Bone tissues are physiologically exposed to mechanical loading such as physical activity or exercise, which contributes to development in growing bone (1, 2). On the other hand, there is a high incidence of stress fractures in young athletes when the bone is subjected to high-impact mechanical loading (3, 4). For example, the majority of stress fractures occur during the growing phase in 13–15-y-old athletes (4). In female athletes, in particular endurance athletes, the female athlete triad, i.e., low energy availability, and functional hypothalamic osteoporosis amenorrhea, is well known to be the main cause of inducing serious bone fragility (5). Energy availability is defined as dietary energy intake minus exercise energy expenditure (6). Low energy availability is caused by insufficient food intake and excessive exercise energy expenditure. Similarly, a previous study reported that male endurance athletes suffered from low bone mineral density (BMD) accompanied by low energy availability (7).

Exercise training, manifested as running exercise, increases cortical thickness in growing bone (8). Mechanical loading-induced bone alteration is achieved by an increase of bone formation and a decrease of bone resorption (9). Moreover, trabecular bone thickness and trabecular bone number increase are induced by exercise (10, 11).

Energy balance and hormonal levels are tightly linked, and alterations in energy and endocrine axes lead to altered feedback and feed-forward in the hypothalamic-pituitary-gonadal (HPG) axis (12). The energy deficit is disrupted to attenuate the pulsatile luteinizing hormone (LH) through low gonadotropin-releasing hormone (GnRH) secretion (13). Low GnRH/LH pulse release

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**Summary** The pathogenesis of bone disorders in young male athletes has not been well understood. We hypothesized that bone fragility is caused by low energy availability, due to insufficient food intake and excessive exercise energy expenditure in young male athletes. To examine this hypothesis, we investigated the influence of food restriction on bone strength and bone morphology in exercised growing male rats, using three-point bending test, dual-energy X-ray absorptiometry, and micro-computed tomography. Four-week-old male Sprague-Dawley rats were divided randomly into the following groups: the control (Con) group, exercise (Ex) group, food restriction (R) group, and food restriction plus exercise (REx) group after a 1-wk acclimatization period. Thirty-percent food restriction in the R and REx groups was carried out in comparison with that in the Con group. Voluntary running exercise was performed in the Ex and REx groups. The experimental period lasted 13 wk. At the endpoint of this experiment, the bone strength of the femurs and tibial BMD in the REx group were significantly lower than those in the Con group. Moreover, trabecular bone volume and cortical bone volume in the REx group were also significantly lower than those in the Con group. These findings indicate that food restriction causes low bone strength and microarchitectural deterioration in exercised growing male rats.

**Key Words** low energy availability, voluntary running exercise, bone strength, bone morphology, bone mineral density
decreases androgen secretion by the testis (12). Androgen deficiency leads to high bone turnover, whereby cortical and trabecular bone loss occurs in growing male mice (14).

Food restriction may have a harmful effect on bone growth under exercise training. A previous study reported that food restriction caused low bone strength and short bone length accompanied by a decreased bone formation and an increased bone resorption, and it decreased cortical and trabecular bone volume during rapid skeletal growth (15). Moreover, it caused abnormal levels of hormones (e.g. androgen) essential for skeletal acquisition (12). However, few studies have examined the influence of a combination of food restriction and exercise training on bone strength and morphology using male animals (16), although many studies have examined either the effect of food restriction on bone tissue (15, 17), or the effect of mechanical loading accompanied with physical activity on bone tissue (8, 18). We reported that the BMD and the bone architecture had been little affected by food restriction combined with exercise training in mature male rats (19). However, it is unknown whether these phenomena are caused by the same conditions in the growing phase. In this study, we hypothesize that low energy availability, due to food restriction, and excessive exercise energy expenditure cause low bone strength and microarchitectural deterioration in the trabecular and cortical bone in growing bones, in spite of the bone tissues being exposed to mechanical loading. To examine this hypothesis, we investigated the influence of food restriction on bone strength and bone morphology in exercised growing male rats.

METHODS

Experimental animals and protocol. Male Sprague-Dawley rats (n=28, 4 wk old) were purchased from CLEA Experimental Animals Supply Co. Ltd. (Tokyo, Japan) and cared for according to the “Guiding Principles for the Care and Use of Animals.” The rats were randomly divided into four experimental groups after a 1-wk acclimatization period: Con (control group; n=7, ad-libitum-fed rats, sedentary), Ex (exercise group; n=7, ad-libitum-fed rats with voluntary running exercise), R (food restriction group; n=7. 30% food-restricted rats, sedentary), REx (food restriction and exercise group; n=7. 30% food-restricted rats with voluntary running exercise). The proximal tibia and diaphysial tibia were assessed by dual energy X-ray absorptiometry (DXA). Bone mineral density (BMD) of the tibia was assessed by dual energy X-ray absorptiometry (DXA; Aloka, DCS-600R, Tokyo, Japan) as previously described (23). The tibia was divided into five divisions, and the first division was considered to be the proximal metaphysis site. The BMD of the tibia at the diaphysis site contains mainly cortical bone. The second to third divisions were considered to be the diaphysis site.

