Dietary Strontium Increases Bone Mineral Density in Intact Zebrafish (*Danio rerio*): A Potential Model System for Bone Research

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Abstract

Zebrafish (*Danio rerio*) skeletal bone possesses properties similar to human bone, which suggests that they may be used as a model to study mineralization characteristics of the human Haversian system, as well as human bone diseases. One prerequisite for the use of zebrafish as an alternative osteoporotic bone model is to determine whether their bone displays functional plasticity similar to that observed in other bone models. Strontium citrate was supplemented into a laboratory-prepared diet (45% crude protein) to produce dietary strontium levels of 0%, 0.63%, 1.26%, 1.89%, and 2.43% and fed *ad libitum* twice daily for 12 weeks to 28-day-old intact zebrafish. Length was determined at 4-week intervals, and both weight and length were recorded at 12 weeks. At 12 weeks, seven zebrafish from each dietary level were analyzed for total bone mineral density by microcomputed tomography. Dietary strontium citrate supplementation significantly ($p < 0.05$) increased zebrafish whole-body and spinal column bone mineral density. In addition, trace amounts of strontium were incorporated into the scale matrix in those zebrafish that consumed strontium-supplemented diets. These findings suggest that zebrafish bone displays plasticity similar to that reported for other bone models (i.e., rat, mouse, and monkey) that received supplements of strontium compounds and zebrafish should be viewed as an increasingly valuable bone model.

Introduction

ALTERNATIVE ANIMAL MODELS are increasingly being utilized to obtain data because of the difficulty of using mammalian trials arising from ethical, practical, and cost concerns. Zebrafish (*Danio rerio*) are considered to be a valuable model organism because they display genetic and experimental amenability, and possess many human developmental and disease gene counterparts. The production of numerous zebrafish mutants has allowed for the study of human diseases such as Alzheimer's disease,¹ congenital heart disease,² Huntington's disease,³ polycystic kidney disease,⁴ and cancer.⁵

Research has suggested that zebrafish may be a good bone model because the endochondral ossification behavior of this species is similar to that of human bone.⁶ Zebrafish have the lamellar structure, the primary features of human osteons, and a hierarchical organization consistent with that described for human long bone.⁶ Ge *et al.*⁶ demonstrated that the characteristics of biomineralization and microstructures of the

zebrafish skeletal bone are similar to those of the human Haversian bone. These structural similarities suggest that the skeletal bone of zebrafish may serve as an effective, simple model to study mineralization characteristics of the human Haversian system as well as human bone diseases.⁶

Recent bone studies utilizing zebrafish have focused on alterations of mineral properties of bone by gene mutation.⁷ No studies have been conducted to determine whether the bone in normal intact wild-type zebrafish can be altered as achieved in other research (i.e., mouse, rat, and monkey) bone models. The determination of whether functional plasticity occurs in zebrafish bone is an important prerequisite for their use as an alternative osteoporotic model.

Strontium is a naturally occurring element and is physically and chemically similar to calcium. These properties make strontium a natural bone-seeking trace element.⁸ Strontium has been shown to increase bone mineral density (BMD) in normal rats,^{9–11} mice,¹² and monkeys.¹³ Strontium administration has also been used to increase BMD in osteoporotic rats^{14,15} and humans.^{16,17} Previous studies involving intact

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monkeys¹⁸ and rats¹⁹ have shown that the amount of strontium incorporated into the skeleton increases in a dose-dependent manner and is directly proportional to intake levels.⁸ These properties make strontium an ideal test element to determine its effect on BMD in zebrafish.

Our objective was to assess the response of zebrafish bones (whole body and spinal) to dietary supplementation of strontium citrate. In addition, we report the effect of strontium on the mineral composition of zebrafish scales, which are involved in the biomineralization process, in this simple model system.

