

Iron deficiency and high-intensity running interval training do not impact femoral or tibial bone in young female rats

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Abstract

In the USA, as many as 20% of recruits sustain stress fractures during basic training. In addition, approximately one-third of female recruits develop Fe deficiency upon completion of training. Fe is a cofactor in bone collagen formation and vitamin D activation, thus we hypothesised Fe deficiency may be contributing to altered bone microarchitecture and mechanics during 12-weeks of increased mechanical loading. Three-week old female Sprague Dawley rats were assigned to one of four groups: Fe-adequate sedentary, Fe-deficient sedentary, Fe-adequate exercise and Fe-deficient exercise. Exercise consisted of high-intensity treadmill running (54 min 3X/week). After 12-weeks, serum bone turnover markers, femoral geometry and microarchitecture, mechanical properties and fracture toughness and tibiae mineral composition and morphometry were measured. Fe deficiency increased the bone resorption markers C-terminal telopeptide type I collagen and tartate-resistant acid phosphatase 5b (TRAcP 5b). In exercised rats, Fe deficiency further increased bone TRAcP 5b, while in Fe-adequate rats, exercise increased the bone formation marker procollagen type I N-terminal propeptide. In the femur, exercise increased cortical thickness and maximum load. In the tibia, Fe deficiency increased the rate of bone formation, mineral apposition and Zn content. These data show that the femur and tibia structure and mechanical properties are not negatively impacted by Fe deficiency despite a decrease in tibiae Fe content and increase in serum bone resorption markers during 12-weeks of high-intensity running in young growing female rats.

Key words: Nutrition: Iron: Exercise: Rodent: Microarchitecture: Mechanical testing: Dynamic histomorphometry

In the USA Military, lower extremity stress fractures remain a burden to health care and financial resources⁽¹⁾. Recruits completing basic military training (BMT) have up to an 18-fold higher risk for developing stress fractures compared with active duty personnel⁽²⁾. Risk factors involved in the aetiology of stress fractures include sex, race and age (non-modifiable) and nutrition status, fitness level and body mass (modifiable)⁽²⁾. Examining how modifiable risk factors impact bone health and contribute to stress fractures is important for developing prevention strategies during BMT.

Fe is required for bone growth during maturation and maintenance in adulthood⁽³⁾. It serves as a cofactor for prolyl- and lysyl-hydroxylases, two enzymes critical for collagen formation. Collagen synthesis begins with the formation of procollagen, a three-dimensional structure composed of glycine and proline. Hydroxylation occurs when ferrous Fe is oxidised in the presence of molecular oxygen, α -ketoglutarate and ascorbic acid⁽⁴⁾.

This new structure undergoes glycosylation and forms collagen, the main constituent of bone. Fe is also a structural component of 25-hydroxyvitamin D₃ 1- α -hydroxylase, the enzyme that catalyses the conversion of calcidiol to the bioactive calcitriol⁽⁵⁾. Calcitriol maintains Ca levels mainly by enhancing intestinal absorption and also by kidney reabsorption and bone resorption⁽⁶⁾.

Fe status falls along a continuum from optimum to Fe deficiency anaemia. Fe deficiency represents the stage between optimum and Fe deficiency anaemia. Fe deficiency is characterised by the depletion of stored Fe and increase in transport proteins, while Fe deficiency anaemia is considered a decrease in the concentrations of Hg and haematocrit⁽⁷⁾. The prevalence of Fe deficiency in women increases by ~20% during BMT^(8,9). While the cause remains unknown⁽¹⁰⁾, it may be due to lower Fe consumption during training, decreasing 22%⁽¹¹⁾. Due to the role of Fe in collagen formation and vitamin D metabolism,

Abbreviations: BMT, basic military training; IAE, iron-adequate exercise; IAS, iron-adequate sedentary; IDS, iron-deficient sedentary; IDE, iron-deficient exercise.

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Fe deficiency may be contributing to changes in microarchitecture and mechanics, leading to stress fractures during periods of increased loading. This has not been studied. Fe deficiency may be contributing to the increased number of stress fractures during BMT; however, this has not been studied.

The objective of this investigation was to measure the impact of Fe deficiency on cortical and trabecular bone of the femur and tibia in sedentary and exercised female rats. To accomplish this, 3-week-old female Sprague Dawley rats were fed Fe-deficient or control diets and completed 12 weeks of involuntary treadmill running, or remained sedentary. Female rats become sexually mature ~ 6 weeks of age and reach peak bone mass within 3 months⁽¹²⁾. This age span includes adolescence and early adulthood, similar to female recruits, i.e. the mean age is 20–22 years old^(11,13). Running exercise was chosen because it comprises a significant portion of physical activity during BMT. Our hypothesis was that Fe deficiency would impair bone development and strength.

