

## THE EFFECT OF DIETARY SALT IN GENETICALLY-DEFINED UNLOADING MODEL

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### ABSTRACT

One of the most serious health hazards to long term space flight is the loss of bone tissue. Today's astronaut may be a veteran of numerous bouts of weightlessness from previous space flights, male or female, and now aging. Shannon Lucid spent more time in space than any other American and any other female. Recovery of lost bone following space flight is crucial to the future of human space flight and most importantly to women who are at higher bone risk. One of the major contributing factors to osteoporosis is theorized to be high dietary salt intake from processed foods. In America, with a large population consuming a typically high salt diet, this may be of particular significance. It may be that initial bone damage from excessive consumption of salt occurs initially in adolescence and to those who are salt sensitive. This has never been tested but is clearly critical information to formulating any effective osteoporosis treatment and prevention plan here in the U.S. Our study focused on the combination of three characteristics in a rat model: 1) young female 2) chronic (42 day) hindlimb underloading / overloading 3) salt sensitivity. The study worked with rats of two genetic groups mirroring salt sensitivity in the American population. The rat bones were examined to quantify bone metabolism in relation to these characteristics. This gave a statistically valid result for the bone damage due to excess salt consumption which is postulated to lead to osteoporosis later in life. Does a modest salt intake adversely affect bone in space or upon return to earth? This study of the effects of two modest levels of salt supplementation to diet on the overloaded and underloaded bones of genetically-defined female growing rats can begin to answer this question.

### INTRODUCTION

The purpose of this study was to determine changes in cortical and trabecular bone in response to a modest dietary salt supplementation and limb underloading / overloading (42 days) in 2 differing genetically-defined female rat models. The time course results point to possible links between salt sensitivity and bone mineral metabolism, irregular patterns of development, and premature bone fragility in an unloaded model. In a 1995 study, Navidi et al. investigated the effect of excess dietary salt in a rat spaceflight model. No further reduction in calcium content was found apart from that induced by unloading the hind limbs. However, work by others on young growing male genetically-defined spontaneously hypertensive rats (SHR) pointed to early adverse bone morphological changes induced by salt that would be missed by standard measurements of bone calcium content. It appears that salt damage to bone may be a structural phenomena, not strictly calcium content modulated. These early adverse structural changes can precede calcium content change leading to fracture. Bone mineral-salt association and salt sensitive hypertension in adult and elderly human populations are difficult to access accurately because genetics, blood pressure therapies and other environmental factors influence reductions in bone mineral and elevations in blood pressure. Our study examined changes in bone in response to a modest 1% and 2% dietary salt supplementation and limb underloading / overloading (42 days) in 2 different, genetically-defined, female rat strains. The effect of modest sodium intake on bone, previously demonstrated

in hypertensive male rats, was studied for the first time in female rats. While some research has been done on normotensive female rats in response to high salt loading (far beyond levels of human consumption), no investigation on female salt sensitive hypertensive rat osteo metabolism and / or its early response to modest salt loading have been done. These results may point to possible links between salt sensitivity and bone metabolism including an accelerated bone turnover rate, irregular patterns of development, and premature bone fragility in an unloaded rat model. In our study the underloaded leg can simulate space flight problems from a hindlimb weightlessness. The other overloaded hindlimb may also help us understand potential problems in astronaut recovery when returning again to earth's gravity from space. Does salt intake adversely affect bone in space or upon return to earth? The effects of this study using two modest levels of salt supplementation to diet on the overloaded and underloaded bones of genetically defined growing rats, will be valuable in identifying threshold parameters of observable adverse effects.

## METHODS

Forty four rats (22 Sprague Dawley wistars and 22 salt sensitive hypertensive rats, SSJrs) at 7 weeks of age, were housed in individual wire-bottomed cages by Laboratory Animal Services at constant room temperature with a standard light-dark cycle. Treated rats drank 1% saline or 2% saline for a 42 day salt supplementation period. Controls drank water. Animals were fed a diet of AIN 93M. All animals were subjected to a hindlimb immobilization bandage procedure for a 42 day period. Systolic blood pressure was measured in restrained unanesthetized rats by the tail-cuff method using an available electrospygmanometer with a physiological recorder in 6th week of study (Pfeffer et al., 1971). Euthanasia was carbon dioxide asphyxiation.

