A Methionine-Restricted Diet and Endurance Exercise Decrease Bone Mass and Extrinsic Strength but Increase Intrinsic Strength in Growing Male Rats

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Abstract

Dietary methionine restriction (MR) has been suggested to be comparable to endurance exercise with respect to its beneficial effects on health. To further investigate the effects of MR and endurance exercise on growing bone, 7-wk-old male Sprague-Dawley rats were fed different L-methionine (Met)-containing diets with or without endurance exercise intervention (Ex; 0.86% Met, 0.52% Met, 0.17% Met, 0.86% Met-Ex, 0.52% Met-Ex, and 0.17% Met-Ex groups). After an 8-wk intervention period, exercise-trained rats had a 9.2% lower body weight (BW) than did sedentary rats (P < 0.05). Additionally, 0.17% Met–fed rats had 32% lower BW when compared with rats fed the other 2 diets (P < 0.05). Serum osteocalcin was lower in the 0.17% Met-Ex group compared with the other 2 exercise groups and the 0.17% Met group (P < 0.05). Serum concentrations of C-terminal telopeptide of type 1 collagen were lower in exercise-trained and 0.17% Met–fed rats than in sedentary rats and rats fed the other 2 diets (P < 0.05 for both). Rats fed the 0.17% Met diet had lower trabecular bone volume, bone mineralization activities, and bone mineral content (BMC; e.g., total, cortical, and spongy BMC) and bone mineral density (BMD; e.g., total and spongy BMD) indices compared with rats fed the other 2 diets (P < 0.05). Exercise-trained rats also had lower bone mineralization activity, trabecular osteoclast density, total BMC, cortical BMC, and total BMD compared with sedentary rats (P < 0.05). In total BMD, only the 0.17% Met-Ex group had values lower than the other 2 exercise groups and the 0.17% Met group (P < 0.05). Compared with rats fed the other 2 diets and sedentary rats, the femora of 0.17% Met–fed and exercise-trained rats, respectively, had smaller size and/or lower extrinsic strength but enhanced intrinsic biomechanical properties (P < 0.05). The results indicate that MR and endurance exercise caused lower whole bone mass, size, and/or strength but might enhance intrinsic bone strength.

Introduction

Living longer and healthily has become a central issue in human well-being-related investigations. Dietary interventions are among the adopted behaviors to improve and preserve human health. Over the past several decades, caloric restriction (CR) has been proven to mitigate the incidence of chronic diseases, optimize energy...
mechanical properties. Histomorphometry, densitometry, tissue dimension, and biochemical properties.

A reduction in dietary Met content has been reported to induce changes in energy metabolism (6) and to extend the life span in rodents by >30% (7–10). Such substantial extension of life expectancy has been primarily attributed to the downregulation of oxidative stress and the delayed onset of aging-associated diseases (11). In fact, MR increases concentrations of the antioxidant glutathione in blood, promotes insulin sensitivity, and decreases visceral adiposity in Fischer 344 rats (4,5).

Although MR extends the life span, there is concern that this dietary regimen could decrease bone strength, because MR-fed rats showed signs of growth arrest and, as a result, were smaller in body size and mass (9). In fact, a recent study revealed that MR reduces bone mass and size in C57BL/6J mice fed a high-fat diet (12), an outcome similar to observations from CR and PR studies (14–16). In contrast to CR and/or PR, MR diets are consumed ad libitum and are designed to be equal in total amino acids and isocaloric with respect to a control diet. Furthermore, long-term MR-fed rats consume more food per unit of body weight (BW) compared with rats in control groups (9). There is therefore the possibility that MR could induce different effects on bone metabolism, bone growth/development, and tissue material properties compared with other dietary restriction regimens. Whether such MR-related reductions in absolute growth and bone mass could be detrimental to bone tissue properties (e.g., bone quality and bone material properties) requires further investigation.

Aside from dietary manipulations, physical activity or exercise is another well-known strategy for improving health. Among various exercise models, endurance exercise has been shown to enhance energy metabolism (17), reduce oxidative stress (18), and prevent chronic diseases (19) in a fashion similar to MR. Accompanied with the apparent efficacy of endurance exercise in BW loss, the accretion of bone mass and/or bone size seems not to be favored. Endurance athletes (e.g., long-distance runners) have normal or subnormal levels of bone mineral density (BMD) and bone mineral content (BMC) (20,21). Similar observations were drawn from animal studies showing that endurance training of growing or adult male rats was associated with a reduction or no change in bone size, mineral accumulation, and intrinsic bone strength (16,22,23). However, when normalized to body mass, healthy endurance athletes and endurance-trained animals had normal or higher BMD or BMC compared with nonexercise control groups (24,25).

In addition, extrinsic and intrinsic bone strength in rats subjected to endurance exercise was not different from that of nontrained animals with larger bones (22). Thus, despite the finding that endurance exercise causes a reduction in bone size and bone mineral accumulation, it does not seem to compromise bone quality. The objective of this study was to investigate the effects of MR and endurance exercise on growing bone quality, which was assessed by examining energy and bone metabolic serum markers as well as bone histomorphometry, densitometry, tissue dimension, and biochemical properties.

