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Moderate zinc deficiency negatively affects biomechanical properties of rat tibiae independently of body composition☆

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Abstract

To guide development of novel nutritional strategies aimed at reducing the incidence of stress fractures, we observed the effects of manipulating dietary zinc (Zn) content on bone integrity in Sprague–Dawley rats fed either a severely Zn-deficient (ZnD; 1 ppm), a moderately Zn-deficient (MZnD; 5 ppm) or a Zn-adequate (ZnAD; 30 ppm) diet for 6 weeks. At the completion of the diet period, body composition, bone mineral content (BMC), bone area (BA) and bone mineral density (BMD) were determined in vivo by using dual-energy X-ray absorptiometry. Following euthanasia, long bones were collected for determination of Zn content and biomechanical strength testing. Despite significant positive correlations between dietary Zn and both body weight (BW) and bone Zn content for the entire cohort ($r=.77$ and $r=.83$, respectively), rats fed MZnD or ZnAD diets did not differ in feed intakes, body composition, BMC, BA, BMD or BW. Tibial bones, but not femur bones, appear to be more responsive to dietary Zn manipulation, as all bone biomechanical strength indices in the ZnAD-fed rats were significantly greater than in rats fed the ZnD diets. Rats fed either MZnD or ZnAD diets had stronger tibiae (129% increase in maximum load and stress at maximum load, $P<.01$) compared with those fed ZnD diets. The load at breakage for the tibial bones of rats fed MZnD diets was not different from the ZnD rats, but lower ($P<.05$) than that of the ZnAD rats. These results suggest that since feed intakes, body composition, BMC, BA, BMD and BW were not significantly different between the MZnD- and ZnAD-fed animals, the reduced bone integrity observed in the MZnD-fed rats resulted from dietary Zn inadequacy, and not as a result of the reduced growth that is typically associated with Zn deficiency.

Keywords: Zinc; DXA; Bone mineral density; Rat

1. Introduction

Zinc (Zn) is a dietary essential trace mineral known to be necessary for normal collagen synthesis and mineralization of bone [1,2]. Zn has been demonstrated to be essential for normal growth of both human and animal skeletal systems [3–12]. In men, dietary Zn intake and plasma Zn both have a positive association with bone mineral density (BMD) [6], and dietary Zn has been shown to improve BMD in humans [6,7,13–15] and rats [9,12,16]. Zn stimulates bone metabolism in rats and bone protein synthesis and bone formation in tissue cultures by increasing the activity of critical enzymes, such as alkaline phosphatase [17,18]. Zn has been shown to augment the anabolic effect of insulin-like growth factor I on osteoblasts, which are responsible for the formation and mineralization of the extracellular matrix of bone during endochondral ossification [19]. In addition, Zn was shown to exert an inhibitory effect on the activity of the osteoclasts responsible for bone resorption in vitro [20,21].

Zn nutriture reflects the balance between Zn intake and Zn excretion. Low Zn intake has been associated with low bone mass in women [13,22]. Possible sources of Zn loss include sweat [23], which increases with exercise, and adjustments in renal excretion, such as occur with extremely low or high intakes of Zn [24]. Thus, mineral-poor diets

Abbreviations: BA, bone area; BMC, bone mineral content; BMD, bone mineral density; BW, body weight; DXA, dual-energy X-ray absorptiometry; FM, fat mass; LBM, lean body mass; Zn, zinc.

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and/or the increased sweat or renal Zn losses might also lead to mild Zn deficiencies, thus increasing the risk of reduced bone mass. Not surprisingly, numerous studies have demonstrated that Zn deficiency leads to the development of osteopenia and osteoporosis [8,15,25,26] and an increase in osteoporotic fractures [5]. Furthermore, reduced serum or plasma Zn concentrations and increased urinary Zn excretion have also been reported in women with osteoporosis [15,25,27]. In animals, Zn deficiency has also been associated with abnormalities in bone growth, bone formation and mineralization [28].