Trabecular and cortical bone microarchitecture by micro-computed tomography (µCT). Assessment of bone microarchitecture was performed by high-resolution micro-computed tomography (µCT: inspeXio SMX-90CT, Shimadzu, Tokyo, Japan), as described previously (24). The proximal tibia and diaphysial tibia were
scanned using an X-ray energy of 70 keV, an integration time of 0.12 s, and a voxel size of 0.025 mm/pix. Three-dimensional (3D) reconstruction of mineralized tissue was performed using the TRI-BONE system (Ratoc System Engineering, Co., Ltd., Tokyo, Japan). For the trabecular bone region in the proximal tibia, we assessed the trabecular bone volume (BV/TV, %), trabecular thickness (Tb.th, μm), trabecular number (Tb.N, N/mm), trabecular separation (Tb.sp, mm), trabecular bone pattern factor (TBPf), and structure model index (SMI). The SMI indicates whether the structure is plate-like or rod-like. Perfect plates have an SMI of 0, and round spheres have an SMI of 4. We also assessed the cortical bone in the proximal tibia and diaphyseal tibia by determining the cortical bone volume (CV, mm³), total bone volume (TV, mm³), cortical bone ratio (CV/TV, %), and cortical thickness (Cb.th, μm).

**Bone histomorphometry to evaluate bone turnover.** Dynamic histomorphometry was assessed as previously described (25). To examine the bone formation rate, calcine labels (8 mg/kg) were injected into the shoulder at 6 and 3 d prior to dissection. Histomorphometric measurements were assessed on the secondary spongiosa of the proximal tibia. For dynamic histomorphometry, the mineral apposition rate (MAR, μm/d) was measured in unstained sections under ultraviolet (UV) light and used to calculate the bone formation rate with a surface referent (BFR, μm²/μm²/year).

**Free testosterone and dihydrotestosterone (DHT) levels.** Serum levels of free testosterone and DHT were determined using sandwich enzyme immunoassay kits (Free Testosterone; IBL International, Hamburg, Germany/DHT; IBL International). These assays were performed in duplicate.

**Statistical analysis.** All data were expressed as the mean ± standard error (SE) and were analyzed with SPSS (version 18.0 J; SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was used to test for statistically significant differences among groups. If a significant difference was detected among groups, these groups were further evaluated using the post-hoc Turkey test. The significance level for major effects was set...
Association bone strength and cortical bone morphology were determined using the Pearson correlation test. The significance level for major effects was set at $p<0.05$.

**RESULTS**

**Body weight, femoral length, running distance, food intake, energy availability, and hormonal levels**

We examined the influence of food restriction on bone strength and bone morphology in exercised growing male rats. Voluntary running training and food restriction were started when the rats reached 5 wk of age. The body weight in the R and REx groups rose more slowly than that in the Con group (Fig. 1A). The rats were fed under alternate-day feeding, whereas an age-matched ad-libitum cohort served as the Con group (Fig. 1B, Table 1). After 13 wk, rats in the R group weighed 29.4 ± 1.4% less, and those in the REx group weighed 53.2 ± 2.0% less, than those in the age-matched Con group. The body weight gain in the REx group was also

| Table 1. Body weight, fat weight, femoral length, running distance, and hormonal levels. |

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>Ex</th>
<th>R</th>
<th>REx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>532.9 ± 16.8</td>
<td>476.0 ± 11.7*</td>
<td>375.0 ± 5.5***</td>
<td>248.6 ± 11.0***‡‡‡†††</td>
</tr>
<tr>
<td>Body weight gain (g/d)</td>
<td>5.9 ± 0.2</td>
<td>5.1 ± 0.2*</td>
<td>3.8 ± 0.1***</td>
<td>3.1 ± 0.8***‡‡‡‡‡</td>
</tr>
<tr>
<td>Abdominal fat weight (g)</td>
<td>25.0 ± 1.2</td>
<td>16.3 ± 0.6</td>
<td>11.6 ± 0.5***</td>
<td>1.4 ± 0.1***‡‡‡‡‡</td>
</tr>
<tr>
<td>Femoral length (cm)</td>
<td>4.08 ± 0.02</td>
<td>4.03 ± 0.01</td>
<td>3.84 ± 0.01***</td>
<td>3.54 ± 0.02***‡‡‡‡‡</td>
</tr>
<tr>
<td>Running distance (km/d)</td>
<td>—</td>
<td>3.6 ± 0.9</td>
<td>—</td>
<td>11.5 ± 1.2‡‡‡</td>
</tr>
<tr>
<td>Food intake (g/d)</td>
<td>21.7 ± 0.5</td>
<td>21.7 ± 0.4</td>
<td>15.5 ± 0.1***</td>
<td>15.6 ± 0.0***</td>
</tr>
<tr>
<td>Energy availability (kcal)</td>
<td>89.2 ± 1.9</td>
<td>83.5 ± 2.1</td>
<td>63.9 ± 0.2***‡‡‡</td>
<td>53.3 ± 1.4***‡‡‡‡‡</td>
</tr>
<tr>
<td>Free testosterone (pg/mL)</td>
<td>14.5 ± 6.2</td>
<td>17.8 ± 9.0</td>
<td>8.7 ± 3.6</td>
<td>5.0 ± 2.1</td>
</tr>
<tr>
<td>Dihydrotestosterone (pg/mL)</td>
<td>606.8 ± 156.7</td>
<td>647.7 ± 250.9</td>
<td>409.6 ± 140.9</td>
<td>346.5 ± 77.7</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE.