Methods

Animals. Male Sprague-Dawley rats (4 wk old) were purchased from the National Cheng Kung University Animal Center. Before starting the experiments, all rats were fed standard Purina Rodent Chow 5001 (Labdiet) containing 0.95% calcium and 1.07% phosphate (wet wt of dry food). The rats were housed 2 to 3 rats per cage under standard conditions of a 21 ± 1°C room temperature and a 12:12-h light-dark cycle. All rats had free access to food and water throughout the pre-experimental and experimental periods. To measure dynamic bone turnover, the fluorescent labels alizarin sodium salt (Alizarin Red S, A5533; Sigma-Aldrich) and calcein (Calcien disodium, 20130; Sigma-Aldrich) were suspended in distilled water and administered through i.p. injection at doses of 30 mg/kg BW and 8 mg/kg BW, respectively, at 10 and 3 d before the rats were killed. At the end of the experiment, the rats were anesthetized with ketamine and xylazine at doses of 80 mg/kg BW and 10 mg/kg BW via i.p. injection, and their body length was measured from the occiput to the sacrum. Whole blood was collected after decapitation, allowed to clot, and centrifuged at 1500 × g for 20 min at 4°C. Serum was collected and immediately stored at −80°C for bone marker analysis. All animal procedures followed the American Physiological Society’s “Guiding Principles in the Care and Use of Animals” and were approved by the Committee of Animal Study at National Cheng Kung University, Tainan, Taiwan (document 99104).

Diet composition. Diets consisting of AIN-76 chemically based diet with protein replaced by amino acid mixtures containing 0.86% Met (control; 519535) and 0.52% Met or 0.17% Met (519539 and 519540, respectively) were purchased from Dyets. The total percentage of amino acids in the 3 diets was kept at 13.84%, and all diets were isocaloric. When Met was decreased in the 0.52%- and 0.17%-Met–containing diets, the content of all other amino acids was changed to make the diets equal in total amino acids (see Supplemental Table 1 for the composition of the 3 diets).

Experimental designs. To investigate the short-term effects of MR on growing bone, 5-wk-old male rats were randomly assigned to 3 short-term (5) (10 d) treatment groups fed 0.86%, 0.52%, and 0.17% Met diets, [0.86%Met (n = 9), 0.52%Met (n = 9), and 0.17%Met (n = 10) groups]. BW, water consumption, and food intake were measured every other day. Data obtained from the 10-d study are included in the Online Supporting Material (see Supplemental Results, Supplemental Figs. 1–5, and Supplemental Tables 2–6).

To examine the long-term effects of MR, 7-wk-old rats were fed the 3 diets for 8 wk. Endurance exercise intervention (Ex) was also applied to a subset of rats. For these studies, the rats were weight-matched and assigned to the following treatment groups: 0.86% Met (n = 12), 0.52% Met (n = 12), 0.17% Met (n = 12), 0.86% Met-Ex (n = 12), 0.52% Met-Ex (n = 12), and 0.17% Met-Ex (n = 12). BW, water consumption, and food intake were measured throughout the entire 8-wk feeding period.

Endurance exercise training. The endurance exercise protocol used was a modification from protocols used in previous studies (22,25). Briefly, the exercise program began at a speed of 12 m/min on a level treadmill, and the treadmill speed was progressively increased to 24 m/min over the first 4 wk. The daily training duration was also progressively increased from 20 to 60 min within the first 2 wk of the training program. The rats were trained on the treadmill 5 d/wk for a total of 8 wk.

Serum analyses. Serum analyses were conducted to examine the effects of the MR diet and endurance exercise on markers of bone turnover, development, energy metabolism, and oxidative stress. Commercial ELISA kits were used to measure serum osteocalcin (Rat-MID ELIA; Immunodiagnostics Systems), C-terminal telopeptide of type 1 collagen (CTX-1; RatLapps Immunodiagnostics Systems), insulin (rat insulin ELISA; Mercodia AB), insulin-like growth factor 1 (IGF-1; Rat/Mouse IGF-1 ELISA kit; Immunodiagnostics Systems), TBARS (TBARS assay kit; Cayman), and leptin (mouse and rat leptin ELISA; Biovendor) following the manufacturers’ procedures. In addition, serum glucose, TGs, and total cholesterol were measured by using commercially available enzymatic kits (Glucose liquidcolor, Triglycerides liquidcolor, and Cholesterol liquidcolor; HUMAN Gesellschaft für Biochemie und Diagnostica).
Bone sample preparation. Bilateral hind-limb bones were removed, and their length was measured by using a caliper with a 0.05-mm precision. Femur length was measured from the most superior point on the head of the femur to the most inferior point on the distal condyle. The distance from the lateral condyles to the tip of the medial malleolus was used for tibia length (26,27). The right tibia of each rat was fixed in a 3.7% neutral paraformaldehyde solution for 24 h and decalcified with a 10% EDTA solution (pH 7.4) at 4°C for 4 wk. Once decalcified, each bone was paraffin embedded, sectioned, and stained for histologic analyses. The left tibia of each rat was dehydrated in gradient alcohol, cleared with xylene, and cut cross-sectionally through the midshaft. The proximal and distal segments of each tibia were embedded in methylmethacrylate and further sectioned for fluorescence microscopy. The right femora were stored in 70% ethanol for micro-computed tomography (μCT) scanning. Finally, the left femora were cleaned of soft tissue, wrapped in gauze, immersed in PBS (pH 7.4), and stored in aluminum foil at −80°C for biomaterial testing.

Growth plate measurements. Serial frontal sections (5 μm in thickness) of each tibia were made by using an Accu-Cut SRM 100 Rotary Microtome (Sakura Finetek), and the sections were processed for hematoxylin and eosin staining as previously described (28). Growth plate (GP) measurements above the proximal metaphysis were performed in the hematoxylin- and eosin-stained sections as previously described (29,30). Briefly, the GP was divided into 3 different zones: the reserved zone (RZ), proliferative zone, and hypertrophic zone; and the mean thickness of each zone and the entire GP were measured. Bone volume over total volume ratio (BV/TV, %) of metaphyseal secondary spongiosa (1.0−3.0 mm below the GP) was measured to examine the correlation between total GP thickness and spongy BV/TV. GP thickness and BV/TV measurements were performed by using the Image Pro Plus software (6.1 version; Media Cybernetics).