Because Zn affects growth, bone turnover and mineralization [8,17,29], the purpose of the present study was to characterize the role of dietary Zn levels on bone quality, particularly aspects relating to biomechanical function of the bone. Much of the research examining the role of Zn status in bone health has focused on frank Zn deficiency—the literature is replete with short-term feeding studies in rats examining bone histomorphometry following Zn-deficient (typically 0–2 ppm) or Zn-adequate (20–50 ppm) diets [8–11,30]. However, the effects of dietary Zn on bone strength in growing rats have not previously been investigated in a dose–response study design, using both a Zn-deficient (1 ppm) and a moderately Zn-deficient diet (5 ppm), for an extended duration (6 weeks). Very few studies have documented the effects of moderate Zn deficiency, which may not be diagnosed clinically because of the lack of reliable Zn status indicators and overt symptoms of deficiency. Moderate Zn deficiency may occur even in developed nations, as classical studies have demonstrated increased growth of Zn-supplemented children from middle to upper socioeconomic classes in the United States and Canada [31,32]. The present study assessed the relationship between dietary Zn status and bone biomechanical parameters in the rat and correlated the changes in dietary Zn status with dual-energy X-ray absorptiometry (DXA) measurements of body composition and bone mineral content. We hypothesized that rats consuming a moderately Zn-deficient diet for an extended period would experience growth retardation, decreased lean body mass and impaired bone quality. In addition, we conducted direct biomechanical testing to determine bone mechanical properties. Our findings indicate that optimizing Zn nutriture may enhance bone integrity and provide justification for a nutritional intervention to reduce stress fractures.

2. Materials and methods

2.1. Animals and diets

Sprague–Dawley rats (male; $n=30$; 125–149 g) from Charles River Laboratories (Wilmington, MA, USA) were housed individually in polycarbonate cages in a constant-temperature ($22\pm2^{\circ}C$) animal room with a 12-h light/dark cycle. The rats were fed a standard diet (LM-485, Harlan Teklad, Indianapolis, IN, USA) for an acclimation period of 14 days and were then divided into three groups ($n=10$ per group) of equal average body weight. The rats were then fed test diets containing a fixed protein level (12% from sprayed dried egg whites) and incremental amounts of Zn carbonate (1, 5 and 30 ppm). Diets were prepared according to the LM-485 formulation (80 g cellulose per kg of diet) [33] by Research Diets (New Brunswick, NJ, USA). All rats were allowed free access to the assigned test diets and deionized water for 45 days, during which body weight and feed consumption were measured at regular intervals (PM 30, Mettler Instruments, Hightstown, NJ, USA). Spilled feed was collected carefully and weighed, and feed intakes were corrected accordingly. At the end of the diet period (Day 46), rats were sacrificed under carbon dioxide. Both legs were dissected away from the hip joint, carefully cleaned of adherent tissue, wrapped with gauze soaked in saline and stored at 4°C until biomechanical testing was initiated. This study was approved by the Institutional Animal Care and Use Committee (IACUC) at USARIEM, and animals were maintained in accordance with IACUC guidelines for the care and use of laboratory animals.

2.2. Zinc analysis

Zn status was assessed in foreleg bones by using flame atomic absorption spectroscopy (AAS; Perkin Elmer 2380, Norwalk, CT, USA). Both foreleg bones and feed (~1.0 g of each diet) samples were diluted eightfold with 5% nitric acid (trace metal grade, Fisher Scientific, Pittsburgh, PA, USA). Zn standards, prepared from a reference solution (Fisher Scientific) in 5% nitric acid, were used as an internal control. All analysis was conducted in acid-washed glassware. Recovery tests were performed to confirm the accuracy of the above-mentioned method, and the recovery of Zn was $108\pm1.1\%$ ($n=5$, CV=2.3%).

2.3. Dual energy X-ray absorptiometry

Immediately prior to and following the 6-week feeding period, body composition, BMC, BA and BMD were assessed in vivo using DXA analysis. Briefly, rats received an anesthetic by intraperitoneal injection of a 1-ml/kg mixture of 40 mg/ml ketamine (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, USA), 10 mg/ml xylazine (Xyla-Ject; Phoenix Scientific, St. Joseph, MO, USA) and 1.5 mg/ml acepromazine (Boehringer Ingelheim Vetmedica, St. Joseph, MO, USA). Once sedation was confirmed, rats were transferred to a Prodigy fan beam densitometer (GE Lunar, Madison, WI, USA) with a small-animal, high-resolution scan module. Small-animal software (enCore Version 7.53.002, 2003, GE Lunar) was utilized for the determination of all body composition measures. All animals were scanned on the same day by the same operator.

