Soy Protein Isolate Suppresses Bone Resorption and Improves Trabecular Microarchitecture in Spontaneously Hyperphagic, Rapidly Growing Male OLETF Rats

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Abstract

Background: Traditionally, milk proteins have been recommended for skeletal health; recently, soy proteins have emerged as popular alternatives. Excess adiposity appears detrimental to skeletal health, as obese adolescents have increased fracture rates compared with healthy controls. However, soy protein effects on skeletal health during excess adiposity remain unknown.

Objective: The study objective was to examine the effects of isocaloric diets containing milk protein isolate (MPI), soy protein isolate (SPI), or a 50/50 combination (MIX) as the sole protein source on metabolic health indicators and bone outcomes in rapidly growing, hyperphagic, male Otsuka Long Evans Tokushima Fatty (OLETF) rats.

Methods: OLETF rats, aged 4 wk, were randomly assigned to 3 treatment groups (MPI, SPI, or MIX, $n = 20$ per group) and provided with access to experimental diets ad libitum for 16 wk.

Results: Body mass did not differ between the groups, but SPI had lower percentage body fat than MPI ($P = 0.026$). Insulin was lower in MPI than in MIX ($P = 0.033$) or SPI ($P = 0.044$), but fasting blood glucose was not different between the groups. SPI significantly reduced serum cholesterol compared with MPI ($P = 0.001$) and MIX ($P = 0.002$). N-terminal propeptide of type I collagen (P1NP) was higher in MIX than MPI ($P = 0.05$); C-terminal telopeptide of type 1 collagen (CTx) was higher in MPI than SPI ($P < 0.001$) and MIX ($P < 0.001$); the P1NP to CTx ratio was significantly higher in SPI and MIX than in MPI ($P < 0.001$). Trabecular separation was reduced in SPI compared with MPI ($P = 0.030$) and MIX ($P = 0.008$); trabecular number was increased in SPI compared with MIX ($P = 0.038$). No differences were seen in cortical geometry and biomechanical properties.

Conclusions: In the context of excess adiposity, soy- and milk-based proteins have comparable effects on cortical bone geometry and biomechanical properties, whereas soy-based proteins favorably affect the trabecular microarchitecture, and the combination of both proteins may offer additional benefits to bone remodeling in rapidly growing male OLETF rats. *Curr Dev Nutr* 2018;2:nzy010.

Keywords: soy protein, milk protein, bone, OLETF rats, trabecular microarchitecture, bone turnover markers

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Abbreviations used: BMD, bone mineral density; BV, bone volume; CCK, cholecystokinin; Conn.D, connectivity density; Ct.Th, cortical thickness; CTx, C-terminal peptide of type I collagen; ER, estrogen receptor; G, shear modulus of elasticity; IGF-1, insulin-like growth factor 1; K_s, torsional stiffness; MIX, 50/50 mixture of soy and milk protein isolate; MPI, milk protein isolate; OLETF, Otsuka Long Evans Tokushima Fatty; P1NP, amino-terminal propeptide of type I collagen; R, robustness; SMI, structural mode index; SPI, soy protein isolate; S_u, ultimate tensile strength or maximal shear stress; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; T_{max}, maximal torque at fracture; Tt.Ar, total area; TV, total volume; μCT, microcomputed tomography.

Introduction

Individuals achieve peak bone mass early in the second decade of life, which makes adolescence a critical time for skeletal growth and bone accrual (1). Peak bone mass is influenced by a variety of lifestyle factors, such as diet, physical activity, and body mass (1). Higher body mass is generally correlated with higher bone mineral density (BMD) owing to increased mechanical loading (2). However, obesity, especially excess adiposity, is associated

with increased systemic inflammation, insulin resistance, and PPAR-γ signaling, which negatively impact the skeleton (3, 4) by increasing osteoclast activity (5) and decreasing osteoblast activity (6), leading to an imbalance in bone remodeling. When adjusted for body mass, obese children tend to have lower whole-body BMD and bone mineral content, as well as higher fracture rates in the lower limbs, compared with lean controls (3, 7). Epidemiologic evidence points to a positive association between dairy intake and bone mass in childhood and adolescence (8–10). In addition, animal studies indicate that soy protein might counteract the detrimental effects of obesity on bone, by reducing PPAR-γ signaling and insulin resistance, and thus correct the imbalance of remodeling (11, 12). However, whether soy- or dairy-based proteins confer greater skeletal benefits in the context of obesity has yet to be determined.

Traditionally, the consumption of cow's milk-based proteins is recommended for optimal skeletal development because dairy milk and dairy products are excellent sources of dietary calcium and vitamin D (13). However, recent evidence suggests that the skeletal benefits of whole dairy products are greater than those derived from calcium and vitamin D supplementation (14). These data support the conclusion that the skeletal benefits of dairy products are due in part to milk protein, in addition to the benefits of calcium and vitamin D. In adolescents, 12–18 mo of milk supplementation resulted in an increase in spine (15) and whole-body BMD (16) compared with those without milk supplementation. A meta-analysis showed that this increase in BMD after an increase in dairy consumption is especially significant in individuals with a history of low intakes (17). Milk consumption during adolescence is also associated with a lower risk of osteoporotic fracture as an adult (18). In healthy men, short-term (i.e., 16 d) milk-protein supplementation increased urinary markers of bone formation and decreased urinary markers of bone resorption compared with baseline (19). Epidemiologic evidence in adolescents (20) supports a strong positive correlation between milk consumption and circulating insulin-like growth factor (IGF-1), which is essential for osteoblast differentiation and bone formation (21, 22). Additionally, short-term supplementation with casein (23) and milk protein increased circulating IGF-1 in prepubescent boys (24). Together, this evidence supports milk-based proteins being a benefit to bone health outside of their micronutrient content.

While milk-based proteins remain popular among consumers, soy-based proteins have emerged as a popular vegetarian, plant-based dairy alternative (25). Unique among plant-based proteins, soy is a high-quality protein equivalent to egg protein (26), which is used as the reference protein in determination of biological value. Not only does soy protein as the sole dietary protein source support positive nitrogen balance in growing humans; data from experimental animal models suggest that soy protein has equivalent or even superior skeletal effects compared with casein in adolescent male animals (27, 28). Soy protein significantly improved femoral BMD, as well as cancellous bone properties, such as trabecular number and bone volume, in male C57BL/6 mice (27). Following soy protein consumption, expression of intestinal calcium transporters, specifically TRPV6, were increased in rats compared with casein controls (28). In young and old men, soy protein intake has been shown to increase circulating IGF-1 concentrations (29). Therefore, a diet containing soy protein isolate should also positively affect skeletal development, although whether animal- or plant-based proteins confer the most benefit is not clear (28).