Dynamic histology. Methylmethacrylate-embedded proximal tibiae were subjected to frontal sectioning (5 μm) by using an automatic rotary microtome (HM 355S; Thermo Scientific). The distal segments of the tibiae were polished at the midshaft cross-sectional surface. Secondary spongiosa of the proximal tibiae (1−3 mm below the GP) and the cross-sectional surfaces of midshaft tibiae were photographed under a fluorescent microscope at 100× and 40× magnifications, respectively. Length measurements of fluorescent labeled/nonlabeled bone surfaces and the distances between 2 fluorescent labels were performed on images using Image Pro Plus. These measurements were subsequently used to calculate 3 standard dynamic histomorphometry indices (31): bone mineralization over bone surface (MS/BS), mineral apposition rate (MAR), and bone formation rate per bone surface (BFR/BS).

In addition, osteoclasts were stained on paraffin sections by using a commercial tartrate resistant acid phosphatase staining kit (387A; Sigma-Aldrich). The multinucleated osteoclast number relative to the trabecular tissue perimeter (N.Oc/BS) of secondary spongiosa (1−3 mm below the GP) was used as a measure of bone resorption (31).

μCT: tissue geometry, static histomorphometry, and densitometry. Right femora that were immersed in 70% ethanol were subjected to μCT scanning (Skyscan 1176) by using the following conditions: Al 0.5-mm filter, 50 kV, 500 μA, 0.5° per picture with 1470 ms exposure time, and pixel size at 9 μm. Various densitometry and histomorphometry analyses were performed by using a CT-Analyzer (version 1.12.0.0; Skyscan). Volumetric BMD and BMC measurements were conducted on whole femur, midshaft cortical bone (transverse slices of 1 mm in thickness) and secondary spongiosa (transverse slices between 0.5 and 3.5 mm below the lowest point of GP at distal metaphysis without cortical bone) of each femur. Histomorphometric indices of BV/TV, trabecular thickness, trabecular number, trabecular separation, connectivity density, and structure model index (SMI) were measured in secondary spongiosa. In addition, a transverse-CT slice between the head of the femur and the distal condyle was acquired to assess cross-sectional parameters including cortical area and 3 indices of cross-sectional moment of inertia (CSMI); polar CSMI (CSMI_p), maximal CSMI (CSMI_max), and minimal CSMI (CSMI_min).

Analyses of biomechanical properties. Biomechanical properties of femora were determined by using a material testing system (MTS-819; MTS System) following methods described previously (22,32). Because bone specimen stored under deep-freezing conditions could reduce bending strength (33), we conducted all biomechanical tests under the same conditions to diminish the possible effects caused by freezing storage. All left femora samples were stored at −80°C for <2 mo and subjected to biomechanical testing on the same day. Briefly, femora samples were thawed and kept wet at room temperature. After length measurement, the midpoint of the anterior surface of each femur was determined and set as the middle spot of 3-point bending. An anteroposterior direction 3-point bending test at a deformation rate of 1 mm/s was then performed on each femur. The spans of the 2 support points were 15 and 20 mm for samples from the 10-d and 8-wk experiments, respectively. Load (unit: N) versus deformation (unit: mm) data acquired by the Team 490 software (version 4.10; Nicolet Instrument Technologies) were used to calculate extrinsic (whole-bone level) biomechanical properties of bone, including parameters of yield load, maximal load, yield load energy, maximal load energy, and stiffness. Furthermore, the intrinsic (tissue-level) biomechanical properties were calculated on the basis of the elastic beam theory (32). Briefly, data from load-deformation curves were transformed into stress-strain data by using the following equations:

\[
\sigma = \frac{FLc}{4l} \\
\epsilon = \frac{12cd}{L^2} \\
E = \frac{F}{d} \times \frac{L^3}{48l}
\]

where \(\sigma\) is longitudinal stress, \(\epsilon\) is longitudinal strain, \(c\) is the maximal distance from the surface subjected to tensional forces to the line crossing the center of mass, \(F\) is the applied load (N), \(L\) is the CSMI_min of the midshaft femora acquired by μCT (see Supplemental Methods for further details on CSMI_min for calculation of intrinsic biomechanical properties), \(E\) is the elastic modulus, \(d\) is the deformation, and \(L\) is the span between the 2 support points of the bending fixture. Because the beam bending theory is valid in the preyield region (32), stress-strain data were only used to determine yield stress, yield toughness, and elastic modulus as parameters of intrinsic biomechanical properties. Yield load and yield stress were determined with the 0.002 strain-offset method described previously (22,32).

Statistical analysis. Results are presented as means ± SDs. One-factor ANOVA was used to compare differences between the treatment groups in the 10-d experiment. For the 8-wk experiment, 2-factor (Met × Ex) ANOVA was used to compare the differences between groups. \(P\) values <0.05 were considered significant. Post-hoc comparisons were conducted by using Fisher’s least significant difference method. Pearson’s correlation analyses were made between total GP thickness and BV/TV, as well as between BW and bone size/mass indices. SPSS statistical analysis software (SPSS 17.0) was used for the data analyses.

Results

Data from the 10-d study are available in the Online Supporting Material (Supplemental Results, Supplemental Figs. 1–5, and Supplemental Tables 2–6). The results presented in the article are primarily from the 8-wk studies.

Food intake and BW. The rats fed the 0.17% Met–containing diet consumed less food per unit of BW during the first week of the study (\(P < 0.05\)). However, in week 8, the food consumption of the 0.17% Met–fed rats was greater than that of those fed the Met–fed controls.
other 2 diets ($P < 0.05$) (Fig. 1A). The 0.17% Met–fed rats were slower in BW gain compared with the other 2 feeding groups ($P < 0.05$). Endurance exercise also reduced BW gain after week 4 of treadmill training ($P < 0.05$) (Fig. 1B).