Materials and Methods

Experimental system

The experimental system consisted of 15 rectangular tanks (35 L volume) connected to a semi-closed 582 L indoor recirculating system (MARS Marineland Retailer Systems; Spectrum Brands, Atlanta, GA). Initial system and replacement water was obtained by filtering tap water through a 1 μ m string filter and activated charcoal filter, followed by reverse osmosis (RO) (Kent Marine Maxxima, Franklin, WI) and then passing the product water through a mixed resin bed deionizer (DI) (Kent Marine Maxxima). The conductivity of the RO/DI water was adjusted to 475 μ S by adding a calcium-free mineral solution containing 348 g/L NaCl, 125 g/L MgCl₂, and 10 g/L KCl to the RO/DI water to produce water with a calcium level (1 mg/L) previously shown to support good zebrafish growth and bone development.²⁰ Daily evaporative loss was replaced with RO/DI water to maintain the conductivity of the system at 475 μ S. Mineral composition of the water was verified by flame atomic absorption spectroscopy (Eurofins Scientific, Inc., Des Moines, IA). A concurrent experiment was also conducted using water with a calcium level of 30 mg/L (data not presented in this article).

Each tank contained 1 cylindrical plastic mesh cage (0.25 m height $\times$ 0.09 m diameter) covered with nylon mesh (Fashion Knee Hi; Americal Corporation, Henderson, NC). Freshwater was pumped from the sump through an ultraviolet sterilizer (Aquafine, Valencia, CA) at a rate of 5.4 L min⁻¹ tank⁻¹ (11,108% daily turnover tank⁻¹ day⁻¹). Woven floss was used for mechanical filtration, while biological filtration was achieved using 82 L of 0.04-m-diameter biofilter balls media. A light:dark photoperiod of 12:12 h was provided by indirect fluorescent lighting. Water temperature was maintained at 28°C ($\pm$ 0.5°C) by a 500-watt heater (Finnex HC-0800 heater and controller) and monitored daily using a thermometer (Fisher Scientific, Pittsburgh, PA). Ammonia-nitrogen, NO₂-N, and pH were measured weekly using an aquarium pharmaceuticals test kit (Aquarium Pharmaceuticals, Inc., Chalfon, PA). Water pH ranged between 8.0 and 8.3, and ammonia-nitrogen and NO₂-N remained below levels that can be detected visually by test-color variation.

Preparation of laboratory diets

The laboratory-prepared diet was adopted from Kovalenko *et al.*²¹ and produced good results in previous studies involving zebrafish.²² The ingredient compositions of the diets are presented in Table 1. Strontium hydrogen citrate was supplemented at levels of 0.0%, 2.0%, 4.0%, 6.0%, and 7.7%, and these amounts were compensated by corresponding de-

creases in the acid-washed diatomaceous earth to produce dietary strontium levels of 0.0%, 0.63%, 1.26%, 1.89%, and 2.43%. Casein, canthaxanthin, fish protein hydrolysate, acid-washed diatomaceous earth, soy lecithin, and wheat gluten were added to a beaker containing distilled water (200 mL/100 g diet) and mixed using a stir bar. Menhaden oil, cholesterol, ascorbylpalmitate, a vitamin premix, betaine, choline chloride, a mineral premix, monopotassium phosphate, and glucosamine were then added and mixed. Egg yolk and strontium hydrogen citrate were then mixed with the other ingredients and homogenized (using a Hamilton Beach 2-speed hand blender [Hamilton Beach Brands, Inc., Washington, NC] for 3 min at high speed). The required amount of alginate was then added, followed by additional homogenization for 2 min at high speed. The resulting thick paste was then spread on plastic-wrapped trays, and fan-dried for 48 h.

TABLE 1. INGREDIENT COMPOSITION AND LEVELS (% DRY WEIGHT) OF THE LABORATORY-PREPARED DIETS USED IN THE STUDY

Ingredient ^a	Inclusion level (g/100 g)				
	0.0	2.0	4.0	6.0	7.7
Ascorbyl-palmitate ^b	0.04	0.04	0.04	0.04	0.04
Betaine ^b	0.15	0.15	0.15	0.15	0.15
Canthaxanthin (10%) ^c	2.31	2.31	2.31	2.31	2.31
Casein ^d	14.70	14.70	14.70	14.70	14.70
Cholesterol ^b	0.12	0.12	0.12	0.12	0.12
Choline ^b	0.38	0.38	0.38	0.38	0.38
Diatomaceous earth ^b	7.70	5.70	3.70	1.70	0.00
Egg yolk ^b	38.45	38.45	38.45	38.45	38.45
Fish protein hydrolysate ^e	15.40	15.40	15.40	15.40	15.40
Glucosamine ^b	0.15	0.15	0.15	0.15	0.15
Menhaden oil ^b	5.63	5.63	5.63	5.63	5.63
Mineral premix ^{d,f}	1.54	1.54	1.54	1.54	1.54
Potassium phosphate ^b	1.15	1.15	1.15	1.15	1.15
Refined soy lecithin ^g	1.90	1.90	1.90	1.90	1.90
Sodium alginate ^h	5.38	5.38	5.38	5.38	5.38
Strontium citrate ⁱ	0.00	2.00	4.00	6.00	7.70
Vitamin premix ^{d,j}	1.15	1.15	1.15	1.15	1.15
Wheat gluten ^b	3.85	3.85	3.85	3.85	3.85