Given the increasing prevalence of excess adiposity and insulin resistance in adolescents and their adverse effects on bone health, the effects of protein source are clinically relevant. To our knowledge, no other studies have looked at protein source and the effect it can have on bone turnover, trabecular microarchitecture, or cortical geometry and biomechanical strength in the context of obesity and insulin resistance. Thus, the current study was performed to compare the effects of milk- and soy-based protein on bone outcomes in the Otsuka Long Evans Fatty (OLETF) rat model of obesity and insulin resistance. We chose the OLETF model because the progression of obesity and insulin resistance relative to skeletal maturity is similar to that of humans (30). We hypothesized that soy-based protein could have an equivalent effect on serum markers of bone turnover, trabecular microarchitecture, and cortical geometry and biomechanical strength in rapidly growing male OLETF rats. The OLETF rat is selectively bred for null expression of cholecystokinin-1 (CCK-1) receptor in the hypothalamus. Because CCK is a gut hormone that signals satiety through interaction with the receptor in the hypothalamus, null expression of the CCK-1 receptor results in hyperphagia (31) and subsequent obesity and insulin resistance with a standard rodent chow diet. Excess adiposity is evident starting at 5 wk of age, followed by the onset of insulin resistance at 12 wk of age, hyperglycemia at 20 wk of age, and frank type 2 diabetes by 40 wk of age (31–33). This progression of disease coincides with skeletal growth, which peaks at around 16 wk of age in the rat, consistent with the development of insulin resistance and obesity relative to skeletal maturity in humans (30).

Methods

Experimental design and animal protocol

In this 16-wk longitudinal study, sixty 4-wk-old, hyperphagic male OLETF rats (Tokushima Research Institute) were randomly assigned to 1 of 3 experimental dietary treatments: milk protein isolate (MPI; Idaho Milk Products), soy protein isolate (SPI; DuPont Nutrition and Health), or a 50/50 combination of MPI and SPI (MIX; Research Diets, Inc.) as the sole protein source (n = 20 per group). All rats were housed individually in a temperature-controlled environment (21°C) with a 0600–1800 light, 1800–0600 dark cycle maintained throughout the experimental period. Rats were allowed ad libitum consumption of the diets from 4 to 20 wk of age. Body weight, food intake, and body composition via EchoMRI were recorded weekly. The animal protocol was approved by the Institutional Animal Care and Use Committee at the University of Missouri and the Harry S Truman VA Subcommittee for Animal Studies. Not all rats were used for all analyses, based on availability of samples.

Experimental diets

Animals were randomly assigned to experimental diets containing MPI, SPI, or MIX as the sole protein source for 16 wks. The MIX group was included to compare the effects of a mixed protein source diet on bone outcomes to the effects of the diets containing only soy- or only milk-based proteins. Each diet was formulated to be isonitrogenous and isocaloric on the basis of the guaranteed analysis provided by the manufacturer, and to meet or exceed the AIN-93G micronutrient requirements for the growing rat, as previously reported (34) **(Table 1)**.

TABLE 1 Composition of experimental diets (34)[1]

Ingredient	Diet, g/kg		
	SPI	MIX[2]	MPI
Cornstarch	240	240	240
Sucrose	100	100	100
Maltodextrin	75	75	75
Cellulose	50	50	50
MPI	0	108.8	217.5
SPI	200	100	0
DL-methionine	3	3	3
Palm oil, bleached, deodorized	52.5	52.5	52.5
Cocoa butter, deodorized	37.5	37.5	37.5
Safflower oil, USP	28.5	28.5	28.5
Sunflower oil	27	27	27
Linseed oil	4.5	4.5	4.5
t-Butylhydroquinone	0.03	0.03	0.03
Mineral mix[3]	10	10	10
Potassium citrate	16.5	16.5	16.5
Dicalcium phosphate	13	13	13
Calcium carbonate	5.5	5.5	5.5
Vitamin mix[4]	10	10	10
Choline bitartrate	2	2	2
Protein, % energy	19	19	19
Carbohydrate, % energy	45	45	45
Fat, % energy	36	36	36
Caloric density (kcal/g)	4.41	4.41	4.41

[1] MIX, a 50/50 mixture of MPI and SPI; MPI, milk protein isolate; SPI, soy protein isolate.
[2] MIX, 50/50 combination of MPI (MPI-85, Idaho Milk Products) and SPI (SUPRO 670, DuPont Nutrition & Health).
[3] Mineral Mix S10026 (Research Diets, Inc) contains (in g/kg of mineral mix): NaCl, 259; MgO, heavy, 41.9; $MgSO_4 \cdot 7H_2O$, 257.6; $(NH_4)_6Mo_7O_{24}$ $4H_2O$, 0.3; $KCrS_2O_8$, 1.925; $CuCO_3$, 1.05; $C_6H_5FeO_7$, 21; CO_3MnH_2O, 12.25; KIO_3, 0.035; NaF, 0.2; Na_2SeO_3, 0.035; $ZnCO_3$, 5.6; sucrose, 399.105.
[4] Vitamin Mix V13401(Research Diets, Inc) contains (in g/kg of vitamin mix): vitamin A palmitate (500,000 IU/g), 0.8; vitamin D_3 (100,000 IU/g) 1.0; menadione sodium bisulfate (62.5% menadione), 0.08; biotin (1%), 2.0; cyanocobalmin (0.1%), 1.0; folic acid, 0.2; nicotinic acid, 3.0; calcium pantothenate, 1.6; pyridoxine-HCl, 0.7; riboflavin, 0.6; thiamin-HCl, 0.6; sucrose 988.42.

Diets differed from AIN-93G in macronutrient composition, in that the diets were higher in fat and contained sucrose. This was done so that the diets would mimic a Western-style diet (35), each providing 19% of energy from protein, 45% from carbohydrate, and 36% from fat. The isoflavone content of the SPI protein (μg aglycone/g protein) was as follows: 453 μg daidzein/g protein, 731 μg genistein/g protein, and 62 μg glycitein/g protein. Isoflavone content of the SPI diet was 90.6 mg of daidzein, 146.2 mg of genistein, and 12.4 mg of glycitein; isoflavone content of the MIX diet was 45.3 mg of diadzein, 73.1 mg of genistein, and 6.2 mg of glycitein. The calcium content (mass %) of the SPI, MIX, and MPI diets was 0.74%, 0.96%, and 1.18%, respectively; the phosphorus content was 0.56%, 0.58%, and 0.60%, respectively. The MPI and MIX diets had higher calcium and phosphorus contents because of the calcium and phosphorus associated with the MPI protein.