**GP and longitudinal growth measurements.** With the exception of RZ thickness, the 0.17% Met–fed rats had smaller bodies, shorter bone lengths, and thinner GP zones compared with the 0.52% and 0.86% Met–fed rats ($P < 0.05$) (Table 1, Fig. 2). Exercise-trained rats also had shorter bones and lower RZ, hypertrophic zone, and total GP thicknesses ($P < 0.05$). GP zones and longitudinal bone growth did not differ between the 0.17% Met and 0.17% Met–Ex groups. However, rats from the 0.86% Met–Ex and/or 0.52% Met–Ex groups had shorter tibiae and smaller GP zones compared with their diet-matched nonexercise groups ($P < 0.05$) (Table 1). The correlation between total GP thickness and BV/TV was significant ($r = 0.32, P < 0.01$).

**Serum markers.** Serum CTX-1, insulin, IGF-1, TBARS, glucose, and TGs were lower in 0.17% Met–fed rats compared with rats fed 0.86% Met and/or 0.52% Met diets ($P < 0.05$). Endurance exercise–trained rats had lower serum CTX-1, insulin, leptin, and TBARS compared with sedentary rats ($P < 0.05$) (Table 2). Osteocalcin was lower in the 0.17% Met–Ex group compared with the 0.17% Met group and the other 2 exercise groups ($P < 0.05$).

**Static/dynamic histomorphometry.** The 0.17% Met–fed rats had lower BV/TV, trabecular thickness, trabecular number, and connectivity density and higher trabecular separation and SMI in the trabecular bone compared with rats fed the other 2 diets ($P < 0.05$) (Figs. 3 and 4). Endurance exercise–trained rats had lower SMI than did sedentary rats ($P < 0.05$) (Fig. 3E). However, there was no difference in SMI between the 2 groups fed the 0.17% Met diet ($P < 0.05$) (Fig. 3E). Dynamic histomorphometry of spongy bone (Table 3) showed that the 0.17% Met–fed rats had lower local bone formation activity (e.g., MAR and BFR/BS) compared with rats fed the other 2 diets ($P < 0.05$). Exercise rats had lower MAR and N.Oc/BS than did sedentary rats ($P < 0.05$).

Cortical bone dynamic histomorphometry revealed that MS/BS, MAR, and BFR/BS indices were lower in the periosteal of 0.17% Met–fed rats compared with rats fed the other 2 diets ($P < 0.05$). Periosteal and endosteal MAR was lower in exercise rats than in nontrained rats ($P < 0.05$) (Table 3).

**Densitometry and cross-sectional measurements.** Rats fed the 0.17% Met diet had lower total and spongy BMD, whereas endurance exercise–trained rats had lower total BMD ($P < 0.05$). However, in total BMD, only the 0.17% Met–Ex group had values lower than the other 2 exercise groups and the 0.17% Met group ($P < 0.05$). BMC indices revealed that rats assigned to the 0.17% Met–containing diet displayed lower total, cortical, and spongy BMC compared with rats fed the other 2 diets, whereas exercise rats had lower total and cortical BMC compared with sedentary rats ($P < 0.05$) (Table 4).

Rats subjected to the 0.17% Met diet and exercise had lower femoral cortical area and 3 CSMI indices compared with those fed the other 2 diets and sedentary rats, respectively ($P < 0.05$). However, there was no difference in CSMI$_p$ and CSMI$_{max}$ between the 2 groups fed the 0.17% Met diet (Table 4).

**Biomechanical properties.** Although yield load energy was not statistically different between the rats fed the 0.17% Met and 0.86% Met diets, bones from the 0.17% Met–fed rats had lower extrinsic bending strength, energy resorption, and stiffness compared with bones from rats fed the 0.86% Met and 0.52% Met diets (Fig. 5A–E). Conversely, 0.17% Met–fed rats had higher intrinsic yield stress, toughness, and elastic modulus (Fig. 5F–H). Bones from exercise-trained rats also displayed higher yield stress and toughness. Among the 3 exercise groups, the 0.17% Met–Ex group had higher yield stress compared with the other 2 exercise groups ($P < 0.05$), whereas no difference was observed in yield toughness. Both yield stress and yield toughness were different between the 3 sedentary groups ($P < 0.05$), with the 0.17% Met and 0.52% Met groups showing the highest and second highest values, respectively. However, only the 0.86% Met–Ex group had higher yield stress and toughness when compared with the 0.86% Met group ($P < 0.05$).

### Discussion

The current study was conducted to gain insight into the effects of MR and endurance exercise on bone development, remodeling,
and biomechanical properties in rats. As in previous studies (9,12), the rats fed the 0.17% Met diet had smaller body size and lower bone mass compared with the 0.86% Met–fed rats, but rats fed the 0.52% Met–containing diet had body weight gain and bone parameters similar to those fed the control (0.86% Met) diet. Smaller bone size or mass in rats subjected to the 0.17% Met–containing diet and endurance exercise was associated with the downregulation of both bone growth and bone turnover. Although the 0.17% Met diet–mediated lower extrinsic strength paralleled the reduction in bone size/mass, this dietary intervention did not compromise bone intrinsic biomechanical properties. In fact, both 0.17% Met diet feeding and endurance exercise appeared to benefit the intrinsic biomechanical properties of bone.

In the current study, short-term MR (0.17% Met) was associated with a lower BW, which was accompanied by a reduction in food intake (Supplemental Fig. 1A). Distinct from the CR model, in which rats are fed lower amounts of food, the MR diet was consumed ad libitum throughout the entire experimental period. Furthermore, food intake per unit of BW in 0.17% Met–fed rats did not differ from those fed the other diets after week 2 of interventions (Fig. 1A). This implies that the long-term MR effects on bone are associated with the restriction of Met and not calories. Supporting this statement are pair-feeding studies showing that rats fed a control (0.86% Met) diet at the amounts consumed by rats fed the 0.17% Met diet attained the same BW as rats with free access to the 0.86% Met diet (7–9). Although the mechanism or mechanisms by which MR decreases BW have not been yet identified, because Met is critical for normal growth and development and plays a key role in protein synthesis, DNA methylation, and polyamine synthesis (5), the MR effects appear to be a phenomenon that could be associated with reduced anabolism and development. In addition, mice fed CR diets had different gene expression patterns from those fed MR diets, implying that there are inherent differences between CR and MR (34,35).