^aLot numbers of ingredients utilized in experimental diets: ascorbylpalmitate (16814DE), betaine (126K0710), canthaxanthin (UT99075290), casein (4822J), cholesterol (017K0152), choline (14003DH), egg yolk (065K0726), glucosamine (057K5314), menhaden oil (116K1837), mineral premix (7850), potassium phosphate (106K0209), refined soy lecithin (108856), vitamin premix (7958), and wheat gluten (037K0095).

^bSigma-Aldrich (St. Louis, MO).

^cDSM Nutritional Products, Inc. (Belvidere, NJ).

^dMP Biomedicals, LLC (Solon, OH).

^eThe Scoular Company (Minneapolis, MN).

^fComposition of the mineral premix (%): calcium carbonate, 2.100; calcium phosphate dibasic, 73.500; citric acid, 0.227; cupric citrate, 0.046; ferric citrate, 0.558; magnesium oxide, 2.500; magnesium citrate, 0.835; potassium iodide, 0.001; potassium phosphate dibasic, 8.100; potassium sulfate, 6.800; sodium chloride, 3.060; sodium phosphate, 2.140; zinc citrate, 0.133.

^gUSB Corporation (Cleveland, OH).

^hTIC Gums (White Marsh, MD).

ⁱDr. Paul Lohmann, Inc. (Huntington Station, NY).

^jComposition of the vitamin premix (%): ascorbic acid, 12.5; butylated hydroxyanisole, 0.1; biotin, 0.1; cellulose, 60.0; calcium pantothenate, 1.5; cobalamin, 0.1; folic acid, 0.5; inositol, 18.0; nicotinic acid, 2.6; para-aminobenzoic acid, 3.0; pyridoxine hydrochloride, 0.3; riboflavin, 0.8; thiamine mononitrate, 0.5.

TABLE 2. MEAN PERCENT SURVIVAL, LENGTH, AND WEIGHT ($\pm$ STANDARD ERROR OF THE MEAN [$N=30$]) OF *DANIO RERIO* FED GRADED LEVELS OF STRONTIUM (AS STRONTIUM CITRATE) *AD LIBITUM* FOR 12 WEEKS

Dietary strontium (%)	Survival (%)	Length (mm)	Weight (mg)
0.0	93.3	25.1 (2.2) ^a	198.1 (56.9) ^a
0.63	86.7	25.3 (2.2) ^a	194.7 (60.4) ^a
1.26	90.0	25.4 (2.4) ^a	194.8 (57.7) ^a
1.89	90.0	24.8 (3.1) ^a	193.5 (72.7) ^a
2.43	90.0	25.5 (3.5) ^a	206.3 (92.3) ^a

Means with different letter designations within columns are significantly different ($p < 0.05$).

to produce dry sheets that were added to a coffee bean grinder (Black and Decker; Appliance Consumer Products, Miramar, FL) and ground to achieve a particle size that passed through a 1 mm² mesh. Diets were then stored at 4°C until used.

Fish

Wild-type zebrafish (*D. rerio*) obtained from a local pet store were spawned as described by Westerfield²³ and reared on rotifers (*Brachionus plicatilis* ["L" type]) fed an enriched diet of *Nannochloropsis* and *Tetraselmis* (Reed Mariculture, Inc., Campbell, CA.) until 28 days postfertilization. To minimize handling stress, these intact juvenile fish were individually photographed in reduced-volume glass containers with reference grids, and lengths (tip of lower jaw to hypural [posterior] notch) were determined by image analysis (Image-J; National Institutes of Health, Bethesda, MD). Fish were then randomly assigned (using Microsoft Excel[®] random number generator) to individual mesh cages (10 fish per mesh cage, 3 mesh cages [replicates] per diet). One of the five laboratory-prepared diets was fed *ad libitum*, twice daily exclusively to fish in each cage. All diets were fed to visible excess ~5 min after feeding and their physical characteristics did not affect feeding. All experimental fish were photographed and measured for length at the end of 4-week intervals and at the 12-week termination of the experiment. At the conclusion of the experiment, total weights (mg) of fish in each replicate were determined and mean individual weight was calculated.