Animal sacrifice and tissue collection

At 20 wk of age, the final body mass was measured, then rats were anesthetized via intraperitoneal injection of pentobarbital (80 mg/kg) and exsanguinated via removal of the heart, as previously described (34). Blood was collected via cardiac puncture, allowed to clot for 20 min at room temperature, then spun at 1500 × *g* for 10 min at 4°C for serum collection. Serum was aliquoted and stored at −80°C for subsequent analysis of metabolic markers and serum markers of bone formation (N-terminal propeptide of type I collagen, P1NP) and resorption (C-terminal telopeptide of type I collagen, CTx). Right tibias and femurs were collected, cleaned of soft tissue, wrapped in 1× PBS-soaked gauze, and frozen at −80°C for subsequent analysis.

Metabolic outcomes

Fasting serum glucose, insulin, free fatty acids, triglycerides, and total cholesterol were measured using commercially available kits as previously described (34).

Serum markers of bone formation and resorption

The concentrations of the bone formation marker P1NP and the resorption marker CTx were measured in serum using commercially available, rodent-specific ELISA kits (ImmunoDiagnostic Systems). The intra-assay CVs were <4% for CTx and <6% for P1NP. All assays were run on the same day to avoid inter-assay variation; all samples were run in duplicate.

Femur calcium and phosphorous contents

Right femurs were cleaned of all soft tissue, weighed, and then defatted in hexane and diethyl ether each for 24 h. Following lipid extraction, femurs were dried to a constant weight at 60°C. Femurs were then placed in a muffle furnace (800°C) overnight to collect ash. The final weight of the ash content was recorded and ashed femurs were dissolved in 12 N HCl for subsequent analysis of calcium and phosphorus contents via inductive coupled plasma-optical emission spectroscopy (University of Missouri, Agricultural Experiment Station Chemical Laboratories). Results are expressed as milligrams of calcium or phosphorus per gram of dry bone (mg/g).

Tibia cortical geometry and trabecular microarchitecture

Microcomputed tomography (μCT) imaging of the tibia was performed using a high-resolution (32-μm slice increment) imaging system (Siemens INVEON Micro SPECT/CT, Siemens Medical). The methods used were in accordance with guidelines for the use of μCT in rodents (36). Scans were acquired using an isotropic voxel size of 0.0316 mm, a peak X-ray tube potential of 80 kVp with a tube current of 500 μA, and a 600-ms exposure at a medium-high magnification using a bin of 2. In a single rotation, 360 projections were collected at 1° increments and calibration images were collected prior to data acquisition. Images were reconstructed in real time using a Feldkamp cone beam filtered back projection algorithm (2D-FDP). Trabecular bone microarchitecture was evaluated in a 1-mm region of interest that started 1 mm below the growth plate of the proximal tibia. Cortical bone cross-sectional geometry was evaluated in the tibia mid-diaphysis, between the crest of the tibia and the distal edge of the tibiofibular joint in a 0.5-mm region of interest 0.25 mm proximal and 0.25 mm distal to the midslice. The optimize threshold function was used to delineate mineralized bone from soft tissue. Segmentation thresholds of 214 mg/cm^3 and 570 mg/cm^3 were used for evaluation of trabecular and cortical bone, respectively. Scans were analyzed using BoneJ software (37), a subset of ImageJ (ver. 1.50d) (National Institutes of Health public domain), and measures of cortical geometry and trabecular microarchitecture were collected. Cortical morphometric outcomes included: tibia

length, total cross-sectional area inside the periosteal envelope (Tt.Ar), marrow area (Ma.Ar), cortical bone area (Ct.Ar), cortical area fraction (Ct.Ar/Tt.Ar, %), average cortical thickness (Ct.Th), and robustness (R, total bone area over tibia length, calculated as R = Tt.Ar/Le). Outcomes for trabecular microarchitecture included: total volume (TV, volume of region of interest), bone volume (BV, volume of region segmented as bone), bone volume fraction (BV/TV), connectivity density (Conn.D, degree of trabeculae connectivity normalized to TV), trabecular number (Tb.N, mean number of trabeculae per unit length, calculated as 1/(Tb.Th + Tb.Sp) (38)), trabecular thickness (Tb.Th, mean trabecular thickness), trabecular separation (Tb.Sp, distance between trabeculae), structural model index (SMI), and degree of anisotropy.

Tibial biomechanical properties

Torsional loading to failure was used to assess the biomechanical properties of the tibia. The distal and proximal ends of the right tibia were embedded in a cylindrical steel holder that was placed in a test fixture. A machined cross bar was used to prevent the proximal end of the holder/tibia from rotating about its long axis while the distal end was rotated about its long axis at a speed of 10 mm/s with a load cell of 5 kg. The machine's control software (Stable Micro Systems) measured the cable force (F, in grams) and the applied torque (T). The load-displacement curve from this analysis is analogous to the torque-twist curve, which was used along with geometrical properties determined from μCT (i.e., length of specimen and polar moment of inertia) to calculate: maximal torque at fracture (T_{max}), torsional stiffness (K_s), shear modulus of elasticity (G), ultimate tensile strength or maximal shear stress (S_u), and energy absorbed to failure (U) as previously described (30).

Statistics

Four-wk-old, hyperphagic male OLETF rats were randomly assigned to 1 of 3 experimental diet groups (MPI, MIX, or SPI). Pearson correlations between metabolic (body weight, body fat percentage, and serum insulin, glucose, triglycerides, and cholesterol) and bone (bone turnover markers, trabecular microarchitecture, and cortical geometry and biomechanical strength) outcomes were performed to determine direct effects of diet compared with indirect effects of metabolic health on bone outcomes. One-way ANOVA was used to test for significant differences between groups for metabolic outcomes, serum bone turnover markers, and trabecular outcomes. Body weight is a strong predictor of cortical bone growth and biomechanical strength, so cortical and biomechanical outcomes were assessed by one-way ANCOVA, with final body weight included as a covariate. When there was a significant difference among groups, post hoc pairwise comparisons were made using the least significant difference technique. Data are means ± SEMs; statistical significance was set at $P < 0.05$. All analyses were performed using SPSS software (SPSS/23.0).

