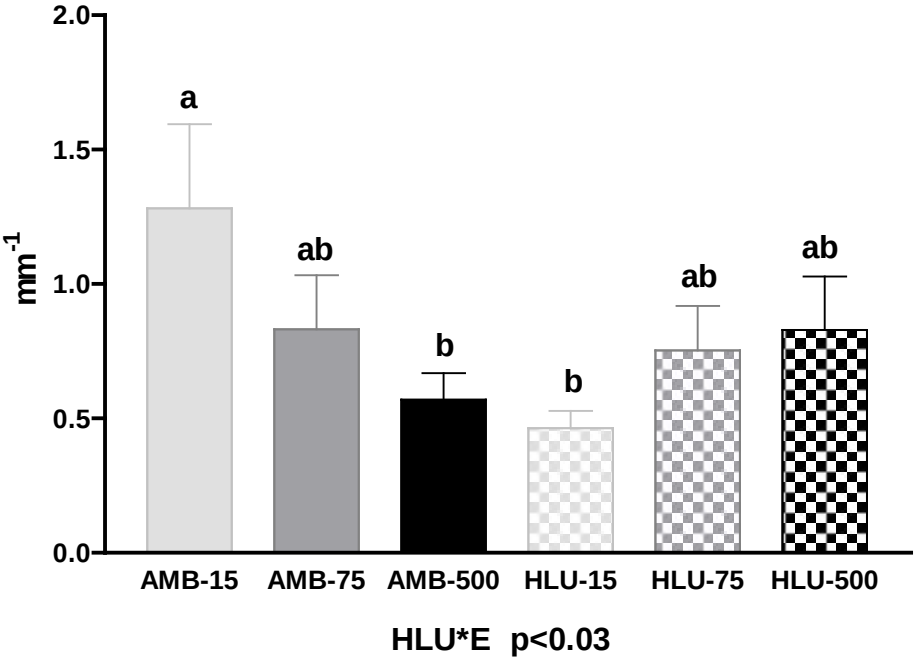
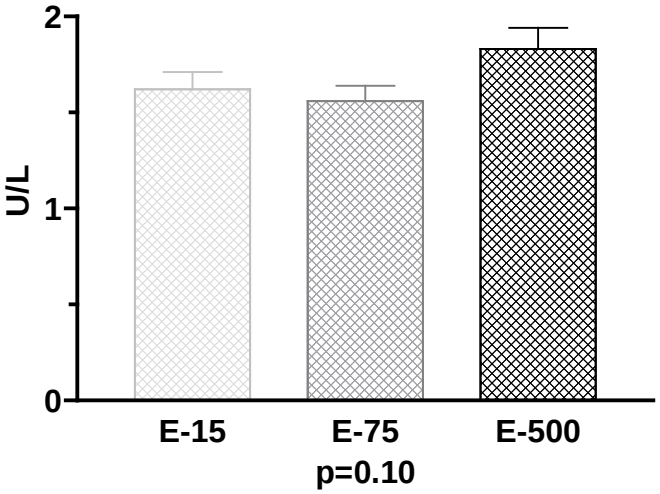


**Figure 18. Trabecular Number**




***Serum markers of bone metabolism.*** Serum ALP, a marker of bone formation, tended to increase with increased vitamin E in the diet (Fig. 19).

**Figure 19. Serum Alkaline Phosphatase**



***Implications:*** The data above (Figures 1-19) indicate that higher doses of vitamin E noticeably improve skeletal health as evidenced by higher bone volume and biomechanical properties in HLU animals. However, contrary to our expectation, vitamin E at higher doses was found to be deleterious to bone in AMB animals. Our group previously observed that higher doses of vitamin E were beneficial in aged mice but had no beneficial effects on the bone of younger mice. Perhaps bone of older animals or of animals undergoing HLU benefits from more vitamin E particularly if these conditions result in greater oxidative damage. Investigations of the mechanisms by which vitamin E acts in mature bone are critical to our understanding of the role of vitamin E in bone health and because of potential for vitamin E to protect against oxygen derived free radicals.

***Items requiring further investigation.*** In these mature animals, dietary vitamin E did not produce significant effects on BMA, BMC, or BMD estimated by DXA. Effects of dietary changes are cumulative over time and are more subtle than the bone response to HLU. Therefore, BMA, BMC, and BMD might not be apparent during the relatively brief time course of this experiment. Studies with nutritional variables may require extended depletion or supplementation periods prior to HLU to enable differences to be seen during the relatively short HLU period.

Also, in these 11-month old animals, urinary DPD at 28 days was not significantly affected by treatment. Previously, we have shown significant differences due to HLU in 5-1/2-month-old rats. However, differences in urinary DPD between HLU and AMB animals were much greater at 14 days than at 28 days suggesting the need for analysis over time. Time series studies examining not only markers of bone resorption but also changes in gene expression due to HLU and/or nutritional or pharmacological interventions could provide insight into alterations in bone metabolism.

