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Simulated weightlessness and synbiotic diet effects on rat bone mechanical strength

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ABSTRACT

This paper reports results on exposure to simulated weightlessness that leads to a rapid decrease in bone mineral density known as spaceflight osteopenia by evaluating the effectiveness of dietary supplementation with synbiotics to counteract the effects of skeletal unloading. Forty adult male rats were studied under four different conditions in a 2×2 factorial design with main effects of diet (synbiotic and control) and weight condition (unloaded and control). Hindlimb unloading was performed at all times for 14 days followed by 14 days of recovery (reambulation). The synbiotic diet contained probiotic strains *Lactobacillus acidophilus* and *Lactococcus lactis lactis* and prebiotic fructooligosaccharide. This paper also reports on the development of a desktop three-point bending device to measure the mechanical strength of bones from rats subjected to simulated weightlessness. The importance of quantifying bone resistance to breakage is critical when examining the effectiveness of interventions against osteopenia resulting from skeletal unloading, such as astronauts experience, disuse or disease. Mechanical strength indices provide information beyond measures of bone density and microarchitecture that enhance the overall assessment of a treatment's potency. In this study we used a newly constructed three-point bending device to measure the mechanical strength of femur and tibia bones from hindlimb-unloaded rats fed an experimental synbiotic diet enriched with probiotics and fermentable fiber. Two calculated outputs for each sample were Young's modulus of elasticity and fracture stress. Bone major elements (calcium, magnesium, and phosphorous) were quantified using ICP-MS analysis. Hindlimb unloading was associated with a significant loss of strength in the femur, and with significant reductions in major bone elements. The synbiotic diet did not protect against these unloading effects. Tibia strength and major elements were not reduced by hindlimb unloading, as was the case for femur, but tibia bone strength was negatively affected by the synbiotic diet. Thus, unexpectedly, the synbiotic diet was associated with null or detrimental effects on bone strength.

1. Introduction

One of the most important health problems to consider on spaceflights is the negative effect of micro-gravity on skeletal bones. Prolonged exposure to low-gravity environments has the tendency to increase spaceflight osteopenia in otherwise fit astronauts, and is not prevented by exercise alone. The resulting changes in turnover due to reduced bone loading are the main cause of osteopenia. In order to combat these health hazards, different diets can be tested to examine whether bone strength can be promoted when gravitational loading is not present. Dietary interventions have included mineral supplementation (e.g., calcium and magnesium), but few studies have evaluated the effectiveness of probiotics and prebiotics in strengthening bone.

To contribute to eventual resolution of the osteopenia problem for permanent human presence in space, it is vital that any intervention effect, beneficial or otherwise, be properly identified using the right equipment and statistical methods. This paper has two goals. First, it describes the construction and use of a three-point bending tester machine to determine the mechanical strength of rat hindlimb bones. Second, the paper seeks to determine if a synbiotic diet can help counter the loss of bone strength due to skeletal unloading. Three-point bending is a common beam loading

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method, with two fixed supports under a beam and a point force in the middle. A free body diagram and the shear and moment diagrams for this type of loading demonstrate that the internal moment reaches a peak value at mid-span.

Since some strains of bacteria are known to be beneficial to the human GI tract, the tester was used to determine if a synbiotic diet (containing probiotics and fermentable fiber) can be an effective treatment to prevent loss of major bone elements (calcium, magnesium, and phosphorous) and prevent increased bone fragility. Both conditions can happen under low-gravity conditions as described by Abram et al. (1988), Garber et al. (2000), Martin (1990), and Sun et al. (2009). Most studies recommend protein-, calcium-, and phosphorus-based diets as reported by Sun et al. (2014) in a recent major review article on rodent microgravity experiments. In another review, Zwart and Smith (2005) focus on human bone condition in gravity and report similar recommendations, but Zhu (2013) reports that rats subjected to simulated weightlessness and fed with Chinese herbal compound had statistically better bone strength compared to the control group. A recent study by Blanton and Gabaldón (2012) showed a moderately protective effect of a synbiotic diet on rat bone microstructural properties in adult rats that were hindlimb suspended for 14 d followed by 14 d of reambulation. However, it was not determined whether the synbiotic diet also improved bone strength. The present study extends on previous findings and tests the hypothesis that bone mechanical strength is also moderately improved by a short-term synbiotic diet (Blanton and Gabaldón, 2012), consisting of fructooligosaccharides (prebiotics) and two probiotic cultures, *Lactobacillus acidophilus* and *Lactococcus lactis lactis*.

The literature provided numerous citations on the potential benefits of a synbiotic diet on bone strength. The proposed mechanisms by which a synbiotic diet may enhance bone strength include: 1) prebiotic-induced improvement in the intestinal absorption of dietary calcium and magnesium (Younes et al., 2001; Coudray et al., 2003), and 2) probiotic-induced correction of microgravity-associated dysbiosis (Lizko, 1991; Gibson and Roberfroid, 1995) and the attendant systemic inflammation that provokes bone resorption (Sonnenfeld, 2002, 2003; Sonnenfeld et al., 2003; Wei et al., 2003; Roller et al., 2004).