Results

Rat characteristics

Metabolic characteristics, including liver function and microbiome composition, of a subset of OLETF rats have been reported previously (34). Initial body mass ($P = 0.808$), final body mass ($P = 0.259$), serum glucose ($P = 0.519$), triglycerides ($P = 0.118$), and free fatty acids ($P = 0.119$) were not significantly different among groups (34). However, percentage body fat was lower in SPI-fed rats ($P = 0.026$) compared with MIX-fed rats, but not MPI-fed rats. Rats consuming the MIX diet had the highest average weekly food intake (181.0 ± 2.3 g) compared with rats consuming the SPI (174.0 ± 2.7 g) and MPI (170.4 ± 2.9 g) diets (34). At time of death, all rats were insulin resistant, as they had elevated fasting insulin but normal glucose concentrations (31). Fasting insulin was significantly different among groups ($P = 0.002$): it was significantly lower in MPI-fed rats than in those fed MIX ($P = 0.006$) and SPI ($P = 0.001$). Rats consuming the SPI diet had significantly reduced serum cholesterol compared with those consuming the MPI and MIX diets ($P = 0.001$ and $P = 0.002$, respectively) (**Figure 1**).

Serum markers of bone formation and resorption

Rats fed the MIX diet had significantly greater P1NP than those on the MPI diet ($P = 0.017$). SPI-fed and MIX-fed rats had significantly lower CTx than MPI-fed rats ($P < 0.001$). Consequently, the ratio of P1NP/CTx was significantly different between groups ($P < 0.001$), with the ratio being significantly greater in rats consuming the MIX diet than on either the SPI ($P = 0.026$) or MPI ($P < 0.001$) diet and the ratio in the SPI group being greater than that in the MPI group ($P < 0.001$) (**Figure 2**).

Femur calcium and phosphorous content

There were no differences in calcium content (milligrams of mineral per gram of dry bone) among groups ($P = 0.105$). However, phosphorus content (milligrams of mineral per gram of dry bone) trended toward group differences ($P = 0.059$), with the MPI group having the highest phosphorus content and the MIX group the lowest (**Figure 3**).

Tibial cortical geometry

Tibia length was not different among groups, suggesting no differences in longitudinal growth. Tt.Ar, Ma.Ar, Ct.Ar, Ct.Th, R, and Ct.Ar/Tt.Ar of the tibia mid-diaphysis were not different among groups, indicating similar bone mass accumulation (36). There were no differences in maximum or minimum moment of inertia (I_{max}, I_{min}) among groups; the I_{max}/I_{min} ratio, which is a measure of the circularity of the diaphysis (39), also showed no difference, indicating similarly shaped tibias among groups (**Table 2**).

Tibia trabecular microarchitecture

BV/TV ($P = 0.364$), Tb.Th ($P = 0.242$), and degree of anisotropy ($P = 0.251$) of the proximal tibia were not different among groups. However, Tb.Sp was significantly lower in rats fed the SPI diet compared with those fed the MIX ($P = 0.014$) and MPI ($P = 0.025$) diets. Tb.N was also significantly increased in the SPI group relative to the MIX group ($P = 0.011$), but not compared with the MPI group ($P = 0.297$). There was also a trend for Conn.D ($P = 0.077$) and SMI ($P = 0.060$) to be different among groups, with the SPI group having the highest Conn.D but the lowest SMI compared with the MIX and MPI groups (**Figure 4**).

Tibial biomechanical properties

Whole-bone (T_{max}, $P = 0.929$; K_s, $P = 0.753$; U, $P = 0.836$) and tissue-level (G, $P = 0.152$; S_u, $P = 0.151$) biomechanical properties of the tibia mid-diaphysis were not different among groups (**Figure 5**).