***Presentations supported by the grant***

Stoecker, B.J., Smith, B.J., Turner, R.T., Evans, G., and Arjmandi, B.H. Effects of skeletal unloading and vitamin E on bone in aged rats. Presentation at the Bioastronautics Investigators' Workshop in Galveston, Texas, January, 2001.

Smith, B.J., Stoecker, B.J. and Arjmandi, B.H. Effects of hindlimb unloading and vitamin E on bone in mature rats. Presentation at the National Institute of Aging Regional Workshop in Oklahoma City, OK, March, 2001.

Smith, B.J., Stoecker, B.J., Turner, R.T., Evans, G., Lucas, E. Martin, S., Riggs, T., and Arjmandi, B.H. Effects of skeletal unloading and vitamin E on bone in aged rats. Poster at Experimental Biology '2000 in Orlando, Florida. April, 2000.

***Published abstracts supported by this grant***

Smith, B.J., Stoecker, B.J., Turner, R.T., Evans, G., Lucas, E. Martin, S., Riggs, T., and Arjmandi, B.H. Effects of skeletal unloading and vitamin E on bone in aged rats. FASEB Journal 15:A979. 2001.

***All other published materials supported by this grant***

Riggs, T. L. Effects of vitamin E and hindlimb unloading on bone composition in aged rats. M.S. Thesis. Oklahoma State University, 83 pp. 2001.

Martin, S.D. Effects of vitamin E and hindlimb unloading on bone density and biomechanical properties of bone in mature rats. M.S. Thesis. Oklahoma State University, 56 pp. 2001.

Stoecker, B.J., Smith, B.J., Turner, R.T., Evans, G. and Arjmandi, B.H. Effects of skeletal unloading and vitamin E on bone in aged rats. Bioastronautics Investigators' Workshop, January 17-19, 2001. (http://www.dsls.usra.edu)

"Researchers study astronauts' bone loss," Stillwater News Press, Stillwater, OK. July 14, 2000.

"Good to the Bone", College of Human Environmental Sciences Magazine, 2000.

"Knees, Knees, Knees……", Oklahoma State University Magazine, 2001.

***Manuscripts and abstracts in preparation from this project***

Smith, B.J., Akhter, M.P., Brusewitz, G., Martin, S., Arjmandi, B.H. and Stoecker, B.J. Characterization of bone changes with hindlimb unloading in 11-month-old male rats. Manuscript in preparation for Journal of Applied Physiology.

Smith, B.J., Turner, R.T., Evans, G., Arjmandi, B.H. and Stoecker, B.J. The protective effects of vitamin E on bone during skeletal unloading. Manuscript in preparation for Journal of Applied Physiology.

Smith, B.J., Riggs, T., Lucas, E.A., Arjmandi, B.H. and Stoecker, B.J. Vitamin E and bone quality. Manuscript in preparation for Journal of Nutrition.

Smith, B.J., Brackett, D., Lerner, M., Arjmandi, B.H. and Stoecker, B.J. Alterations in gene expression in bone induced with vitamin E and skeletal unloading. Manuscript in preparation for Journal of Bone and Mineral Research.

***Undergraduate students, graduate students and postdoctoral students supported by the grant***

| | |
|---|---|
| Postdoctoral Student: | Brenda J. Smith |
| M.S. Students: | Tracy Riggs<br>Sloan Martin |
| Undergraduate Students: | Andrea Beaver<br>Rachel Bell<br>Jarrod King |

***Related publications and abstracts presented in 1999-2001:***

Smith, B.J., King, J.B., Lucas, E.A., Akhter, M.P., Arjmandi, B.H. and Stoecker, B.J. Detrimental effects of skeletal unloading and dietary copper depletion on bone quality in mature rats. (Journal of Nutrition – tentatively accepted, responses to reviewers submitted 9/26/01)

Smith, B.J., King, J.B., Lucas, E.A., Akhter, M.P., Arjmandi, B.H., and Stoecker, B.J. Alterations in vertebral bone quality related to skeletal unloading and copper depletion in mature rats. FASEB J. 14:A224. 2000.

Smith, B.J., Lerner, M.R., Brackett, D.J., Wronski, T.J. and Stoecker, B.J. Alterations in gene expression induced by chronic exposure to mechanical unloading in vivo. J. Bone Min. Res. 15:S207. 2000

Smith, B.J., King, J., Lucas, E., Brusewitz, G., Arjmandi, B.H., and Stoecker, B.J. The effect of dietary copper depletion on bone metabolism in mature tail-suspended rats. J. Bone Min. Res. 14:S391. 1999.

Smith, B.J., King, J., Lucas, E., Stoecker, B.J. Tail suspension and copper depletion affect bone in mature male rats. FASEB J. 13:A578. 1999.