Methods

Animals

This study was approved by the Institutional Animal Care and Use Committee at the Uniformed Services University (USU) under protocol MEM-17–020. Animals were maintained in accordance with the Guide for Animal Care and Use of Laboratory Animals as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources.

Forty-two 3-week-old (51–75 g) female Sprague Dawley rats (strain code 400) were purchased from Charles River (Wilmington, MA). Rats were pair-housed in a temperature and humidity controlled room within the USU Department of Laboratory and Animal Resources facility on a reverse 12-h light and 12-h dark schedule and allowed free access to food and water. After 72 hours of acclimation to the facility, animals began treadmill habituation and remained on standard chow. Body mass, feed and water consumption were measured weekly throughout the study. At the end of the experiment, rats were euthanised by carbon dioxide exposure. Blood was collected via cardiac puncture, and plasma or serum was isolated by centrifugation (3800 g for 15 min at 4°C) and stored at –80°C until analysis. Right and left femurs and tibiae were dissected and prepared/stored as described below.

Treadmill habituation

All animals were habituated to running on a three lane rodent motorised treadmill (Columbus Instruments) and VO₂ peak testing procedures on a single lane modular enclosed metabolic treadmill (Oxymax, Columbus Instruments). Animals performed a total of six habituation sessions on non-consecutive days over the course of 2 weeks. During habituation, animals ran at speeds of 10–25 m/min for a total of 10 min at a 10° incline. A mild electrical shock (0.34 mA) was used for negative reinforcement during all treadmill sessions, and one chocolate chip was provided to each animal after all treadmill sessions as a means of positive reinforcement. After habituation, animals were divided into exercise or sedentary groups.

Diet

After treadmill habituation, half of the exercise and sedentary animals were randomly assigned to either a Fe-deficient or Fe-adequate control diet. The experimental diets were based on a modified AIN-93G and contained either no Fe (Research Diets Inc., New Brunswick, NJ, Product ID D03072501) or 0.212 g ferric citrate /kg (Product ID D03072502). In total, there were four experimental groups: Fe-adequate sedentary (IAS, *n* 10), Fe-adequate exercise (IAE, *n* 11), Fe-deficient sedentary (IDS, *n* 10) or Fe-deficient exercise (IDE, *n* 10).

VO₂ peak testing and exercise training

The Oxymax system was calibrated to manufacturer specifications before each VO₂ peak test. For the VO₂ peak test, animals completed a 10 min warm-up with the velocity increased from 15 to 25 m/min at an incline of 10°. After warm up the velocity was increased by 2.0 m/min every 2 min until VO₂ leveled-off or animals refused to run, defined as shock lasting longer than 5 s. A VO₂ peak test was performed every 2 weeks to adjust running speed. Animals achieving a velocity within 2 m/min during VO₂ peak testing were grouped together for training. Each session consisted of an 18 min warm-up at 50 % VO₂ peak, six alternating intervals consisting of 4 min at 85–90 % VO₂ peak and 2 min at 50 % VO₂ peak. A 10° incline was maintained throughout all training sessions. Animals completed three training sessions per week on non-consecutive days for 12 weeks. BMT includes both physical training as well as military training exercises.

Iron status and bone biomarkers

Fe status was determined in whole blood using an automated complete blood count (VRL Laboratories). ELISA kits were used to measure serum tartrate-resistant acid phosphatase 5b (TRAcP 5b, SB-TR102), c-terminal telopeptides type I collagen (CTx, AC-06F1), osteocalcin (OC, AC-12F1), procollagen type I N-terminal propeptide (PINP, AC-33F1) (Immunodiagnostic Systems) and parathyroid hormone (PTH, 50–155–358) (Fisher Scientific). The coefficient of variation was < 10 % for all assays.

Bone mineral analysis

Tibiae were harvested, cleaned of tissue, wrapped in phosphate-buffered saline-soaked gauze, placed in 5 ml conical scintillation vials and stored at –80°C prior to shipping to the University of Delaware (Newark, DE). Tibiae were thawed to room temperature, dried at 60°C degree for 24 h, weighed and digested in trace metal grade nitric acid. Mineral composition (Fe, Ca, Zn and P) was measured using inductively coupled plasma optical emission spectrometry⁽¹⁴⁾.