2. Methods

Animals and study design. A detailed description of the study design, animals and care, hindlimb unloading protocol, and diet ingredients are reported by Blanton and Gabaldón (2012). In brief, forty 6-mo-old Sprague-Dawley male retired breeder rats (Simonsen Laboratories, Santa Clara, CA) were randomly assigned into four groups in a 2 × 2 factorial design with main effects of diet [synbiotic (SYN) or control (CON)] and loading condition [hindlimb-unloaded (HLU) or normally loaded (L)]. The two normally loaded groups ($n = 10$ each) remained weight-bearing for the 28 day experiment, and are referred to as LC (loaded, fed control diet) and LS (loaded, fed synbiotic diet). The two groups of hindlimb-unloaded rats ($n = 10$ each) were maintained on either the control diet (ULC) or synbiotic diet (ULS). Both groups were tail-suspended with a harness for 14 days to simulate weightlessness of the hind limbs, following the protocol developed by NASA, as described by Morey-Holton and Globus (2002). The rats were then returned to a normal weight-bearing condition for 14 days of reambulation. The purpose of reambulation was to simulate recovery from microgravity (skeletal unloading). The protocols were based on previous studies showing that bone formation rates are significantly decreased by 14 days of unloading (Hefferan et al., 2003; Patterson-Buckendahl et al., 1989) and improve after 14 days of return to weight-bearing (Lafage-Proust et al., 1998; Wronski and Morey, 1983). The animals were housed at Idaho State University, Pocatello, and cared for by Dr. Blanton. The experimental protocol was approved by Idaho State University Animal Welfare Committee. The animals were inspected for health on a daily basis and the study was conducted under veterinarian supervision.

Diets. The CON and SYN diets were based on a powdered form of the American Institute of Nutrition (AIN)-93M purified laboratory rat diet (Dyets, Inc., Bethlehem, PA). The complete diet ingredients and modification for the SYN diet are described in Blanton and Gabaldón (2012, Table 1). In brief, the two diets were isocaloric, containing an equal number of calories per gram of food eaten. The SYN diet was formulated by adding prebiotics (fructooligosaccharide) and lyophilized probiotic cultures (1×10^{11} CFU/g of equal parts *Lactobacillus acidophilus* and *Lactococcus lactis lactis*), which were supplied by Nutraceutix, Redmond, WA. The unloaded rats were fed *ad libitum*, and their average daily food intakes were measured. The loaded rats were pair-fed to match the daily food intakes of unloaded rats. The results for food intake and body weight are reported in Blanton and Gabaldón (2012, Table II).

Bone dissection. On experimental day 29, rats were euthanized using carbon dioxide. Bones from the hindlimbs (femora and tibiae) were carefully removed and prepared accordingly for the various tests. Bones from the right side of the body were used for μCT scanning (Blanton and Gabaldón, 2012), followed by ashing for elemental analysis as described below. Bones from the left side were wrapped in saline-soaked gauze and stored frozen at −70 °C in cryogenic storage vials until mechanical strength testing as described below.

Bone ICP-MS analysis. The femora and tibiae were ashed in a Jelenko Accu-therm II 1000 muffle furnace for 24 h at 800 °C (Martin, 1990). Ashed bones were then pulverized using a mortar and pestle, and dissolved in concentrated, trace metal grade nitric acid. Digestion of ash was completed in a Milestone Ethos EZ microwave oven. Aliquots of the digested samples were analyzed by ICP-MS analysis using an Agilent 7500ce. The major bone elements of interest were calcium, phosphorous, and magnesium. The ICP-MS analysis method used was EPA 6020a; the internal standards were ^{6}Li, ^{45}Sc, ^{72}Ge, ^{115}In, ^{159}Tb, and ^{209}Bi. All samples were analyzed by the Chemistry Department at Colorado State University–Pueblo.

Statistical analysis. The analysis of bone strength data was performed by means of two-way ANOVA with main effects of diet and loading condition as described above. Logarithmic and square root transformations of response variables were performed where appropriate to satisfy the assumptions of the ANOVA model. The results of the analysis were also confirmed by means of nonparametric tests. In addition, two bone strength measures, Young's modulus and fracture stress, were related to each other by means of regression models for both femur and tibia. Such models allow one bone strength measure in the presence of the other and help reduce the number of measurements taken overall.

2.1. Construction of three point bending tester

Testing equipment for three-point bending was designed and constructed by students and faculty in the CSU–Pueblo Engineering department. Initial plans for bone testing included the use of a fulcrum and moment-arm device. However, during the process of reviewing the documents pertaining to other similar experiments, it was discovered that there was a specific set of guidelines governing all such experiments, ASAE Standard: ASAE S459 (ASAE PPAPC, 1992). These procedural guidelines must be followed for an experiment to be considered valid. Per these standards, the three-point bending test must be performed using equipment that can supply a constant feed rate of up to 10 mm per minute that is