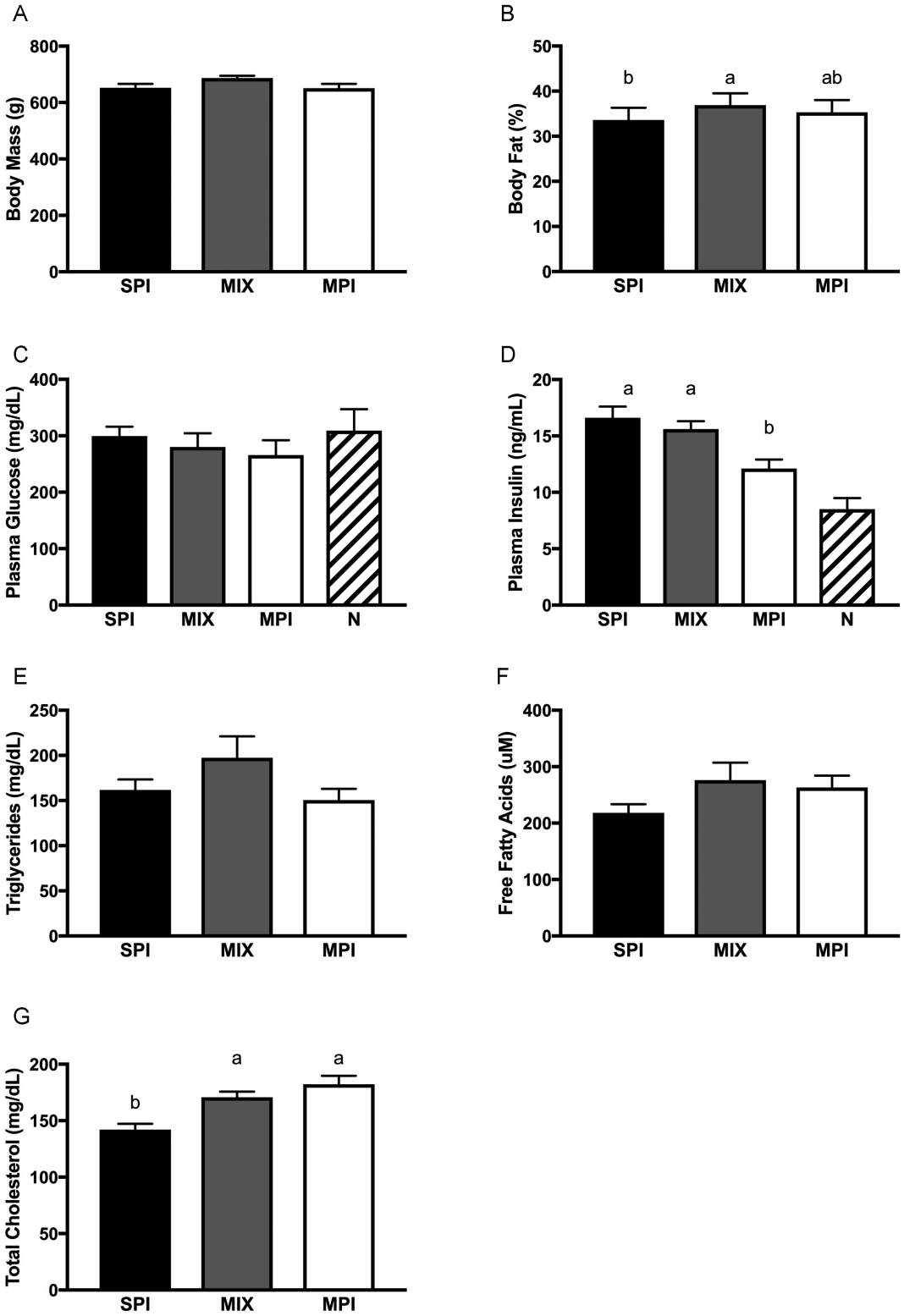

FIGURE 1 Final body weights and fasting metabolic characteristics in OLETF rats fed a Western-style diet with SPI, MPI, or MIX. Body mass (A), body fat percentage (B), plasma glucose (C), plasma insulin (D), triglycerides (E), free fatty acids (F), and serum total cholesterol (G). Data are means ± SEMs; $n = 9–10$ rats/group. Different letters denote significance, $P < 0.05$. For reference, normoglycemic and normoinsulinemic values have been provided. MIX, a 50/50 mixture of MPI and SPI; MPI, milk protein isolate; N, normoglycemic and normoinsulinemic; OLETF, Otsuka Long Evans Tokushima Fatty; SPI, soy protein isolate.

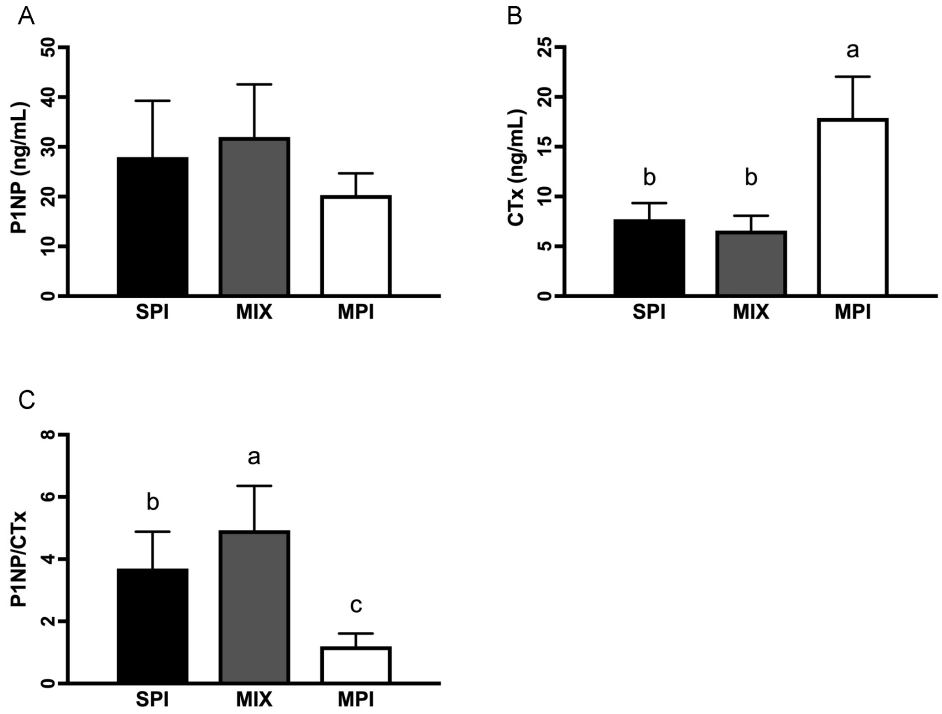

FIGURE 2 Serum markers of bone turnover in OLETF rats fed a Western-style diet with SPI, MPI, or MIX. P1NP (A), CTx (B), and P1NP/CTx (C). Data are means ± SEMs; $n = 9$–10 rats/group. Different letters denote significance, $P < 0.05$. CTx, C-terminal telopeptide of type I collagen; MIX, a 50/50 mixture of MPI and SPI; MPI, milk protein isolate; OLETF, Otsuka Long Evans Tokushima Fatty; P1NP, N-terminal propeptide of type I collagen; SPI, soy protein isolate.

Pearson correlations

Body weight was significantly positively correlated with Ct.Th ($r = 0.260$, $P = 0.047$), T_{max} ($r = 0.436$, $P = 0.003$), and K_s ($r = .0481$, $P = 0.001$), and trended toward significance with G ($r = .0257$, $P = 0.098$) and S_u ($r = 0.250$, $P = 0.102$). Body fat percentage was significantly positively correlated with T_{max} ($r = 0.496$, $P = 0.019$), K_s ($r = .0568$, $P = 0.006$), G ($r = 0.460$, $P = 0.031$), and Tb.Sp ($r = 0.392$, $P = 0.036$), and trended toward significance with S_u ($r = 0.411$, $P = 0.058$). There was a significant negative correlation between body fat percentage and Conn.D ($r = -0.398$, $P = 0.033$). There was a significant positive correlation between serum insulin and serum P1NP ($r = 0.437$; $P = 0.020$). No other correlations were significant.

Discussion

In this study, we examined the effects of 3 experimental diets (MPI, MIX, and SPI) on cancellous and cortical bone outcomes in obese, insulin-resistant rapidly growing male OLETF rats. We chose the OLETF rat model because the progression of obesity and insulin resistance relative to skeletal maturity in that model is similar to that of humans (30). We hypothesized that soy- and milk-based proteins would have an equivalent effect on bone outcomes during a time of rapid growth. In support of our hypothesis, the diets had a similar effect on cortical geometry and biomechanical properties of the tibia. However, rats fed the SPI diet showed significant improvements in trabecular microarchitecture, specifically trabecular spacing and trabecular number, and rats fed the MIX diet showed an increased ratio of bone formation to bone resorption, as measured by serum markers. Taken together, these results suggest that, in a male rodent model of young obesity, soy- and dairy-based proteins are comparable for cortical bone geometry and biomechanical strength, soy protein might favorably affect cancellous bone microarchitecture, and a mix of both proteins might benefit bone remodeling.

Rats fed the MPI diet had significantly greater serum CTx than rats fed the SPI and MPI diets (104% and 172% higher, respectively), suggesting a suppression of bone resorption with the presence of soy protein. This supports previous studies where soy protein suppresses bone resorption through decreased levels of receptor activator of nuclear factor-κB ligand (RANKL), a known promoter of osteoclastogenesis (40). Additionally, this significant increase of CTx in the MPI diet group is clinically relevant, as evidence points to a fracture relative risk of 2.1 owing to a 25% increase in serum CTx (41). While soy protein independently improved bone remodeling, the effect of combining soy- and milk-based proteins resulted in a significant increase in bone formation relative to resorption based on serum markers, which is consistent with a previous report on the combination of milk protein and soy isoflavones in protection against the loss of BMD in hind-limb unloading through an increase in osteogenic genes in the bone marrow (42). This implies that the combination of soy- and milk-based proteins might be most beneficial to bone remodeling in rapidly growing rats. This is significant, considering that an imbalance in bone remodeling in favor of resorption could be one of the primary mechanisms behind the impaired bone health seen in childhood obesity (43).