Dynamic histomorphometry

Calcein injections (25 mg/kg) were administered subcutaneously 9 and 2 d prior to euthanasia to permit measurement of bone remodelling rates. Tibiae were harvested, cleaned of tissue and placed in 10 % formalin for 48 h before changing to 70 % ethanol. Bones were kept at 4°C and shipped to the Indiana University School of Medicine (Indianapolis, IN). Tibiae were



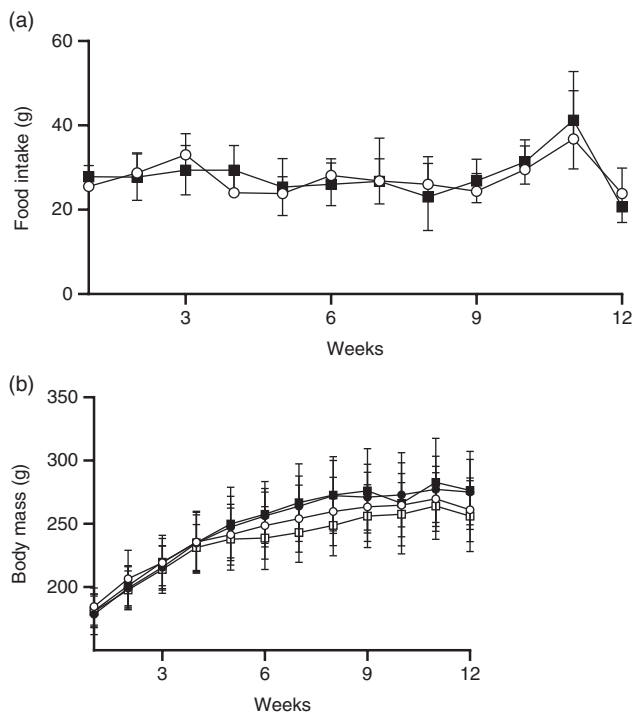


Fig. 1. (a) Weekly food intake. Values are means \pm SD per cage, 2 rats per cage, n 20–21 per group. IA, iron adequate; ID iron deficient. \circ , IA, \blacksquare , ID. (b) Weekly body mass response to exercise and iron intake. Values are means \pm SD, n 10–11 per group. IAS, iron-adequate sedentary; IAE, iron-adequate exercise; IDS, iron-deficient sedentary; IDE, iron-deficient exercise. \circ , IAS, \blacksquare , IAE, \square , IDS, \square , IDE.

subjected to serial dehydration and embedded in methyl methacrylate. Sections (4 μ m) were cut using a microtome, fixed to slides and cover-slipped unstained. Trabecular bone surfaces were measured for single, double and no label; regions with double label were further analysed to determine the distance between labels. The mineral apposition rate⁽⁹⁾, mineralising surface/bone surface and bone formation rate were calculated. All measures, calculations and terminology were adopted from the American Society for Bone and Mineral Research⁽¹⁵⁾.

Micro-computed tomography

Femurs were harvested, cleaned of tissue, wrapped in phosphate-buffered saline-soaked gauze, placed in 5 ml conical scintillation vials and stored at 4°C prior to shipping to the Indiana University School of Medicine (Indianapolis, IN). Cortical and trabecular bone scans and analyses were conducted according to standardised procedures^(16,17). In brief, whole femora were scanned using an 18 μ m isotropic voxel size on a SkyScan 1176 micro-computed tomography (micro-CT) system (Bruker, Billerica, MA). At the mid-diaphysis (~50% total length), cortical (Ct.) bone area, cortical cross-sectional thickness and mean polar moment of inertia were quantified. At the distal femur metaphysis (1-mm region located ~0.5 mm proximal to the growth plate) trabecular bone volume/tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N) were quantified.

Mechanical testing

Three-point bending tests were completed with the same femora (left) that were used for micro-CT scans according to standardised procedures^(16,18). In brief, bones were placed posterior side down on bottom fixtures that were 18 mm apart. The upper fixture point was positioned, centred at midpoint of the bone. Bones were displaced at a rate of 2 mm/min until failure. Force and displacement data were collected at 15 Hz. Mechanical parameters were calculated using a custom MATLAB program as previously published^(16,18). Structural parameters included maximum load, yield load, stiffness, total work and post-yield work. Material-level properties were calculated using beam-bending equations and geometry data from micro-CT analysis to estimate ultimate stress, yield stress, modulus and toughness.