FIG. 1. Mean length of *Danio rerio* fed *ad libitum* levels of an experimental diet supplemented with graded levels of strontium over 12 weeks.

Seven randomly selected fish from each treatment group were euthanized in cold MS-222 and fixed with 10% neutral buffered formalin for subsequent microcomputed tomography (μ CT) analysis.

μ CT analysis

Whole zebrafish were scanned using a Scanco μ CT 40 desktop cone-beam micro-CT scanner (Scanco Medical, Basersdorf, Switzerland) set at 70 kVp, an intensity of 114 μ A, and an integration time of 200 ms. Zebrafish were placed on their side in a 36-mm-diameter sample holder and scanned at a resolution of 18 μ m. Scans were automatically reconstructed into two-dimensional slices and the region of interest was outlined in each slice using the μ CT Evaluation Program (v5.0A; Scanco Medical). Scan threshold of the zebrafish bone was the same (107) for all scans. A three-dimensional (3D) reconstruction was then performed to obtain 3D images of the bones. To assure accurate density measurements, two levels of quality control were run on the Scanco μ CT40. A weekly quality control was performed using a phantom containing five cylinders of differing densities. The monthly quality

TABLE 3. MEAN TOTAL BONE MINERAL DENSITY, BONE VOLUME, AND CALCULATED BONE PARAMETERS ($\pm$ STANDARD ERROR OF THE MEAN [$N=7$]) OF *DANIO RERIO* FED GRADED LEVELS OF STRONTIUM (AS STRONTIUM CITRATE) *AD LIBITUM* FOR 12 WEEKS

Dietary strontium (%)	Bone density (mg HA/ccm)	Bone volume (mm ³)	Bone density/length (mm)	Bone density/weight (g)	Bone density/bone volume
0.0	471.83 (15.20) ^a	7.86 (1.47) ^a	17.76 (2.00) ^a	2.24 (0.71) ^a	61.49 (9.02) ^a
0.63	475.08 (18.93) ^{a,b}	8.09 (1.91) ^a	18.56 (1.63) ^a	2.60 (0.87) ^a	61.41 (13.90) ^a
1.26	503.90 (22.96) ^{b,c}	10.97 (1.87) ^a	19.97 (3.32) ^a	3.14 (1.55) ^a	47.04 (8.13) ^a
1.89	504.57 (20.51) ^{b,c}	10.33 (2.75) ^a	21.70 (3.66) ^a	3.53 (1.68) ^a	53.02 (18.89) ^a
2.43	516.55 (16.78) ^c	11.29 (3.36) ^a	19.94 (2.65) ^a	2.72 (0.97) ^a	48.84 (12.70) ^a

Means with different letter designations within columns are significantly different ($p < 0.05$).

TABLE 4. MEAN TOTAL BONE MINERAL DENSITY OF SPINAL COLUMNS ($\pm$ STANDARD ERROR OF THE MEAN [$N=5$]) OF *Danio rerio* FED GRADED LEVELS OF STRONTIUM (AS STRONTIUM CITRATE) *AD LIBITUM* FOR 12 WEEKS

Dietary strontium (%)	Bone density (mg HA/ccm)	Bone volume (mm ³)	Bone density/bone volume
0.0	394.60 (31.53) ^a	0.218 (0.02) ^a	1881.25 (203.82) ^a
2.43	460.32 (23.80) ^b	0.253 (0.03) ^a	1916.01 (207.21) ^a

Means with different letter designations within columns are significantly different from those within the same environmental condition ($p < 0.05$).

control was performed on a different part of the same phantom containing three fine aluminum wires to check the alignment (geometry) of the scanner.