The 10-d feeding period of the 0.17% Met–containing diet (the 0.17% Met group) caused a significant (38%) reduction in spong BV/TV when compared with the 0.86% Met diet (Supplemental Fig. 3A). This difference in BV/TV was maintained (39% lower) over the course of the 8-wk study (Fig. 3A), implying that there was not a continuous loss of spong bone in MR-fed rats. Serum bone markers and the local dynamic histology analyses from the 10-d and 8-wk experiments instead suggested that MR caused a downregulation in bone turnover rate. In fact, in typical osteopenia/osteoporosis animal models (e.g., ovariectomy), there is simultaneous upregulation of both osteoblastic and osteoclastic activities (36). According to the densitometry analyses in 10-d and 8-wk studies, MR mainly caused less bone mineral accumulation as shown by BMC indices. However, the slightly but significantly lower spong BMD in 0.17% Met–fed rats and the total BMD in the 0.17% Met-Ex group could imply compromised mineralization in response to MR or the combination of MR and exercise.

TABLE 1  Linear growth measurements and GP thickness in young male rats fed diets containing 0.86%, 0.52%, and 0.17% Met with or without exercise training for 8 wk.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body length, cm</th>
<th>Femur length, mm</th>
<th>Tibia length, mm</th>
<th>RZ thickness, μm</th>
<th>PZ thickness, μm</th>
<th>HZ thickness, μm</th>
<th>Total GP thickness, μm</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.86% Met</td>
<td>26.9 ± 0.9</td>
<td>40.4 ± 0.7</td>
<td>44.6 ± 1.1</td>
<td>36 ± 6</td>
<td>78 ± 8</td>
<td>75 ± 8</td>
<td>190 ± 17</td>
</tr>
<tr>
<td>0.52% Met</td>
<td>26.7 ± 0.9</td>
<td>40.6 ± 0.9</td>
<td>45.1 ± 0.7</td>
<td>36 ± 5</td>
<td>72 ± 7</td>
<td>67 ± 7</td>
<td>175 ± 12</td>
</tr>
<tr>
<td>0.17% Met</td>
<td>27.0 ± 1.1</td>
<td>39.8 ± 0.6</td>
<td>43.8 ± 0.8**</td>
<td>30 ± 6*</td>
<td>71 ± 7*</td>
<td>62 ± 4**</td>
<td>188 ± 11</td>
</tr>
<tr>
<td>0.86% Met-Ex</td>
<td>27.1 ± 1.0</td>
<td>39.3 ± 0.6</td>
<td>41.2 ± 0.86*</td>
<td>33 ± 6</td>
<td>76 ± 6*</td>
<td>56 ± 6</td>
<td>168 ± 10</td>
</tr>
<tr>
<td>0.52% Met-Ex</td>
<td>25.0 ± 0.7</td>
<td>36.5 ± 0.9</td>
<td>41.6 ± 0.96</td>
<td>36 ± 7</td>
<td>62 ± 6</td>
<td>56 ± 6</td>
<td>155 ± 10</td>
</tr>
<tr>
<td>0.17% Met-Ex</td>
<td>25.0 ± 0.6</td>
<td>36.0 ± 1.9</td>
<td>0.15 ± 0.02</td>
<td>32 ± 6</td>
<td>62 ± 6</td>
<td>56 ± 6</td>
<td>150 ± 14</td>
</tr>
</tbody>
</table>

Values are means ± SDs, n = 12. Within the exercise or sedentary groups, labeled means without a common letter differ, P < 0.05. *Different from corresponding sedentary group, P < 0.05. Ex, endurance exercise intervention; GP, growth plate; HZ, hypertrophic zone; PZ, proliferative zone; RZ, reserve zone; 0.17% Met, 0.17% methionine diet group; 0.17% Met-Ex, 0.17% methionine diet and exercise group; 0.52% Met, 0.52% methionine diet group; 0.52% Met-Ex, 0.52% methionine diet and exercise group; 0.86% Met, 0.86% methionine diet group; 0.86% Met-Ex, 0.86% methionine diet and exercise group. 2 Main effect of Met: the 0.17% Met-fed rats differed from rats fed the other 2 diets, P < 0.05.
The MR-mediated lower spongy bone mass in growing rats appeared to be associated with less linear growth. It is known that metaphyseal spongy bone volume is derived from endochondral ossification of cartilaginous matrix left by dead GP chondrocytes (37). Thus, MR-related lower metaphyseal spongy bone volume could be a response to decreased chondrocyte activity in the GP of 0.17% Met–fed rats during the early growth phase. Moreover, dynamic histomorphometry analyses conducted in bones from the 8-wk study revealed that the lower metaphyseal bone volume was partially caused by a reduction in bone formation activity during a later growing phase. Similar observations were reported in PR (38) and CR (14) studies demonstrating that growing rats fed these diets had thinner GPs or reduced linear growth and lower spongy bone volume.

Endurance exercise alone caused a minor reduction in long bone growth and GP thickness as reported in previous treadmill or voluntary running training studies (16,39). Exercise not only downregulated systemic bone resorption markers but also downregulated local bone formation activity and osteoclast density. The observed lower bone turnover rate and smaller GP thickness (linear growth) led to no change in spongy bone of exercise-trained rats compared with the nonexercise rats. Moreover, exercise enhanced trabecular structure (e.g., SMI) in rats fed the 0.86% and 0.52% Met–containing diets, which would be associated with higher biomechanical strength of spongy bone (40).