Fracture toughness

Fracture toughness of right femurs was measured using a linear elastic fracture mechanics approach⁽¹⁹⁾. Femurs were notched approximately one-quarter of the way through the posterior side of the mid-diaphysis with a low-speed sectioning saw equipped with a diamond wafering blade (width of 0.012") and then the notch tip was sharpened by hand with a razor blade lubricated with a 1 μ m diamond suspension to a final depth not exceeding one-third of the anterior–posterior diameter. Each bone was then loaded to failure in 3-pt bending (support span of 16 mm) at a displacement control rate of 0.001 mm/s with the notched surface in tension. After failure, the distal end of each femur was cleansed of bone marrow with a water pick and dehydrated using an ethanol gradient (70–100%) and a vacuum desiccator. Following sputter-coating with gold, the cross-sectional fracture surface was imaged with a scanning electron microscope. The angles of stable and unstable crack growth were obtained from the images and, along with geometric properties from micro-CT data, a custom MATLAB script calculated stress intensity factors for crack initiation, maximum load and fracture instability⁽²⁰⁾.

Statistical analysis

A power analysis was conducted using G*Power for a factorial ANOVA with 2 \times 2 between-subject factors, power set at 0.8 and α at 0.05. In order to detect a large effect size ($f = 0.51$), 32 total rats (n 8/group) were needed to complete the study. Data were analysed using IBM SPSS 27.0 (SPSS Inc.). Main effects of diet, activity and the interaction were determined using a 2-factor ANOVA. Post hoc pairwise comparisons were made using Bonferroni to adjust for multiple comparisons. We accepted a $P < 0.05$ as being statistically significant. All data are presented as mean \pm SD.

Results

Iron status

Weekly food intake (Fig. 1(a)) and final body weight did not differ between treatment groups (Fig. 1(b)). Fe deficiency reduced Hg, haematocrit, mean corpuscular hemoglobin and mean

Table 1. Haematological response to exercise and iron intake

	IAS		IAE		IDS		IDE		Diet	Exercise	Diet × Exercise
	Mean	SD	Mean	SD	Mean	SD	Mean	SD			
Hgb, g/dl	18.3	1.3	18.0	1.3	15.1	1.5	14.9	2.3	< 0.001	–	–
Hct, %	58.1	5.3	57.5	5.5	49.5	5.8	49.5	6.5	< 0.001	–	–
MCH, pg	18.1	0.9	18.6	0.8	15.6	1.0	14.9	1.3	< 0.001	–	0.062
MCHC, g/dl	31.5	1.2	31.7	1.2	30.4	1.0	30.1	0.7	< 0.001	–	–
RDW, %	12.1	0.6	12.5	0.5	13.7	1.0	15.4	1.5	< 0.001	0.002	0.036

Values are means \pm SD, n 10–11. IAS, Fe-adequate sedentary; IAE, Fe-adequate exercise; IDS, Fe-deficient sedentary; IDE, Fe-deficient exercise; Hct haematocrit; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; RDW, red cell distribution width.