2.4. Bone biomechanical testing

Femur and tibia bones were kept at 4°C until determination of breaking strength using a 5-kN Flexure Fixture,

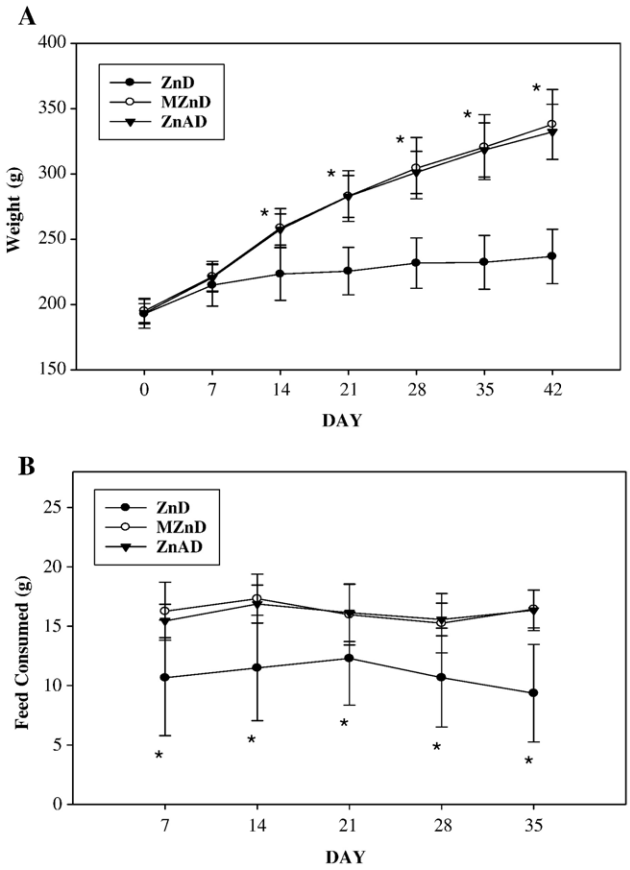

Fig. 1. Effect of dietary Zn on body weight gain (A) and 24-h feed intake (B) in rats fed a Zn-deficient (ZnD, 1 ppm), a moderately Zn-deficient (MZnD, 5 ppm), or a Zn-adequate (ZnAD, 30 ppm) diet for a period of 6 weeks. Asterisks (*) indicate significant differences ($P<.05$) between the ZnD and other dietary treatment groups. Values are means±S.D. ($n=10$ rats per group).

configured for three-point bend tests and attached to an Instron Universal Testing Machine Model 4502 equipped with a 10-kN load cell (Instron, Canton, MA, USA), as previously described [34]. The crosshead speed was 50 mm/min, and the data sampling rate was 10 samples/s. Maximum load, stress at maximum load, load at failure and stress at failure were determined in femurs and tibias using Series IX, v 8.08.00 software (Instron).

Table 1
Zinc content of the diets and foreleg bone

	Diets groups		
	ZnD ($n=10$)	MZnD ($n=10$)	ZnAD ($n=10$)
Zinc (mg/kg feed) stated	1.0	5.0	30.0
Zinc (mg/kg feed) measured	0.3±1.0	5.0±0.9	27.9±0.5
Zinc in forelegs (μg/g tissue)	84.2±14.8[a]	95.3±10.5[a]	156.7±22.3

Values are mean±S.D. Abbreviations for the diet groups are the same as those in Fig. 1.
[a] $P<.05$ vs. ZnAD.

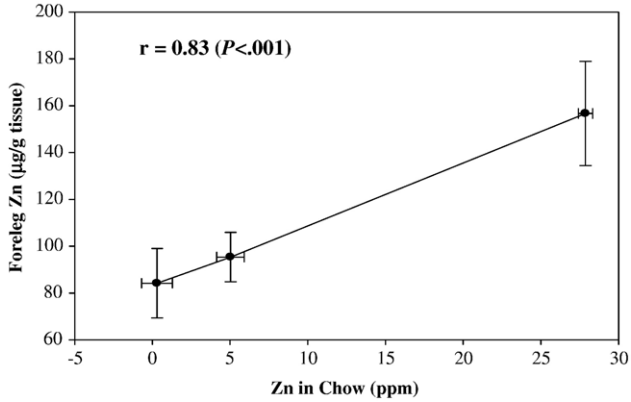

Fig. 2. Effects of varying dietary Zn on foreleg zinc levels (micrograms per gram tissue) in rats fed ZnD, MZnD or ZnAD diets for a period of 6 weeks. Values are means±S.D. ($n=10$ forelegs per group; $n=3$ feed samples per group).