Femur, tibia, ulna and vertebral and humeral bones were dissected, freed of soft tissue, and prepared for analysis and storage. The wet weight (g) of the right and left femur was measured to the nearest mg on a analytical balance. The length of both femurs was measured from the tip of greater trochanter and ball of femur to the distal femur. Both femurs of each animal were removed from storage, and thawed at room temperature for 24 hours. A three-point bending test using standardization set forth by American National Standards Institute for three-point bending test of animal bone (ASAE Standards, 1995), was applied to each femur sample in an Instron Universal Testing Machine (Instron Corp., Canton, MA). Immediately after the breaking strength force measurement, bone was cut transversely, one mm from breaking point ( fracture location) toward proximal end for additional cross-sectional measurements used to calculate moment of inertia, ultimate bending stress and the apparent modulus of elasticity. A 5 mm high cylinder section was cut from distal end to be used for mineral content and density measurements. The wet weights of each 5 mm sections, obtained in air and under degassed water, were measured. Formulas for the calculation of bone volume and density were determined according to Archimedes principle (Donahue et al., 1988). The 5 mm sections of cortical bone were defatted. After drying, the fat free dry weight was recorded, followed by solubilizing with 6 N HCL (Metz et al., 1990). Tissue mineral was analyzed by inductively coupled plasma mass spectrometry (Thermo Jarrell Ash, Atom Scan 16, Franklin, MA). In order to examine the micro-structure damage, atomic force microscopy has been chosen as a tool to measure surface morphology at a sub-nanometer resolution. Because the surface of bone at this resolution is not well characterized, at least five scans at each site will be necessary , so that detected differences reflect that created by physiochemistry.

Three collected urine samples from each rat (stored at -80 °C) will be thawed and assayed for deoxypyridinoline crosslinks using Ppylinks-D ELISA kit when funding and time become available. This marker is used clinically, as well as in the rat model, and has potential in monitoring astronauts' bone loss and rehabilitative efforts upon returning to gravity. Recently, Lalande et al.

(1998) has reported in response to an 8% NaCl supplementation to food in male spontaneously hypertensive (SHR) rats, a reduction in trabecular number and increased trabecular bone separation, indicating bone resorption. Surprisingly, no significant difference in the pyridinoline/creatinine ratio was detected using the enzyme-linked immunosorbent assay after 8% NaCl was administered to male rats. Both deoxypyridinoline and pyridinoline are sensitive to bone resorption, but we do not know if this method will be sensitive enough to detect changes from a 1% and 2% saline treatment in female rats.

Both the right tibia, left tibia and vertebrae bone samples were stored separately in 100% ethanol at 4°C, followed by immediate embedding in glycol methacrylate. Protocols for subsequent sectioning (developed by Patsy Ruegg and Dr. William Huffer, Metabolic Bone Biopsy Service at the University of Colorado Health Sciences Center) of glycol methacrylate blocks and staining of sections were strictly followed (Huffer et al., 1994). Eight 5 micron sections from the proximal tibia were cut and placed on charged slides to air dry. Four 5 micron sections were placed in deionized water. Wet sections were used for Van Kossa and hematoxylin-eosin staining to identify insoluble calcium. Air dried sections were used for tartrate resistant acid phosphatase and sirrus red stains to identify osteoclasts and bone remodeling units. Lastly, three 5 micron sections were mounted to uncoated slides and air dried. These unstained sections were used for examining calcein uptake under UV light. Calcein was administered by intraperitoneal injection on separate days (day 3 and day 1 before rat autopsy). Determination of % bone volume, % osteoid surface, % osteoclast surface and % mineralizing surface will be assessed from 5 micron section images by available epifluorescent and brightfield microscopes. Image analysis and processing of bone will be measured by IPTK 2.1 Tool Kit (John C. Russ, 1999) with Photoshop 5.0 in accordance with the standardization of the American Society of Bone Mineral Research Histomorphometric Nomenclature Committee (Parfitt et al., 1987). Bone histomorphometry results of the 1% saline treated animals are ongoing pending final instruction in image analysis and processing workshop in the last week of May.