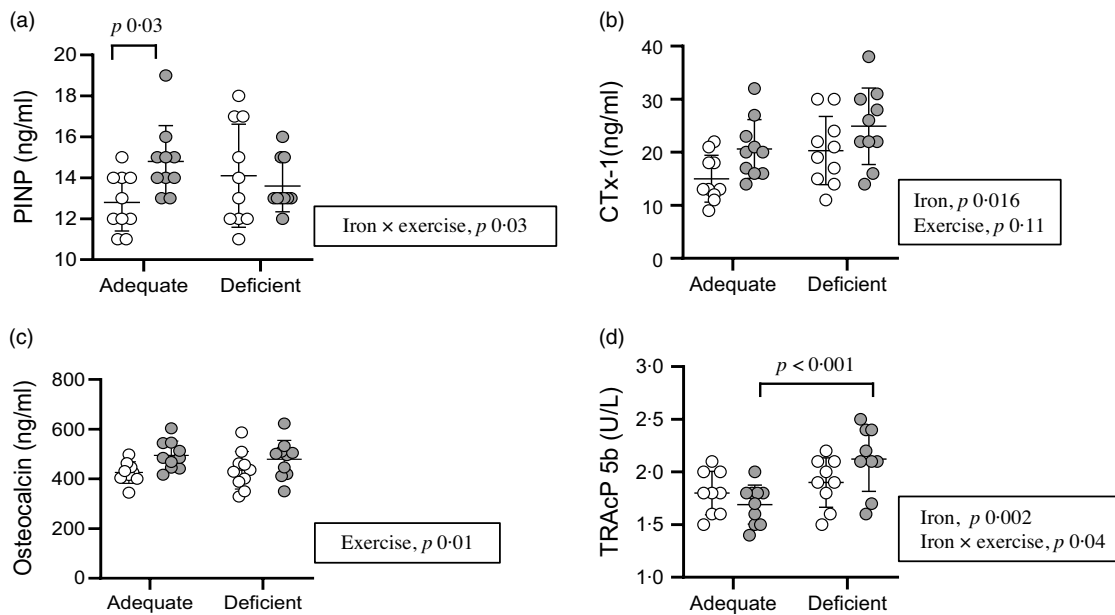


Fig. 2. Serum biomarkers of bone formation and resorption. Values are means \pm SD, n 9–10 per group. (a) Procollagen type I N-terminal propeptide (PINP); (b) C-terminal telopeptide type I collagen (CTx-1); (c) osteocalcin; (d) tartrate-resistant acid phosphatase 5b (TRAcP 5b). \circ , Sedentary, \bullet , exercise for figures 2–6.

corpuscular Hg concentration and increased red cell distribution width (Table 1). Exercise further increased red cell distribution width within Fe-deficient rats.

Serum bone biomarkers

Fe deficiency increased the bone resorption markers TRAcP 5b (15%) and CTx (27%) (Fig. 2). In exercised rats, Fe deficiency further increased TRAcP 5b (25%). In Fe-adequate rats, exercise increased the bone formation marker procollagen type I N-terminal propeptide by 16%. Also, exercise increased osteocalcin levels by 13%. The concentration of parathyroid hormone was unaffected by Fe deficiency or exercise: (IAS: 139.7 ± 20.3 , IAE: 114.6 ± 8.7 , IDS: 119.1 ± 14.3 , IDE: 139.6 ± 16.0 pg/ml), $P > 0.05$.

Tibiae histomorphometry

Independent of exercise, Fe-deficient rats had higher mineral apposition rate (MAR, 24%) and bone formation rate (BFR, 30%) (Fig. 3).

Femoral micro-computed tomography

The microarchitecture of femoral trabecular and geometrical properties of cortical bone are shown in Fig. 4. Of the trabecular microarchitectural properties (Fig. 4(a)–(d)), exercise led to higher bone volume/tissue volume (22%) and Tb. Th (9%). In cortical bone (Fig. 4(e)–(h)), exercise led to higher Ct. Th by 5%.

Femoral three-point bending

Structural and material properties of the femur are shown in Table 2. Exercise increased maximum load by 7%.

Femoral fracture toughness

Measures of fracture toughness did not differ between groups (Fig. 5).

Tibiae bone mineral analysis

Tibiae bone mineral composition is shown in Fig. 6. Independent of activity, Fe deficiency resulted in a 57% lower

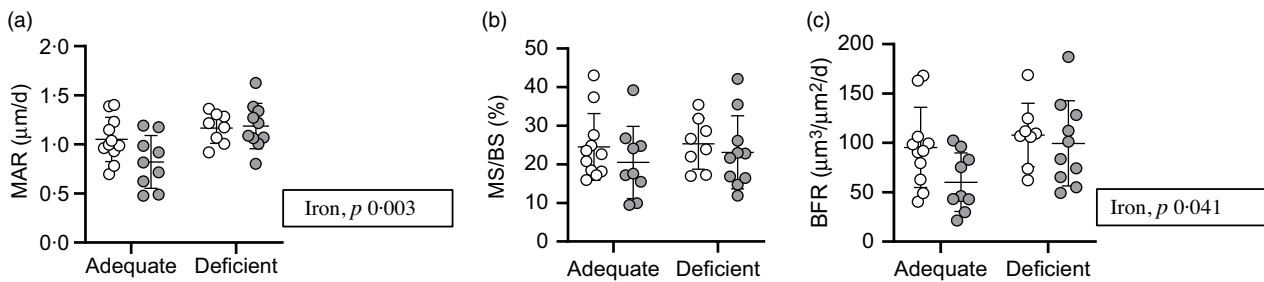


Fig. 3. Dynamic histomorphometry analyses determine at the proximal tibia metaphysis. Values are means \pm SD, n 8–11 per group. (a) Mineral apposition rate (MAR); (b) mineralized surface (MS/BS); (c) bone formation rate (BFR).