Energy-dispersive X-ray analysis

Energy-dispersive X-ray analysis was performed on representative scales obtained from the lateral line of zebrafish randomly selected from those fed a diet containing either 0% or 2.43% strontium ($n=3$ per dietary level). Analysis was performed using a Philips 515 scanning electron microscope, set to a 30 kv accelerating voltage, fitted with a Kevex Quantum EDS Detector with PGT Avalan Acquisition System with PGT Spirit Version 1.07.05. Spectra were collected for a live time duration of ~ 80 – 90 s. The counts per second ranged from 1200 to 2500 and the dead time for the detector ranged from 20% to 30%.

Statistical analysis

Length, weight, and bone mineral parameters were compared using analysis of variance (v12.0; SPSS, Chicago, IL) to determine if significant differences existed among the diets. Where significance was indicated, Tukey's HSD inequality was used to determine which treatment pairs were significantly different ($p < 0.05$). Bone mineral parameters of the spinal column were statistically compared using the inde-

pendent-samples *T*-test. Tests for normality and constant variance were performed before all statistical analysis.

Results

Zebrafish within all treatment populations actively consumed feed and grew rapidly, throughout the duration of the experiment, suggesting that they were healthy. Survival was between 86.7% and 93.3% for those zebrafish fed the 0.63% and 0.0% (control) strontium containing diets, respectively (Table 2). Neither mean length nor mean weight of zebrafish was significantly different among the different dietary strontium citrate treatments at week 12 (Table 2). Mean length of zebrafish fed diets supplemented with different strontium levels followed a linear growth pattern over time (Fig. 1). As dietary strontium levels increased, mean BMD of zebrafish increased; BMD values of zebrafish fed the 2.43% strontium diet were significantly higher than those of zebrafish fed diets containing 0% or 0.63% strontium (Table 3). Bone volume and other calculated bone parameters were not significantly different among the different dietary treatments. Spinal column BMD in zebrafish that consumed the 2.43% strontium diet was significantly greater than that of those that consumed the control diet (Table 4). Strontium was incorporated into the scale matrix of those zebrafish that consumed strontium-containing diets (Figs. 2 and 3). No noticeable differences in bone structure were determined in those fish that consumed the control and the strontium-containing diets (Figs. 4–6).

Discussion

Dietary strontium increased BMD in a dose-dependent manner without producing noticeable defects in bone structure, similar to that reported in intact rats,^{9–11} mice,¹² and monkeys.¹³ Intact rats fed strontium ranelate, a distrontium salt, up to 900 mg/kg body weight/day displayed dose-dependent increases of bone mass of the vertebral body and of the midshaft femur.¹⁹ Administration of strontium chloride (0.5 mmol strontium injected once per week for 6 weeks) increased bone formation in normal rats,¹¹ whereas the feeding of strontium ranelate (1.6 mmol strontium/kg body weight/day for 8 weeks) increased trabecular number and reduced

FIG. 2. Energy-dispersive X-ray spectrum of a representative lateral line scale from *D. rerio* fed 0% strontium (control) *ad libitum* at the end of a 12-week growth trial. Letters represent periodic elements.

FIG. 3. Energy-dispersive X-ray spectrum of a representative scale from *D. rerio* fed 2.43% strontium *ad libitum* at the end of a 12-week growth trial. Letters represent periodic elements.

trabecular separation in intact rat tibia, indicating increased bone formation.²⁴ The feeding of strontium ranelate at levels of up to 2.91 mmol strontium/kg body weight/day to intact adult monkeys over a 6-month period resulted in a dose-dependent decrease in bone resorption and a corresponding increase in the magnitude of mineralization surfaces, without negative effects on bone mineralization.¹³ These data indicate that strontium is readily incorporated into vertebrate bone and further suggest similar mechanisms of bone formation among vertebrates.

The lack of a difference in BMD between the zebrafish in this study reared under 1 mg/L water calcium and those reared at 30 mg/L water calcium (unpublished data) suggests that water-soluble calcium, which is readily absorbed across zebrafish gills,²⁵ does not noticeably interfere with the absorption of dietary strontium. This lack of interference is important because fish absorb calcium from their aquatic environment, a major difference in comparison to other terrestrial bone models, and an environmental source of calcium could have hypothetically interfered with strontium uptake as observed with dietary calcium. High levels of dietary calcium decrease the active intestinal absorption of strontium^{26,27} because the common carrier system for calcium and strontium in the intestinal wall has a greater affinity for calcium.^{28,29} In the human gut, absorption of strontium is only 25%–30% when provided exclusively³⁰ and is absorbed to a lesser extent when administered with calcium.^{31,32} These data further suggest the utility of zebrafish as a bone model, despite their simultaneous absorption of environmental and dietary calcium.