Data from the underloaded vs overloaded limbs will be analyzed by a Student's t-test for paired observations. Results between groups will be analyzed by analysis of variance. Post hoc Duncan's Multiple Range tests will be used to determine significant differences between groups. Null hypotheses will be rejected at  $P < 0.05$  level of significance. Correlations will be by linear or polynomial regression analysis using Macintosh Minitab software. Using a one way ANOVA, the effects of strain and modest 1% salt supplementation added to drinking water have been determined on recorded data as food intake, fluid intake, body weight and urine volume. Additionally, strength, density, volume, length, weight, mineral content, etc., differences of significance follow.

## RESULTS AND DISCUSSION

Effects of the 1% saline were not as marked as anticipated, as a 1% treatment may represent a threshold effect. In addition, an unexpected confounding factor was highly variable salt appetites between rats. No rat was restricted in the amount that it drank. Since the added NaCl was in the drinking water, each rat consumed a different amount of water: Thus, each dose of salt differed between individual rats. Because the dose-response was not as marked as hoped, we extended the dose-response relationship increasing the salt modestly in the drinking water from 1 to 2% in second half of study. Two percent saline in drinking water is still well within the range of salt consumption in processed foods in humans in America and Japan. Unlike previous studies (Furuse et al., 1992) which found significant differences in saline fluid intake between wistar and salt sensitive rats, no significant differences in saline fluid intake were found here in response to 1% saline supplementation. Previous studies of saline vs water intake were mostly

short term with male rats. Body weight in wistar rats was significantly higher than in salt sensitive rats ( $p < 0.03$ ). Body weight in saline treated rats was not significantly different than that in untreated rats. Food intake per day in wistar rats was not significantly different from salt sensitive rats. Food intake per day in the saline treated rats was significantly higher than in the untreated rats ( $p < 0.01$ ). Fluid intake per day per rat in wistar rats was not significantly different from salt sensitive rats. Fluid intake per day in the saline treated rats was significantly higher than in the untreated rats ( $p < 0.001$ ). Tail cuff measurements of blood pressure and heart rate were significantly higher in salt sensitive strain, however, no significant difference was found in response to the 1% saline supplementation. Significantly higher blood pressure readings were found in response to a 2% salt supplementation ( $p < 0.005$ ) in both wistar and salt sensitive strain. As in the 1% treatment, the salt sensitive strain had significantly higher blood pressure ( $p < 0.000$ ) when compared to normotensive wistar strain. Sodium intake is considered a risk factor for both hypertension and osteoporosis (Goulding and McParland, 1990; Law et al., 1992). Researchers estimate 30-60% of hypertensives and 25-40% normotensives are salt sensitive.

Mineral differences were found both in the underloaded and overloaded limb examinations. Femoral cortical weight in the underloaded limb was significantly heavier than the overloaded limb, however, bone calcium and phosphorous content in the overloaded limb was significantly higher than the underloaded limb ( $p < 0.011$  and  $p < 0.025$ , respectively). Calcium and phosphorus are important minerals for bone strength. Bone sodium, potassium and boron content in the underloaded limb was significantly higher than the overloaded limb. ( $p < 0.004$ ,  $p < 0.047$ , and  $p < 0.018$  respectively).

In our study, as in Furuse's study of male spontaneously hypertensive (SHR) rats, no differences in calcium content between those on NaCl and those on water were found. However, we tested for a variety of minerals associated with calcium balance in bone. In general, 1% saline decreased two important inorganics. Bone magnesium content in the overloaded femur of the saline treated rat was significantly reduced when compared to the overloaded femur of the untreated rat. ( $p < 0.001$ ). Magnesium deficiency has an adverse effect on bone (Kenny et al., 1994). Bone boron content in the overloaded femur of the saline treated rat was reduced when compared to the overloaded femur of the untreated rat ( $p < 0.09$ ). Additionally, the length of the underloaded femur of the saline treated rats was shorter than the underloaded femur of the untreated rat as measured from the distal end of femur to ball of femur ( $p < 0.08$ ). Since this may indicate an important alteration in shape, we will place the proximal end of the femur in glycol methacrylate, slice it longitudinally and apply Van Kossa stain to 5 micron sections to examine how calcium is distributed in this area. This may indicate increased mineral redistribution in saline treated rats with the potential to change size and shape in the final analysis.