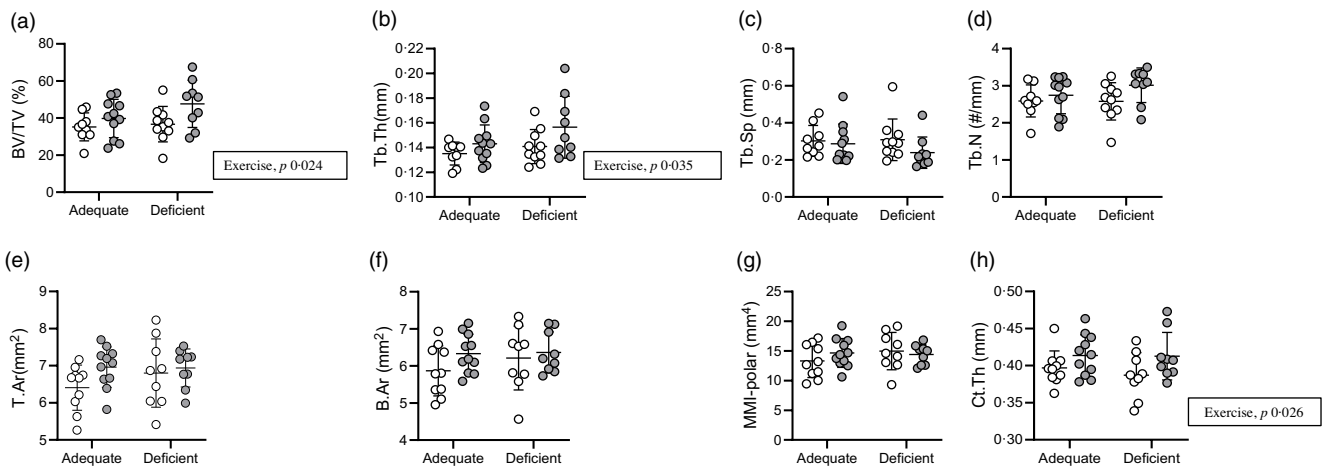


Fig. 4. Femur cortical geometric properties and cancellous microarchitecture determined by micro-CT. Cortical properties were determined at the mid-diaphysis. Values are means \pm SD, n 9–11 per group. (a) Bone volume/total volume (BV/TV); (b) trabecular thickness (Tb. Th); (c) trabecular separation (Tb. Sp); (d) trabecular number (Tb.N); (e) mean total cross-sectional tissue area (T.Ar); (f) mean total cross-sectional bone area (B.Ar); (g) mean polar moment of inertia (MMI-polar); (h) cortical thickness (Ct. Th).

Table 2. Biomechanical properties of the femur determined by three-point bending tests

	IAS	IAE	IDS	IDE	Diet	Exercise	Diet \times Exercise
Structural properties							
Stiffness, n /mm	284.2	22.5	298.6	33.2	281.2	42.0	275.0
Yield load, n	86.1	7.6	85.0	5.3	83.2	10.6	85.2
Maximum load, n	102.6	7.9	114.3	11.4	104.7	13.0	107.7
Total work, mJ	37.09	10.83	45.73	10.19	41.04	11.26	42.53
Post-yield work, mJ	22.91	12.09	32.48	10.61	27.65	11.03	28.23
Material properties							
Modulus, GPa	0.36	0.09	0.35	0.06	0.34	0.08	0.31
Yield stress, MPa	24.6	6.0	24.1	4.5	23.5	6.0	22.3
Ultimate stress, MPa	29.2	7.2	32.2	5.7	29.5	7.0	28.3
Toughness, MPa	2.34	0.72	3.08	0.94	2.70	0.85	2.54

Values are means \pm SD, n 9–11 per group. IAS, Fe-adequate sedentary; IAE, Fe-adequate exercise; IDS, Fe-deficient sedentary; IDE, Fe-deficient exercise.

skeletal concentration of Fe. Bone Zn levels were 9% higher in Fe-deficient rats and 5% in exercised rats. These responses were driven by exercise in Fe deficiency (9%) and Fe deficiency in exercised rats (14%). Independent of Fe, exercise increased bone Ca and phosphorous concentrations in bone by 2%.