The amount of strontium added to the diets spanned a range above and below levels that have been reported to promote increased BMD in normal rats,^{9–11} mice,¹² and monkeys,¹³ when adjusted for size and estimated consumption. Although consumption, and therefore dietary strontium intake, was not determined in this study, lack of significant differences in weight among the dietary treatments suggests that rates of consumption of diets were similar. While accurate determination of consumption may pose a limitation in pharmacological kinetic analysis, the mode of action by which strontium (or other bone enhancing compounds) increases BMD can still be determined. This potential value is evident because the precise cellular and molecular mechanisms of strontium action on bone cells have yet to be determined.³³

In this study, whole zebrafish were scanned by μ CT, thereby permitting the determination of BMD of total body as well as specific bone (i.e., a section of the spinal column) from a single scan. Researchers conducting studies that involve mice and rats typically scan a specific section of bone that they believe will be affected by the treatment. While this approach may be adequate for some studies, the potential to miss detrimental (or positive) effects in other bones is present, especially when compounds that have unknown physiological actions are used. Whole-body μ CT scans also permitted a 3D reconstruction of the zebrafish skeletal system, and these 3D images were visually scanned for defects or differences among treatments. This capability represents a significant advantage in utilizing zebrafish because it is not always feasible when using larger animals.

FIG. 4. Microcomputed tomography (μ CT) of a representative zebrafish (*D. rerio*) fed the 0% (control) strontium diet at the end of a 12-week growth trial.

FIG. 5. μ CT of a representative zebrafish (*D. rerio*) fed the 1.26% strontium diet at the end of a 12-week growth trial.

FIG. 6. μ CT of a representative zebrafish (*D. rerio*) fed the 2.43% strontium diet at the end of a 12-week growth trial.

In addition to bone, biomineralization in zebrafish also occurs in the scales, which have a chemical composition similar to that of other skeletal tissues, but a calcium metabolism that differs from bone.²⁵ The scales are covered by scleroblasts, cells hypothesized to be utilized in mineralization and matrix production, which allows them to be a site of labile calcium storage.²⁵ The incorporation of trace amounts of strontium into the zebrafish scales suggests that strontium, like calcium, can be transported to and stored in the scales. Although this scale system is not found in humans, alternative systems have previously been utilized to obtain a greater understanding of a biochemical system.³⁴ Therefore, zebrafish scales may be another means to investigate the mechanism by which strontium or other chemical effectors influence mineral metabolism.

Although an osteoporotic zebrafish bone model does not currently exist, results of previously conducted research suggest that strontium produces similar bone effects on normal and osteoporotic organisms. Strontium ranelate (0.30–1.20 mmol strontium/kg body weight/day) fed to ovariectomized rats prevented trabecular bone loss induced by estrogen deficiency during the course of the 8-week study.¹⁴ Strontium carbonate (0.52 mmol strontium/kg body weight/day) administered to ovariectomized rats 3 months after ovariectomy reduced the increase in bone turnover experienced by estrogen deficiency and restored the bone mineral content lost after ovariectomy.¹⁵ Considering the role of calcium and strontium in normal zebrafish growth, further evaluation of zebrafish as a simple model of osteoporosis is warranted.

The results of this study demonstrate a functional plasticity of zebrafish bone, similar to that observed in other bone models when fed graded levels of dietary strontium. The previously stated advantages of this model, combined with their reduced genome size, ease of genetic manipulation, short generation time, easy breeding, large brood size, and reduced maintenance cost, suggest that zebrafish can be used as a simple and effective model to study the formation and repair of bone. Refinement of feed production technologies described in this study will promote evaluation of the mode of action of those compounds or drugs involved in bone metabolism.

Acknowledgments

This project was supported in part by the Center for Metabolic Bone Disease, NIH Grant P30AR046031, UAB Core Center for Basic Skeletal Research (CCBSR), NIH/NIAMS Grant 5P30 AR046031, Comprehensive Training Grant in Bone Biology and Disease, NIH/NIAMS Grant 2T32 AR047512, and the UAB Small Animal Phenotyping Core (P30AR046031 and P30DK56336, and DK079626).

Disclosure Statement

No competing financial interests exist.

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