2.5. Statistical analysis

Power analyses were used to confirm our ability to discern significant differences between and within groups. Numeric data were statistically analyzed using ANOVA and Tukey's post hoc tests. Pairwise comparisons were made using Student's unpaired t test. Results are presented as means±S.D. For body weight data, differences were assessed using ANOVA with repeated measures. We set our level of significance at $\alpha=.05$.

3. Results

3.1. Body weight and feed intake

Growth rates (Fig. 1A) and 24-h feed intakes (Fig. 1B) were not different between the MZnD and ZnAD groups during 6 weeks of the feeding trial. Within 12 days of consuming the ZnD diet, growth rates of the ZnD rats were significantly affected as compared with MZnD and ZnAD rats. At all times, rats fed ZnD diets consumed significantly less feed than rats fed either the MZnD or the ZnAD diets ($P<.05$), and their feed efficiency [total weight gain (g)/total feed consumed (g)] was significantly less than rats fed either the MZnD or the ZnAD diets ($P<.05$, data not shown). AAS analysis of digested rat chow confirmed the Zn concentrations of the diets, as stated by the manufacturer (Table 1). The mean daily Zn intakes from the three experimental diets were highly correlated with foreleg Zn levels ($r=.83$, $P<.05$, Fig. 2).

3.2. Body composition

Initially, no differences existed between any group for BW, LBM, FM, BMC, BA or BMD (Table 2). Following the 6-week feeding trail, there were no differences in these body composition indices between the MZnD and ZnAD diet groups. However, rats consuming the ZnD diets exhibited significantly lower values for BW, LBM, FM, BMC, BA and BMD when compared with rats from the MZnD and ZnAD diet groups ($P<.05$).

Table 2
Effect of varying Zn diets on body composition as assessed in vivo using DXA analysis

	Groups			ANOVA
	ZnD ($n=10$)	MZnD ($n=10$)	ZnAD ($n=10$)	
BW (g)				
Week 0	192.9±11.0	195.1±9.8	193.5±7.3	.857
Week 6	236.8±20.8[a]	337.9±26.7	332.2±21.1	<.001
LBM (g)				
Week 0	155.3±10.4	156.9±8.6	155.4±7.2	.904
Week 6	175.2±17.2[a]	245.2±21.6	238.5±17.4	<.001
FM (g)				
Week 0	29.4±7.3	31.9±4.7	31.7±8.4	.650
Week 6	41.3±7.1[a]	71.8±8.2	71.8±11.8	<.001
Whole-body BMC (g)				
Week 0	3.83±0.33	3.84±0.16	3.75±0.20	.686
Week 6	6.28±0.59[a]	8.10±0.57	7.99±0.54	<.001
Whole-body BA (cm^2)				
Week 0	34.33±2.57	34.70±1.75	34.20±1.95	.846
Week 6	44.17±2.62[a]	53.50±3.34	52.80±3.43	<.001
Whole-body BMD (g/cm^2)				
Week 0	0.111±0.003	0.110±0.003	0.110±0.001	.475
Week 6	0.142±0.006[a]	0.151±0.004	0.151±0.003	<.001

Values are mean±S.D. Abbreviations for the diet groups are the same as those in Fig. 1.
[a] $P<.001$ vs. MZnD and ZnAD.

3.3. Bone mass, length and strength

Rat tibial bones demonstrate significant Zn-sensitive changes in bone biomechanical indices. Bones from rats consuming the ZnD diet exhibit significantly ($P<.05$) lower maximum load, stress at maximum load, load at breakage, stress at breakage and energy to breakage than those from rats fed the ZnAD diet (Fig. 3). While the maximum load, stress at maximum load and stress at breakage of tibial bones from the MZnD rats were not different from those seen among ZnAD bones, tibiae of MZnD-fed rats had significantly lower ($P<.05$) load at breakage than those from Zn-adequate animals. The load at breakage for the tibiae of MZnD and ZnD rats were not significantly different. Dietary Zn levels did not significantly affect the energy to break of the tibiae, although the trend toward a higher energy to break was observed between the MZnD and ZnD dietary groups ($P=.06$). Rat femur bones appear to be less sensitive to dietary Zn levels, as femurs from rats fed the ZnD diets show no mechanical deficit when compared to femurs from rats fed the MZnD or ZnAD diets (Table 3).