Discussion

Two known functions of Fe in bone metabolism include the synthesis of collagen and vitamin D activation. Whether Fe deficiency affects these processes and impairs bone maturation during increased mechanical loading is unknown and the focus

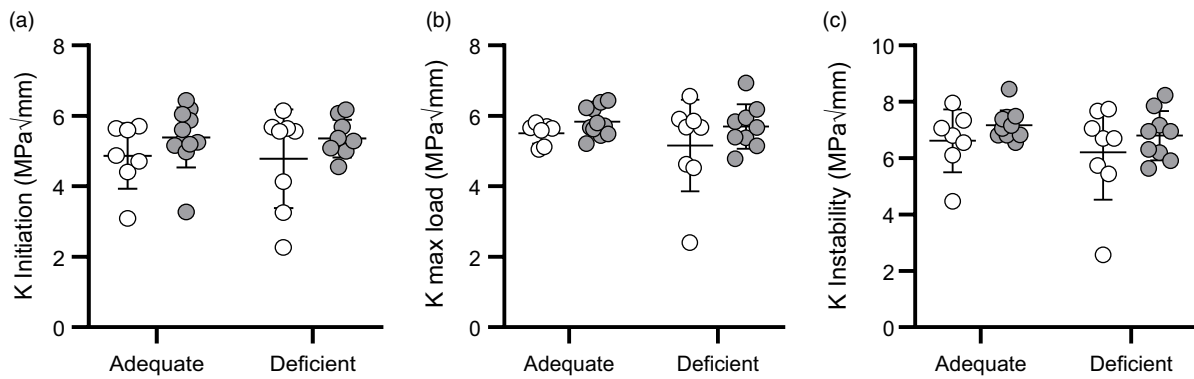


Fig. 5. Fracture toughness analyses determine at the femur. Values are means \pm SD, n 7–10 per group.

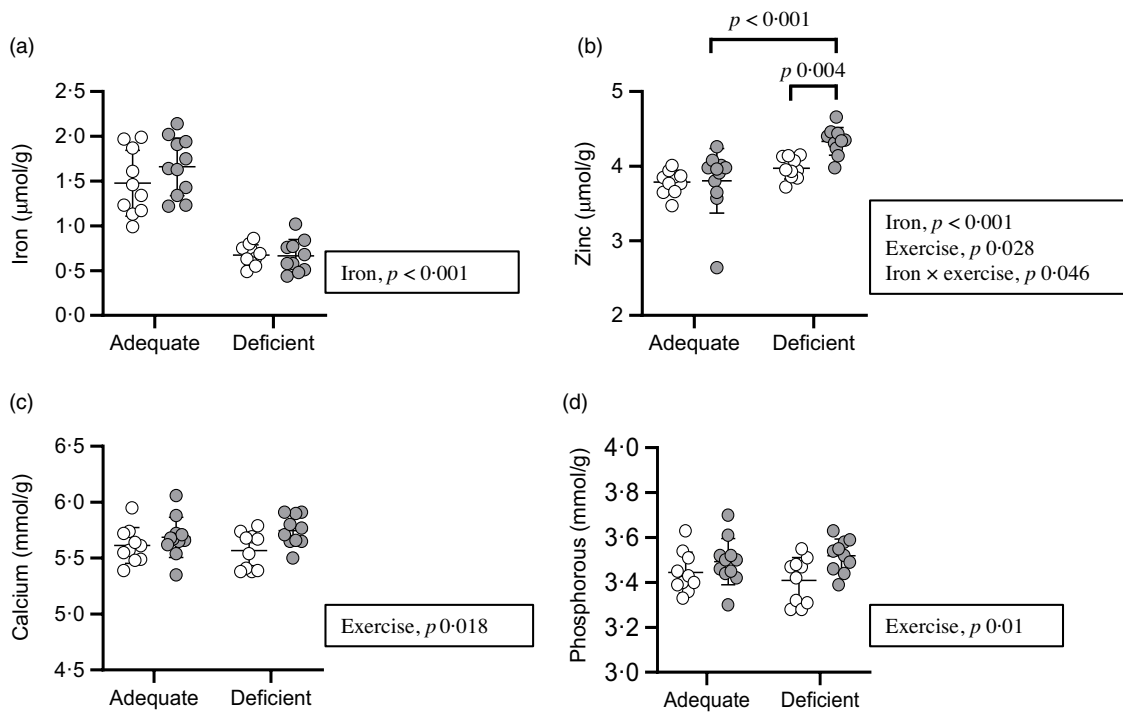


Fig. 6. Bone mineral composition of the tibia. Values are means \pm SD, n 9–11 per group.

of this research. In young female Fe-deficient rats, we report two novel findings: (1) trabecular bone may be more sensitive to changes in Fe status given the increase in MAR and bone formation rate, irrespective of exercise and (2) Zn accumulates in the tibia to a greater degree when combined with exercise.