MR also downregulated bone appositional growth as shown by lower periosteal bone formation activity (Table 3) and

### TABLE 2 Serum markers of bone metabolism, energy metabolism, and aging in young male rats fed diets containing 0.86%, 0.52%, and 0.17% Met with or without exercise training for 8 wk

<table>
<thead>
<tr>
<th>Met</th>
<th>0.86% Met diet</th>
<th>0.52% Met diet</th>
<th>0.17% Met diet</th>
<th>P (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.86% Met</td>
<td>0.86% Met-Ex</td>
<td>0.52% Met</td>
<td>0.52% Met-Ex</td>
</tr>
<tr>
<td>Osteocalcin, μg/L</td>
<td>474 ± 212</td>
<td>549 ± 186*</td>
<td>426 ± 218</td>
<td>561 ± 256*</td>
</tr>
<tr>
<td>CTX-1, μg/L</td>
<td>44.7 ± 6.7</td>
<td>36.7 ± 13.7</td>
<td>42.5 ± 9.4</td>
<td>35.7 ± 11.3</td>
</tr>
<tr>
<td>Insulin, μg/L</td>
<td>0.34 ± 0.10</td>
<td>0.27 ± 0.06</td>
<td>0.42 ± 0.14</td>
<td>0.32 ± 0.12</td>
</tr>
<tr>
<td>IGF-1, μg/L</td>
<td>942 ± 168</td>
<td>930 ± 262</td>
<td>953 ± 166</td>
<td>1010 ± 318</td>
</tr>
<tr>
<td>Leptin, μg/L</td>
<td>3.42 ± 2.83</td>
<td>3.08 ± 1.98</td>
<td>3.90 ± 2.71</td>
<td>2.62 ± 3.05</td>
</tr>
<tr>
<td>TBARS, μmol/L</td>
<td>39.1 ± 16.9</td>
<td>24.9 ± 14.4</td>
<td>36.1 ± 10.1</td>
<td>18.9 ± 10.4</td>
</tr>
<tr>
<td>Glucose, mmol/L</td>
<td>1.71 ± 0.20</td>
<td>0.86% Met diet</td>
<td>0.52% Met diet</td>
<td>0.17% Met diet</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>1.29 ± 0.30</td>
<td>1.22 ± 0.17</td>
<td>1.44 ± 0.34</td>
<td>1.19 ± 0.19</td>
</tr>
</tbody>
</table>

1 Values are means ± SDs, n = 11 or 12. Within the exercise or sedentary groups, labeled means without a common letter differ, P < 0.05.*Different from corresponding sedentary group, P < 0.05. CTX-1, C-terminal telopeptide of type 1 collagen; Ex, endurance exercise intervention; IGF-1, insulin-like growth factor 1; 0.17% Met, 0.17% methionine diet group; 0.86% Met, 0.86% methionine diet group; 0.52% Met, 0.52% methionine diet group; 0.17% Met-Ex, 0.17% methionine diet and exercise group; 0.86% Met-Ex, 0.86% methionine diet and exercise group.

2 Main effect of Met: for CTX-1, insulin, IGF-1, and glucose, the 0.17% Met–fed rats differed from rats fed the other 2 diets, P < 0.05. Within the exercise or sedentary groups, labeled means without a common letter differ, P < 0.05. For TGs, the 0.17% Met–fed rats differed from 0.86% Met–fed rats, P < 0.05; for TGs, the 0.17% Met–fed rats differed from 0.52% Met–fed rats, P < 0.05.

### FIGURE 3 Histomorphometry of secondary spongiosa in young male rats fed diets containing 0.86%, 0.52%, and 0.17% Met with or without exercise training for 8 wk. Architectural indices including BV/TV (A), Tb.Th (B), Tb.Sp (C), Tb.N (D), SmI (E), and Conn.Dn (F) were acquired from μCT analyses in the distal metaphysis of the femur. Values are means ± SDs, n = 12. Labeled dietary groups without a common letter had overall means that differ, P < 0.05. Within the exercise or sedentary groups, labeled means without a common letter differ, P < 0.05. *Different from corresponding sedentary group, P < 0.05. BV/TV, bone volume over total volume ratio; Conn.Dn, connectivity density; Ex, endurance exercise intervention; SmI, structure model index; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; μCT, micro-computed tomography.
smaller dimension (e.g., cross-sectional area, CSMI indices) and bone mineral accretion (e.g., cortical BMC) (Table 4). On the other hand, exercise caused a minor, but significant, down-regulation of bone mineral apposition (e.g., MAR) and size-related measurements. Similar findings have been reported for other nutritional manipulation studies (e.g., CR and PR) (14–16) as well as endurance interventions (16,22). Because BW is known to be associated with bone size, we further conducted a Pearson’s correlation between BW and various size-related indices. In the 10-d and 8-wk studies, long bone cross-sectional measurements and BMC indices consistently correlated with BW (Supplemental Table 6 and Supplemental Table 7). Furthermore, the lack of change in cortical BMD (Table 4) among groups suggests that the MR diet and endurance exercise did not compromise cortical bone mineralization quality. The smaller bones in MR-fed or exercise-trained rats are more likely the result of a body-size–related phenomenon.