Many genetics differences were recorded. Femoral weight in the overloaded and but not underloaded limb of the wistar rat was significantly heavier than the salt sensitive rat, when the effect of body weight factored in ( $p < 0.007$ ). The overloaded and underloaded femur bone of the wistar rat was significantly longer than the overloaded and underloaded femur of the salt sensitive rat. On the other hand, the lesser-weightbearing ulna bone of wistar rats was not found to be significantly different from the salt sensitive rat in weight or length. Additionally, no significant differences were found in mineral content in the lesser weightbearing ulna bone between the wistar and salt sensitive rats. This is a particularly important measurement as many human measurements to determine the condition of bone quality for prevention of osteoporosis, use this site for testing bone. It may prove to be an inappropriate site for preventive measurements, as weight-bearing bone sites are more at risk for change and or respond to adverse change more quickly. Higher calcium content has been observed in female spontaneously hypertensive femur bone tissue previously by Lau's et al. long term study. Higher concentrations of inorganics retained in bone tissues of spontaneously hypertensive rats over control rats have been associated with

various anomalies in hypertension (Gelinas and Schmit, 1994). Inorganic retention of minerals and metals have been found in male hypertensives and is believed to effect health adversely. Higher concentrations of inorganics were observed in this study in the underloaded bone of salt sensitive hypertensive rats when compared to normotensive rats. Bone calcium and phosphorus content in the underloaded limb of the salt sensitive rat was significantly higher than the underloaded limb of the wistar rat. ( $p < 0.001$ ,  $p < 0.013$  respectively). Trends for elevated levels were also found in magnesium, manganese and iron concentrations in the underloaded leg. On the other hand, in the exercised femur of salt sensitives, there was little difference in all inorganics measured (calcium, sodium, potassium, boron, manganese, iron, copper, zinc, and aluminum). The exception was magnesium in which salt sensitives had significantly lesser concentrations than wistars in weightbearing leg. This is important as a deficiency in magnesium affects bone adversely (Kenny et al., 1994).

### CONCLUSION

Today's experienced astronaut may be a veteran of numerous bouts of weightlessness from previous space flights, male or female, and 35-60 years of age. In response to the new astronaut, planners of future space missions, may need to individualized astronaut's diet in a prescriptive manner while training both on the ground and in orbit to insure skeletal adequacy for repeated missions. This study suggests that dietary countermeasures applied while in space, in ground training, and throughout an astronaut's work career to deter the adverse effect of weightlessness bouts on bone, are of great importance. In addition, this study may provide information on the selection of future candidates for long term flight careers further enhancing the assessment of bone prior to beginning one's career. Furuse et al.'s examination of young growing SHR male animals pointed to early adverse bone morphological changes induced by 1% salt supplementation that would be missed by standard measurements of bone calcium content. The authors suggest that salt damage to bone may be a structural phenomena, not strictly calcium content modulated. These early adverse structural changes precede calcium content change, leading to fracture. In our female animal model, the effects on bone from unloading/overloading and genetic strain differences were more apparent than 1% saline treatment effects on bone on parameters tested to date. Significant genetic differences between the normotensive wistars and salt sensitive hypertensive strains as well as unloading/overloading differences were found in length, weight, and mineral content. However, differences that were found to date in response to the 1% saline treatment, point to additional adverse bone effects that may have an additive adverse effect. This accumulation of adverse changes from weightlessness bouts, from salt sensitive individuals and/or those whose salt intake is not minimal, could escalate fracture risk and should be taken into consideration in orbit, in training and post flight.

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