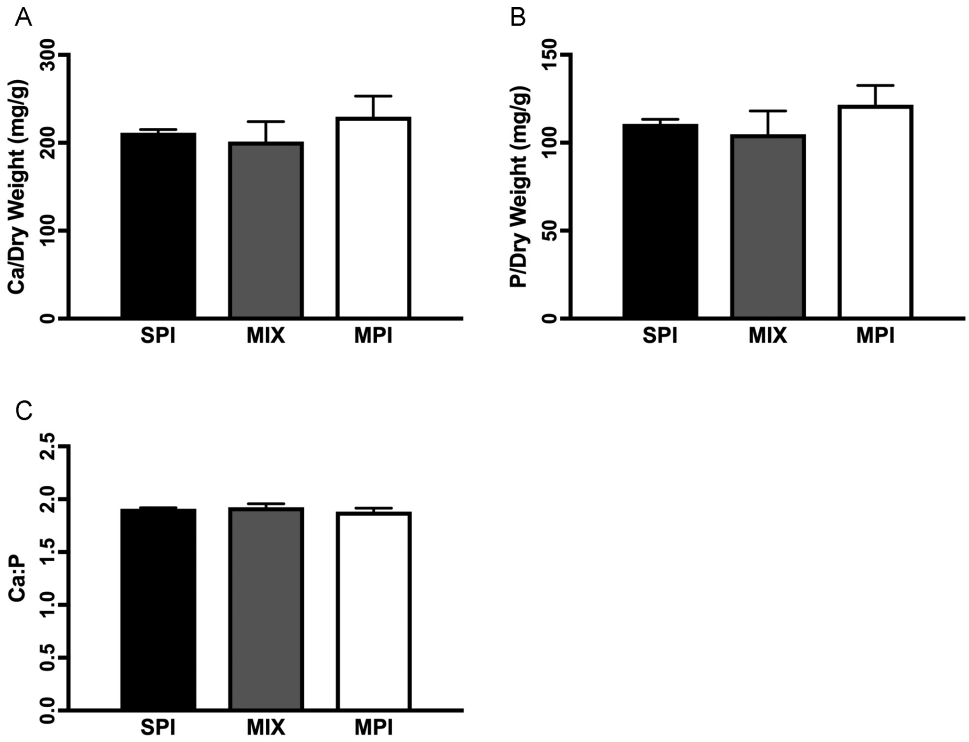

FIGURE 3 Mineral content of the femur in OLETF rats fed a Western-style diet with SPI, MPI, or MIX. Ca/dry weight (A), P/dry weight (B), and Ca-to-P ratio (C). Data are means ± SEMs; $n = 5$ rats/group. Different letters denote significance, $P < 0.05$. MIX, a 50/50 mixture of MPI and SPI; MPI, milk protein isolate; OLETF, Otsuka Long Evans Tokushima Fatty; SPI, soy protein isolate.

A proposed mechanism behind the effects of soy on bone are the estrogen-like actions of soy isoflavones on the estrogen receptor (ER), specifically ERβ (44), and the effects of soy protein intake on bone health in women have been studied extensively (45–47). Fewer studies have examined the skeletal effects of soy in men, partly because of concerns surrounding increased levels of estrogen-like molecules in males and the possibility that bioactive phytoestrogens could have an effect on reproductive hormones in men (48). However, most studies have shown that soy intake has no effect on reproductive hormone levels in either adults or children (49, 50). Additionally, 1 study in older rats showed that soy protein could attenuate orchidectomy-induced bone loss (51), and estrogen levels are generally a stronger predictor of BMD in males than testosterone (52). In addition to its estrogen-like actions (53), other studies show that soy protein may decrease levels of calveolin-1 (54), which could lead to a decrease in osteoblast senescence through a decrease in PPAR-γ (11). SPI could also prevent bone deterioration induced by a high-fat diet by preventing loss of insulin signaling in the bone (12). Additional studies in this area are warranted.

TABLE 2 Cortical geometry of the tibia mid-diaphysis in OLETF rats fed a Western-style diet with SPI, MPI, or MIX[1]

	SPI	MIX	MPI	P-value (Diet)[2]
Tibia length, mm	44.60 ± 0.30	45.04 ± 0.36	44.46 ± 0.32	0.43
Tibia diameter, mm	3.93 ± 0.08	3.99 ± 0.08	3.92 ± 0.08	0.78
Tt.Ar, mm^2	9.75 ± 0.11	9.89 ± 0.11	9.91 ± 0.10	0.43
Ma.Ar, mm^2	2.52 ± 0.06	2.69 ± 0.06	2.59 ± 0.06	0.11
Ct.Ar, mm^2	7.22 ± 0.12	7.20 ± 0.11	7.32 ± 0.12	0.74
Ct.Ar/Tt.Ar, mm^2	0.74 ± 0.01	0.73 ± 0.01	0.74 ± 0.01	0.30
Ct.Th, mm^2	1.02 ± 0.01	1.02 ± 0.01	1.01 ± 0.01	0.69
I_{max}, mm^4	7.61 ± 0.09	7.55 ± 0.10	7.50 ± 0.10	0.72
I_{min}, mm^4	4.70 ± 0.10	4.78 ± 0.11	4.74 ± 0.11	0.86
I_{max}/I_{min}	1.63 ± 0.03	1.58 ± 0.03	1.60 ± 0.03	0.40
K, mm^4	13.8 ± 0.55	12.9 ± 0.56	13.2 ± 0.57	0.53
R, mm	.219 ± 0.003	.220 ± 0.003	.223 ± 0.003	0.48

[1]Data are means ± SEMs adjusted with final body weight as a covariate; $n = 18$–20 rats/group. Ct.Ar, cortical area; Ct.Ar/Tt.Ar, cortical volume fraction; Ct.Th, cortical thickness; I_{max}, maximum moment of inertia; I_{min}, minimum moment of inertia; I_{max}/I_{min}, ratio of maximum to minimum moment of inertia; K, polar moment of area; Ma.Ar, marrow area; MIX, a 50/50 mixture of MPI and SPI; MPI, milk protein isolate; R, robustness (Tt.Ar/length); SPI, soy protein isolate; Tt.Ar, total area.
[2]*P*-values are a one-way ANCOVA with body weight as a covariate.