During development, body mass and bone mass/size are influenced by endocrinal and nutritional factors. Among the multiple endocrinal factors associated with growth, IGF-1 plays a critical role in body size (41) and bone size/mass (42). Insulin and leptin are also correlated with linear bone growth and bone mass (43,44). Thus, the lower IGF-1, insulin, or leptin concentrations observed in MR and exercise rats could be the major

<table>
<thead>
<tr>
<th>Metaphysis</th>
<th>0.86% Met</th>
<th>0.86% Met-Ex</th>
<th>0.52% Met</th>
<th>0.52% Met-Ex</th>
<th>0.17% Met</th>
<th>0.17% Met-Ex</th>
<th>P (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS/BS, %</td>
<td>50.5 ± 4.5</td>
<td>52.6 ± 7.7</td>
<td>50.8 ± 6.6</td>
<td>53.2 ± 7.6</td>
<td>51.6 ± 6.7</td>
<td>46.8 ± 7.7</td>
<td>0.35 0.95 0.16</td>
</tr>
<tr>
<td>MAR, μm/d</td>
<td>2.55 ± 0.60</td>
<td>2.29 ± 0.28</td>
<td>2.46 ± 0.45</td>
<td>2.28 ± 0.31</td>
<td>2.23 ± 0.26</td>
<td>2.00 ± 0.28</td>
<td>0.02 0.02 0.94</td>
</tr>
<tr>
<td>BFR/BS, μm²/μm² · d</td>
<td>1.29 ± 0.35</td>
<td>1.20 ± 0.18</td>
<td>1.24 ± 0.27</td>
<td>1.21 ± 0.23</td>
<td>1.15 ± 0.21</td>
<td>0.94 ± 0.28</td>
<td>0.02 0.08 0.51</td>
</tr>
<tr>
<td>N,μc/BS, 1/mm</td>
<td>2.81 ± 1.75</td>
<td>2.42 ± 0.98</td>
<td>3.07 ± 1.12</td>
<td>2.83 ± 1.10</td>
<td>4.48 ± 2.44</td>
<td>2.72 ± 0.75</td>
<td>0.08 0.03 0.17</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Periosteum</th>
<th>0.86% Met</th>
<th>0.86% Met-Ex</th>
<th>0.52% Met</th>
<th>0.52% Met-Ex</th>
<th>0.17% Met</th>
<th>0.17% Met-Ex</th>
<th>P (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS/BS, %</td>
<td>65.2 ± 15.3</td>
<td>72.5 ± 14.7</td>
<td>68.6 ± 16.6</td>
<td>74.6 ± 18.2</td>
<td>41.3 ± 16.2</td>
<td>28.9 ± 15.3</td>
<td>&lt;0.01 0.94 0.07</td>
</tr>
<tr>
<td>MAR, μm/d</td>
<td>3.24 ± 0.33</td>
<td>2.51 ± 0.88</td>
<td>2.95 ± 0.43</td>
<td>2.81 ± 0.50</td>
<td>2.28 ± 1.25</td>
<td>1.79 ± 0.89</td>
<td>&lt;0.01 0.02 0.41</td>
</tr>
<tr>
<td>BFR/BS, μm²/μm² · d</td>
<td>2.11 ± 0.53</td>
<td>1.89 ± 0.78</td>
<td>2.03 ± 0.61</td>
<td>2.11 ± 0.61</td>
<td>1.03 ± 0.68</td>
<td>0.55 ± 0.40</td>
<td>&lt;0.01 0.15 0.29</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Endosteum</th>
<th>0.86% Met</th>
<th>0.86% Met-Ex</th>
<th>0.52% Met</th>
<th>0.52% Met-Ex</th>
<th>0.17% Met</th>
<th>0.17% Met-Ex</th>
<th>P (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS/BS, %</td>
<td>33.3 ± 9.3</td>
<td>27.7 ± 10.8</td>
<td>25.7 ± 9.6</td>
<td>32.2 ± 13.9</td>
<td>33.0 ± 10.7</td>
<td>34.7 ± 15.4</td>
<td>0.37 0.77 0.25</td>
</tr>
<tr>
<td>MAR, μm/d</td>
<td>2.75 ± 0.96</td>
<td>1.82 ± 0.74</td>
<td>2.43 ± 0.74</td>
<td>1.77 ± 1.21</td>
<td>2.27 ± 1.13</td>
<td>2.12 ± 0.81</td>
<td>0.80 0.02 0.37</td>
</tr>
<tr>
<td>BFR/BS, μm²/μm² · d</td>
<td>0.97 ± 0.38</td>
<td>0.51 ± 0.35</td>
<td>0.59 ± 0.18</td>
<td>0.68 ± 0.47</td>
<td>0.80 ± 0.42</td>
<td>0.68 ± 0.30</td>
<td>0.51 0.06 0.08</td>
</tr>
</tbody>
</table>

1 Values are means ± SDs, n = 10–12. BFR/BS, bone formation rate per bone surface; Ex, endurance exercise intervention; MAR, mineral apposition rate; MS/BS, bone mineralization over bone surface; N,μc/BS, number of osteoclasts relative to the trabecular tissue perimeter; 0.17% Met, 0.17% methionine diet group; 0.17% Met-Ex, 0.17% methionine diet and exercise group; 0.52% Met, 0.52% methionine diet group; 0.52% Met-Ex, 0.52% methionine diet and exercise group; 0.86% Met, 0.86% methionine diet group; 0.86% Met-Ex, 0.86% methionine diet and exercise group.

2 Main effect of Met: the 0.17% Met-fed rats differed from rats fed the other 2 diets, P < 0.05.

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factors contributing to the observed smaller body and bone size, and therefore the lower extrinsic bone strength.

In contrast to the absolutely smaller bone size and lower extrinsic bone strength, the results of biomechanical analyses indicated that 8 wk of MR and endurance exercise interventions tended to benefit bone material by enhancing the intrinsic biomechanical properties. It has been previously reported that dietary manipulations (e.g., CR or PR) and endurance exercise by CR or PR diets disappeared, and bones of exercise rats showed higher toughness (22,45,46). Taken together, these dietary manipulations (e.g., CR or PR) and endurance exercise interventions and therefore the lower extrinsic bone strength.