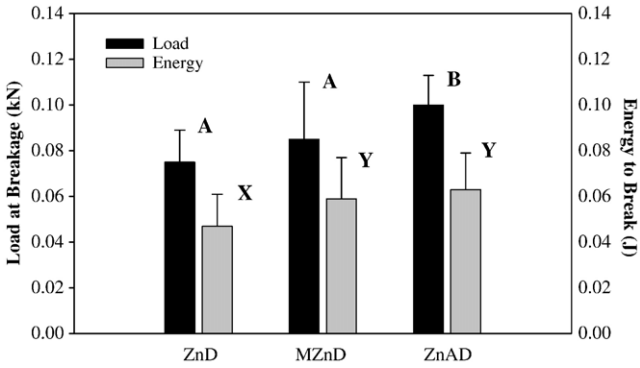

Fig. 3. Effects of varying dietary Zn on load at breakage (left axis) and energy to break (right axis) in tibiae. Biomechanical testing of tibial bones were determined with an Instron machine, and the load at breakage and energy to break measures were determined by using Series IX, v 8.08.00 software (Instron). Values with different superscripts differ significantly ($P<.05$). Values are means±S.D. ($n=10$ tibiae per group).

4. Discussion

The aim of this study was to evaluate the effects of varying levels of dietary Zn deficiency on growth, body composition and bone parameters in rats. Many previous studies of Zn deficiencies in rats indicate that severely Zn-deficient diets cause a decrease in BW and long bone growth [8,9,11] that has been attributed to diminished caloric intake. Our results indicate that rats consuming a moderately Zn-deficient diet for an extended period do not

Table 3
Effect of varying Zn diets on bone biomechanical data determined postsacrifice on long bones

	Groups							
	Tibia/Fibula				Femur			
	ZnD ($n=10$)	MZnD ($n=10$)	ZnAD ($n=10$)	ANOVA	ZnD ($n=10$)	MZnD ($n=10$)	ZnAD ($n=10$)	ANOVA
Maximum load (kN)	0.080±0.012[a]	0.103±0.008	0.106±0.012	<.001	0.119±0.022	0.122±0.015	0.126±0.014	.744
Displacement at maximum load (mm)	0.559±0.090	0.631±0.094	0.632±0.071	.097	0.904±0.189	0.923±0.200	0.897±0.207	.963
Stress at maximum load (MPa)	0.708±0.104[a]	0.909±0.068	0.935±0.103	<.001	1.054±0.193	1.080±0.133	1.113±0.125	.734
Load at auto break (kN)	0.075±0.014[b]	0.085±0.025	0.100±0.013	.016	0.114±0.035	0.118±0.020	0.108±0.032	.785
Displacement at auto break (mm)	0.673±0.346	0.739±0.309	0.626±0.132	.671	1.093±0.562	0.930±0.224	0.954±0.302	.656
Stress at auto break (MPa)	0.666±0.127[b]	0.746±0.219	0.889±0.104	.010	1.004±0.307	1.046±0.173	0.953±0.285	.784

Values are mean±S.D. Abbreviations for the diet groups are the same as those in Fig. 1.
[a] $P<.001$ vs. MZnD and ZnAD.
[b] $P<.01$ vs. ZnAD, but not different from MZnD.

experience growth retardation or anorexia. In fact, the visible effects of Zn deficiency, such as hair loss, hyperkeratosis of the paws and dermatological changes [10,35], did not appear in the MZnD rats, despite the reduced Zn status, as indicated by the foreleg Zn concentrations. However, our data suggest that moderate Zn deficiency impairs bone quality, and that the effect of dietary Zn on bone biomechanical measures appears to be more specific for tibial bones.

As expected in rats fed MZnD diets, Zn concentrations in the foreleg (Fig. 2) were significantly lower than in rats fed ZnAD diets. As noted by Eberle et al. [8], there is a possibility that, because the rats had free access to the diets, the observed skeletal effects might be due not to Zn depletion per se, but rather to retarded growth and reduced feed intake, with subsequently lowered energy and protein intake in the Zn-deficient animals. However, Fernandez-Madrid et al. [36] demonstrated by comparing Zn-deficient rats with pair-fed and ad libitum-fed controls that the impairment in protein and collagen synthesis observed in Zn-depleted rats was in fact due to Zn deficiency and not caloric intake differences. In this study, the feed intakes in the MZnD rats were not different from the ZnAD rats (Fig. 1B); thus, we conclude that the bone biomechanical effects were specific to Zn inadequacy and not the reduction of calories consumed.