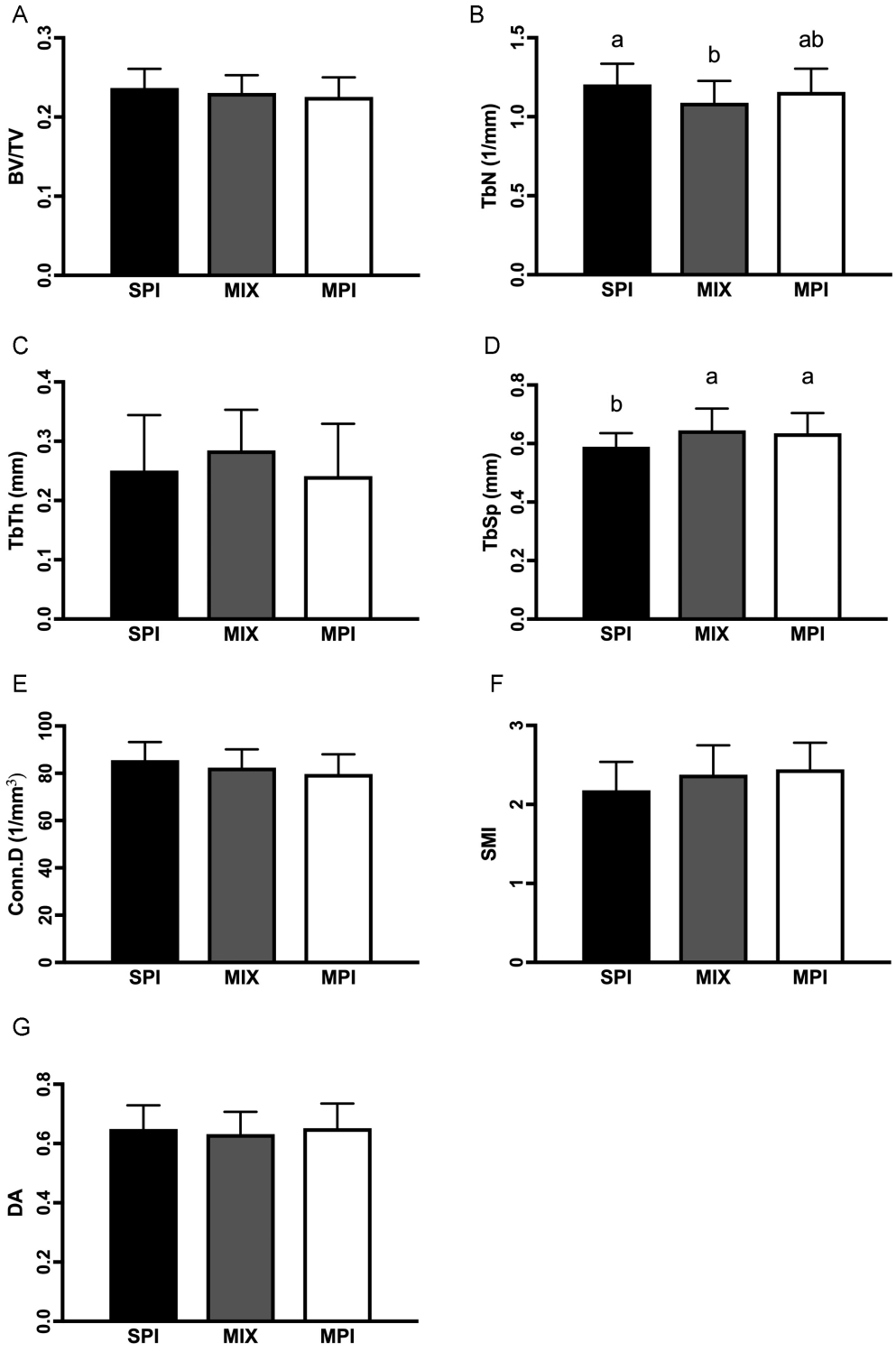

FIGURE 4 Trabecular microarchitecture of the proximal tibia in OLETF rats fed a Western-style diet with SPI, MPI, or MIX. BV/TV (A), TbN (B), TbTh (C), TbSp (D), Conn.D (E), SMI (F), and DA (G). Data are means ± SEMs; $n = 19–20$ rats/group. Different letters denote significance, $P < 0.05$. BV/TV, trabecular bone volume fraction; Conn.D, connectivity density; DA, degree of anisotropy; MIX, a 50/50 mixture of MPI and SPI; MPI, milk protein isolate; OLETF, Otsuka Long Evans Tokushima Fatty; SMI, structural mode index; SPI, soy protein isolate; TbN, trabecular number; TbSp, trabecular separation; TbTh, trabecular thickness.