In this study, we did not observe dose-response effects on bone properties or serum markers in rats in the 0.52% Met and 0.86% Met groups. Interestingly, compared with the 0.86% Met diet, the 0.52% Met diet is capable of mitigating mitochondrial oxidative stress in the liver and brain without compromising the whole-body growth of Wistar rats (47,48). This suggests that a 40% MR (0.52% Met diet) could be sufficient to generate some beneficial effects at the cellular level, but more robust MR effects are mostly seen with the feeding of 0.17% Met–containing diet. An MR diet has been already translated to humans (35). In fact, fat oxidation significantly increased in obese adults who were administered a diet in which Met was limited to 2 mg/(kg·d) (49). A potential natural strategy to achieve MR could be the consumption of vegetarian or vegan diets because plant protein contains less Met than does animal protein (4,5). Indeed, vegetarian and vegan diets are 38–47% lower in dietary Met content (50). Although it has been suggested that low-Met-containing vegetarian/vegan diets could be beneficial to slowing down aging (4,51), users should consume a vitamin B-12 supplement, because this nutrient is lacking in plant food.

![Image](https://academic.oup.com/jn/article-abstract/144/5/621/4578275)

**FIGURE 5** Bone biomechanical properties of femora in young male rats fed diets containing 0.86%, 0.52%, and 0.17% Met with or without exercise training for 8 wk. Extrinsic biomechanical properties: yield load (A), maximal load (B), yield load energy (C), maximal load energy (D), and stiffness (F). Indices of intrinsic biomechanical properties: yield stress (F), yield toughness (G), and elastic modulus (H). Values are means ± SDs, n = 12. Labeled dietary groups without a common letter had overall means that differ, P < 0.05. Within the exercise or sedentary groups, labeled means without a common letter differ, P < 0.05. *Different from corresponding sedentary group, P < 0.05. Ex, endurance exercise intervention; GPa, gigapascal; MPa, megapascal.

**TABLE 4** Densitometry and cross-sectional measurements in bones of young male rats fed diets containing 0.86%, 0.52%, and 0.17% Met with or without exercise training for 8 wk.

<table>
<thead>
<tr>
<th>0.86% Met diet</th>
<th>0.52% Met diet</th>
<th>0.17% Met diet</th>
<th>P (ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.86% Met</td>
<td>0.86% Met-Ex</td>
<td>0.52% Met</td>
<td>0.52% Met-Ex</td>
</tr>
<tr>
<td>Total BMD, g/cm³</td>
<td>0.927 ± 0.014</td>
<td>0.928 ± 0.018</td>
<td>0.941 ± 0.014</td>
</tr>
<tr>
<td>Total BMC, mg</td>
<td>492 ± 46</td>
<td>471 ± 37</td>
<td>509 ± 46</td>
</tr>
<tr>
<td>Cortical BMD, g/cm³</td>
<td>1.215 ± 0.022</td>
<td>1.216 ± 0.015</td>
<td>1.217 ± 0.020</td>
</tr>
<tr>
<td>Cortical BMC, mg</td>
<td>10.7 ± 0.9</td>
<td>9.7 ± 0.6</td>
<td>10.8 ± 0.8</td>
</tr>
<tr>
<td>Spongy BMD, g/cm³</td>
<td>0.612 ± 0.019</td>
<td>0.626 ± 0.027</td>
<td>0.622 ± 0.024</td>
</tr>
<tr>
<td>Spongy BMC, mg</td>
<td>10.3 ± 2.2</td>
<td>10.8 ± 2.3</td>
<td>9.9 ± 2.0</td>
</tr>
<tr>
<td>Cortical area, mm²</td>
<td>8.8 ± 0.8</td>
<td>7.9 ± 0.5</td>
<td>8.9 ± 0.6</td>
</tr>
<tr>
<td>CSMI&lt;sub&gt;max&lt;/sub&gt;, mm&lt;sup&gt;4&lt;/sup&gt;</td>
<td>29.6 ± 4.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.6 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>28.6 ± 3.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CSMI&lt;sub&gt;min&lt;/sub&gt;, mm&lt;sup&gt;4&lt;/sup&gt;</td>
<td>18.9 ± 2.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.3 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.6 ± 2.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CSMP&lt;sub&gt;max&lt;/sub&gt;, mm&lt;sup&gt;4&lt;/sup&gt;</td>
<td>10.8 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.3 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.0 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

1 Values are means ± SDs, n = 11 or 12. Within the exercise or sedentary groups, labeled means without a common letter differ, P < 0.05. *Different from corresponding sedentary group, P < 0.05. **Different from corresponding Met group, P < 0.05. 

P Ex, exercise intervention; Met<sup>2</sup>, Met<sup>2</sup> methionine diet; Met<sup>2</sup>-Ex, Met<sup>2</sup> methionine diet and exercise group; Met<sup>2</sup>, Met<sup>2</sup> methionine diet group; Met<sup>2</sup>-Ex, Met<sup>2</sup> methionine diet and exercise group; Met<sup>2</sup>, Met<sup>2</sup> methionine diet group; Met<sup>2</sup>-Ex, Met<sup>2</sup> methionine diet and exercise group.

2 Main effect of Met: the 0.17% Met–fed rats differed from rats fed the other 2 diets, P < 0.05.
In conclusion, MR and endurance exercise downregulated bone and energy metabolic indices, bone size, and/or extrinsic bone strength in growing rats, which appears to be part of an adaptive response to changes in energy metabolism in the entire organism. In contrast, MR and endurance exercise did not compromise intrinsic bone biomechanical properties. There is the possibility that MR and endurance exercise could reduce bone aging by slowing bone turnover and enhancing energy metabolism. Future studies will be therefore geared toward investigating the long-term effects of MR in mature and aging bone.

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