As expected, the experimental diet (Fe deficiency) resulted in reduced markers of Fe status without inducing anemia. The model was, therefore, appropriate for studying the effects of Fe deficiency on bone. Rodent models of severe Fe deficiency anaemia have consistently resulted in increased bone resorption coupled with a decrease in bone mineral content and strength^(21–25). However, research focused on Fe deficiency and bone health have led to disparate findings and may be due to differences in animal strain, sex, age, physical activity and diet^(26,27). For example, no differences in femoral

bone strength were measured in 10-week-old male Sprague Dawley rats fed diets similar to the present study and completed 12 weeks of motorised wheel running⁽²⁶⁾. In contrast, sedentary weanling (3 weeks) female Long-Evans rats fed an Fe-deficient diet for 10 weeks demonstrated lower maximum load and stiffness during femoral three-point bending tests⁽²⁷⁾. In the present study, while the femoral and tibiae composition and maximum load were unaffected by Fe deficiency and treadmill running, the higher serum TRAcP 5b and CTx levels indicate bone resorption was higher at the time of measurement. This suggests that other skeletal sites were negatively affected by our experimental protocol. A similar observation was reported by Parelman *et al.*⁽²⁷⁾, i.e. there was no effect of Fe deficiency on femoral trabecular volume or cortical porosity, but a trend for reduced force and size-independent stiffness in the L-4 vertebral core. Research

directed towards understanding the bones' response to nutrient deficiencies and exercise should, therefore, consider measuring loaded and control bone.

The results presented here and the work by others^(21–25) suggest that bone responses to Fe are dependent upon the magnitude of deficiency. Fe deficiency anaemia reduces osteoblast activity, whereas Fe deficiency enhances proliferation, differentiation and mineralisation function of osteoblasts and reduces apoptosis⁽²⁸⁾. Our data corroborate these findings because Fe deficiency enhanced dynamic bone formation in the proximal tibia.

Similar to Fe, Zn is an essential trace mineral. When Fe intake is low, Zn absorption is enhanced since they compete for intestinal absorption⁽²⁹⁾. We found that Fe deficiency reduced Fe while increasing Zn in the tibia, and it was highest in exercised rats. The observation that Fe deficiency causes reduced Fe and increased Zn in bone has been previously reported in the femur of older (22-week) male rats⁽²⁶⁾. Zn stimulates gene expression of type I collagen, alkaline phosphatase and osteocalcin, indicative of bone formation⁽³⁰⁾, and inhibits osteoclast differentiation⁽³¹⁾. Taken together, increased Zn deposition may confer protection against structural deficits during Fe deficiency and load-bearing exercise. Whether Zn compensates for Fe during extended periods of Fe deficiency is unknown.

The increase in bone Ca and phosphorous with exercise training further supports the benefits of physical activity on bone modelling during maturation and may be more important than Fe in female rats. Weanling rats experience a rapid increase in whole body bone mineral density between 2 and 3 months of age followed by stabilisation up to 36 months⁽¹²⁾. The introduction of treadmill running at 6 weeks of age leads to further increases in tibial bone mineral content compared with sedentary controls⁽³²⁾, supporting the general consensus that weight-bearing activity has a positive influence on skeletal health⁽⁶⁾. However, we cannot exclude the possibility that Fe deficiency progressively leads to bone fragility, especially at unloaded sites, and may be exacerbated during longer running training programmes.

In summary, femoral or tibiae morphology and mechanical properties are not negatively impacted by 12 weeks of Fe deficiency and running exercise in young female rats. Protection may be attributed to marginal Fe deficiency and increased Zn, and the positive effects of load-bearing exercise. These novel findings do not implicate Fe deficiency as causative of bone fragility and questions a relationship between Fe deficiency and stress fractures in female recruits completing BMT.

We, however, cannot exclude the possibility of rat-human differences, and if prolonged Fe deficiency does lead to bone loss. In addition, non-loaded sites may have been impacted by Fe deficiency and provides justification for measuring different skeletal sites in future investigations.

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The authors' contributions are as follows: J. M. S. and H. G. G. were co-principal investigators, contributed to the study design, data collection, data analyses and wrote the manuscript. E. A. S., C. E. M., R. K., A. J. S., J. M. W. and M. R. A. contributed technical expertise, data analyses, and assisted with critical manuscript revisions. All authors read and approved the final version of the manuscript.

The authors have no conflicts of interest.

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