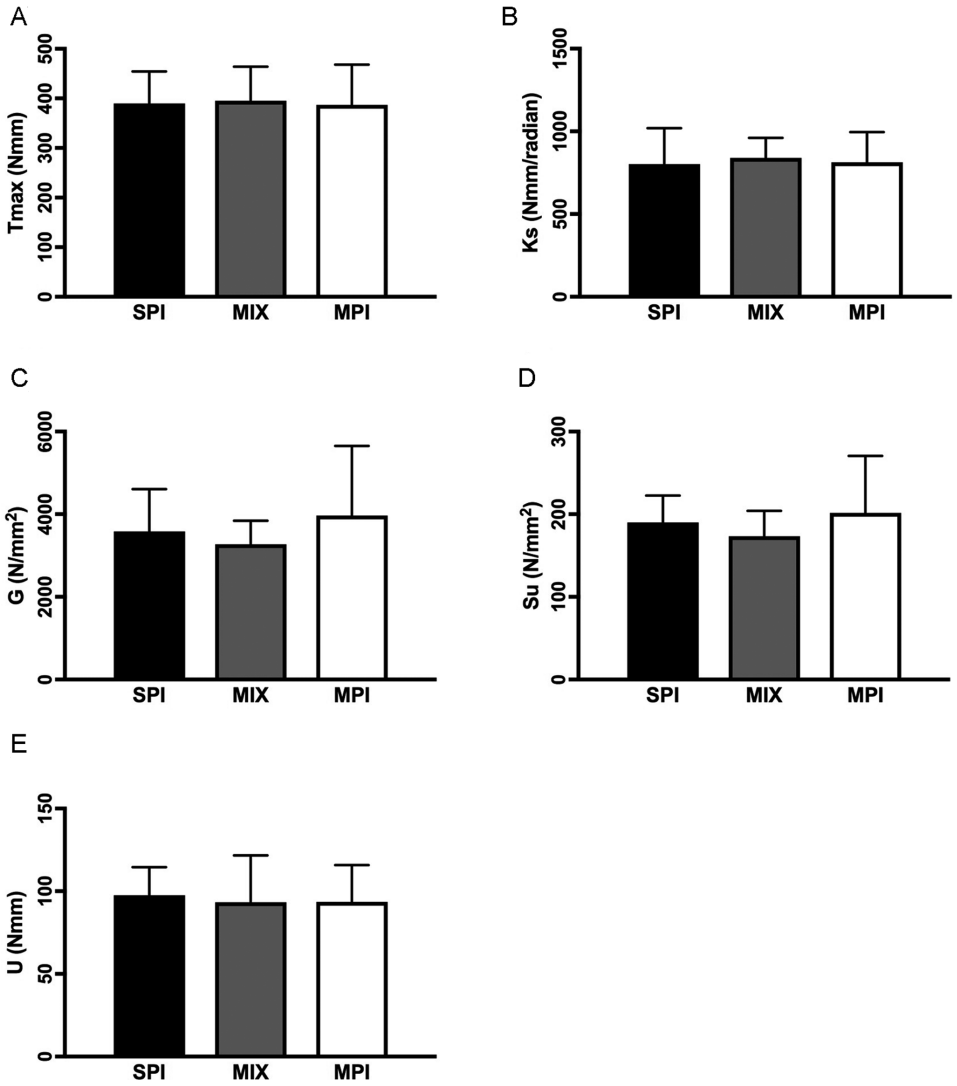

FIGURE 5 Biomechanical strength measures of the tibia mid-diaphysis in OLETF rats fed a Western-style diet with SPI, MPI, or MIX. T_{max} (A), K_s (B), G (C), S_u (D), and U (E). Data are means ± SEMs adjusted with final body weight as a covariate; $n = 19–20$ rats/group. Different letters denote significance, $P < 0.05$. G, shear modulus of elasticity; K_s, torsional stiffness; MIX, a 50/50 mixture of MPI and SPI; MPI, milk protein isolate; OLETF, Otsuka Long Evans Tokushima Fatty; SPI, soy protein isolate; S_u, ultimate tensile strength or maximal shear stress; T_{max}, maximal torque at fracture; U, energy absorbed to failure.

Cancellous bone and cortical bone responded differently to 16 wk of a soy-protein diet. Cortical geometry and biomechanical properties were not significantly different among the dietary treatments, but the trabecular microarchitecture was affected. Specifically, Tb.Sp was significantly decreased and Tb.N was significantly increased in rats fed the SPI diet. One explanation for the effects of soy protein on cancellous, but not cortical, bone is the distribution of the ER isoforms α and β. Soy isoflavones preferentially bind to the ER-β subspecies (44), and cancellous bone has greater expression of ER-β compared with cortical bone (55). Additionally, the rate of turnover in trabecular bone is considerably higher than that of cortical bone (56), owing to an increase in active surface area (57). Other researchers have also shown that soy preferentially protects trabecular bone through increases in bone formation (58), which would be consistent with our findings. However, the bone markers that we measured represent the whole body, and cannot distinguish between cancellous and cortical bone. Loss of individual trabeculae and thinning of existing trabeculae (59), as well as a lower trabecular bone volume (60), significantly contribute to loss of bone strength. Thus, data from the present study suggest that soy protein isolate might increase cancellous bone strength. However, we did not have the capacity to test the compression strength of cancellous bone and further study is warranted.

Finally, we measured the calcium and phosphorus contents of the femur, as mineral content is an important determinant of BMD and bone strength in growing rats (61). While there were small differences in the calcium and phosphorous contents of the MPI and SPI proteins and, therefore, the respective diets, all of the diets met or exceeded the calcium and phosphorous recommendations for growing rodents (62). Thus, it was not surprising that there were no differences in calcium content of the femur. While there was a trend toward a difference in

total femoral phosphorus content, with the MPI group being the highest, the ratio of calcium to phosphorus was not different among groups, indicating that th eprotein source did not significantly affect the relative composition of the 2 primary minerals in bone during skeletal growth.

Because overweight and obesity are now linked to poor bone heath and increased fracture risk (3, 7), there is potential for dietary protein intake to indirectly improve bone health in overweight adolescents through improvements in metabolic health. Epidemiologic evidence suggests that a dietary pattern incorporating more low-fat dairy products might lower the risk of type 2 diabetes (63) and hypertension (64). In adolescents, dairy consumption is inversely associated with central adiposity (65). Soy protein has also been widely studied for its reported metabolic health benefits (66, 67). In adults, soy consumption results in a clinically significant decrease in circulating total and LDL cholesterol (68, 69) and triglycerides (53). In this study, there were few metabolic differences among the dietary treatments. However, the rats fed the SPI diet had a lower body fat percentage compared with the rats fed the MIX diet, and had decreased circulating cholesterol compared with those fed MIX or MPI. Additionally, the rats fed SPI had significant improvements in liver function (34), which is often used as a surrogate measure of metabolic health, indicating improved metabolic health following a soy-based diet. This improvement in metabolic health and decrease in circulating cholesterol have significance for skeletal health, as both excess adiposity (4) and hypercholesterolemia (70) have detrimental effects on bone, and mouse models show that adiposity induced by a high-fat diet especially affects cancellous bone (71).

All groups showed insulin resistance, but rats in the SPI group had higher serum insulin than those in the MPI group. We observed a significant positive correlation between insulin and P1NP. This result was contrary to our hypothesis that insulin resistance would be detrimental to bone health. However, insulin is a major anabolic hormone that can have significant effects on bone growth through osteoblast activity (72), and previous studies have shown that insulin resistance can be beneficial to trabecular microarchitecture (73, 74). Additionally, we showed positive correlations between body weight and body fat percentage and all biomechanical strength outcomes, as well as a significant positive association between body weight and cortical thickness. This was unsurprising, considering body weight is a strong determinant of cortical bone growth and strength (75). Finally, we showed a positive correlation between body fat percentage and Tb.Sp and a negative correlation between body fat percentage and Conn.D. These results support our hypothesis that dietary protein intake could indirectly affect bone health through actions on metabolic health.

In summary, our findings suggest that, in the context of excess adiposity, soy-based and milk-based proteins have comparable effects on cortical bone geometry and biomechanical properties, while soy-based proteins favorably affect trabecular microarchitecture, and the combination of both proteins may offer additional benefits to bone remodeling in young, rapidly growing male OLETF rats. These findings are of key significance as many people consume protein from a variety of animal- and plant-based sources.

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