Bone quality can be defined by qualitative and quantitative factors contributing to bone fragility. Qualitative factors include fatigue damage accumulation, architectural deterioration, increased bone turnover and osteocyte deficiency [37,38], and our findings cannot address these factors. However, our study does indicate that dietary Zn change mediated effects on quantitative factors that include material properties (e.g., BMC, BMD) and structural characteristics (e.g., size, shape, cortical thickness and trabecular architecture) [39]. Low BMD is associated with increased fracture risk [40]. In agreement with bone densitometry studies suggesting that increasing dietary Zn improves BMD in rats [9,12,16,41], nonhuman primates [42,43] and humans [6,7,13–15], we found that whole-body BMD increased significantly in rats fed the MZnD and ZnAD diets over the study period. BMD increased less in rats fed ZnD diets compared with rats fed MZnD and ZnAD diets. The relative increase in BA was 29% for the ZnD group and 54% for both the MZnD and ZnAD groups, whereas the relative increase in BMC was 64% for the ZnD group and 112% for both the MZnD and ZnAD groups (Table 2). BMD was not impaired in MZnD rats as it was with ZnD rats, which may be related to growth patterns experienced by these animals. Compared with ZnAD rats, the MZnD rats showed no differences in growth rates throughout the study, despite consuming six times less than the recommended intake of Zn for growing rats. Following 2 weeks on the 1-ppm diet, ZnD rats exhibited compromised growth rates, with no signs of recovery or "catch-up growth" occurring. There is likely a metabolic adaptation to the low Zn intake or decreased Zn requirements with age that may help support bone development that was compromised in ZnD rats, but not in MZnD rats.

Coupling the findings of the densitometric measurements and the morphometric measurements, we conclude that bone mineral deposition was unable to maintain bone growth in the ZnD group, but femur bones adapted through an increase in size, possibly through periosteal apposition that maintains the cross-sectional area of the bone and, thus, its strength [44] in the femurs, despite less mineral being deposited. Zn-deficient diets have been demonstrated to reduce femur BMD in rats [9,12,45]. In rats fed ZnD diets, total BMD was significantly reduced. The additional 4 ppm Zn added to the MZnD diets significantly affected these parameters (BMC, BA and BMD), as there was no measurable difference between the MZnD and the ZnAD diet groups. Despite the lack of difference between the MZnD and the ZnAD groups for BMD, BA, BMC, maximum load and stress at maximum load, tibial bones from rats fed ZnAD diets had higher load at failure, and thereby could be more resistant to stress fractures.

Extrapolated to humans, it can be seen that even moderate Zn deficiency may impair certain biomechanical properties of long bones, and that improving Zn nutriture might be beneficial. Early data from the ongoing ZENITH study indicates there is some evidence of a relationship between Zn nutritive status and bone turnover in the older adult participants aged 55–87 years [46]. In postmenopausal women (aged 50–76 years), Nielsen and Milne [47] showed that low dietary Zn (3 mg/day) increased serum calcitonin, which is considered "unfavorable," as it is associated with increased hip bone loss in humans [48] and with suppressed bone formation and stimulated bone resorption in rats [49]. In the present study, the stress at auto break reflects the rigidity of bone as a whole, whereas the slope of the linear region of the stress vs. strain curve (elastic modulus or Young's modulus) reflects the intrinsic stiffness of rigidity and material properties of bone. High modulus may indicate bone to be more rigid, whereas low modulus could mean the bone is more ductile [50]. Compared to the ZnAD group, tibial bones from the MZnD group had the highest mean value for displacement at auto break. However, the stress at maximum load and stress at auto break were maximal in the tibial bones taken from rats consuming ZnAD diets (Table 3).

Finally, although other studies have shown that Zn-deficiency has detrimental effects on many bone biomechanical indices [41–43], the present study demonstrates that bone health is affected by moderate Zn deficiency independent of feed intake or body weight. The exact involvement of Zn and its clinical significance in bone health needs to be further elucidated in animal and human models, and conclusions about the effects of a single nutrient on bone mass must be given cautiously, taking into account its interaction with others. Clearly, the rat model shows that Zn is essential to bone health and that

when Zn is suboptimal for extended periods, there are significant effects on bone biomechanical properties that increase the risk of stress fractures. Although rodent models can contribute valuable information in this area, it is clear that more appropriate animal models and human studies are of paramount importance. The need to address the value of supplementation of Zn on bone health is evident and long overdue for greater attention.

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