* $p<0.05$, *** $p<0.001$ vs. Con group, ‡‡‡ $p<0.001$ vs. Ex group, ††† $p<0.001$ vs. R group.

Rats ($n=28$) were divided randomly into four experimental groups: Con (control group; $n=7$), Ex (exercise group; $n=7$), R (restriction group; $n=7$), REx (restriction and exercise group; $n=7$).

**Fig. 2.** Biomechanical properties of bone. Breaking force (A) and breaking energy (B) of the femoral diaphysis were determined by the three-point bending test. Food restriction decreased bone strength, although bone tissues were exposed to mechanical loading by running exercise. Values are mean ± SE. * $p<0.05$, *** $p<0.001$ vs. Con group. † $p<0.05$ vs. R group. ‡‡‡ $p<0.001$ vs. Ex group.

**Fig. 3.** BMD at tibia. BMD at the proximal tibia (A), and BMD at the diaphyseal tibia (B). BMD was measured by dual energy X-ray absorptiometry. Food restriction decreased BMD, although bone tissues were exposed to mechanical loading by running exercise. Values are mean ± SE. *** $p<0.001$ vs. Con group, † $p<0.05$, ††† $p<0.001$ vs. R group, ‡‡‡ $p<0.001$ vs. Ex group.
lower than that in the Con group (Table 1).

The femoral length in the REx group was lower than that in the Con group (Table 1).

The energy availability in the REx group was lower than that in the Con group (Table 1).

Running distance in the REx group was higher than that in the Ex group (Table 1).

Hormonal levels (free testosterone and dihydrotestosterone) in the REx group tended to be lower than these in the Con group (Table 1).

Mechanical bone strength of the femoral diaphysis, BMD of the proximal and diaphysial tibia

Assessment of the bone strength of the femoral diaphysis was measured by the three-point bending test. The bone strengths of the femoral diaphysis in the R group and REx group were significantly lower than that in the Con group (Fig. 2A and B). The BMD at the proximal and diaphysial tibia in the R group were significantly lower than that in the Con group (Fig. 2A and B). The BMD at the proximal tibia and the BMD at the diaphysial tibia in the R group were significantly lower than that in the Con group (Table 1).

Table 2. Bone morphology and bone turnover: Trabecular bone at proximal tibia.

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>Ex</th>
<th>R</th>
<th>REx</th>
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</thead>
<tbody>
<tr>
<td>Bone volume/Tissue volume (%)</td>
<td>12.0±1.6</td>
<td>14.4±0.5</td>
<td>10.6±0.5</td>
<td>8.5±0.4***‡‡‡</td>
</tr>
<tr>
<td>Trabecular thickness (mm)</td>
<td>74.2±3.2</td>
<td>70.9±1.6</td>
<td>70.9±1.6</td>
<td>69.4±2.9</td>
</tr>
<tr>
<td>Trabecular number (N/mm)</td>
<td>1.7±0.2</td>
<td>1.5±0.1</td>
<td>1.5±0.1</td>
<td>1.2±0.0***</td>
</tr>
<tr>
<td>Trabecular separation (µm)</td>
<td>585.6±94.8</td>
<td>609.0±44.3</td>
<td>609.0±44.3</td>
<td>753.0±28.4**‡‡‡</td>
</tr>
<tr>
<td>SMI</td>
<td>2.4±0.1</td>
<td>2.4±0.1</td>
<td>2.4±0.1</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>TBPf (1/mm)</td>
<td>6.9±0.7</td>
<td>6.6±0.5</td>
<td>6.6±0.5</td>
<td>6.6±1.1</td>
</tr>
<tr>
<td>MAR (µm/d)</td>
<td>2.8±0.1</td>
<td>2.6±0.1</td>
<td>2.1±0.4</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>BFR/BS (mm³/mm²/y)</td>
<td>0.4±0.0</td>
<td>0.4±0.0</td>
<td>0.3±0.1</td>
<td>0.3±0.0</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE.

*p<0.05 vs. Con group, †††p<0.001 vs. Ex group.

Rats (n=28) were divided randomly into four experimental groups: Con (control group; n=7), Ex (exercise group; n=7), R (restriction group; n=7), REx (restriction and exercise group; n=7).

Table 3. Bone morphology: Cortical bone at proximal tibia and diaphysial tibia.

<table>
<thead>
<tr>
<th></th>
<th>Con</th>
<th>Ex</th>
<th>R</th>
<th>REx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical bone volume (mm³)</td>
<td>15.6±0.6</td>
<td>15.0±0.5</td>
<td>12.7±0.2***</td>
<td>9.8±0.3***††††††</td>
</tr>
<tr>
<td>Total bone volume (mm³)</td>
<td>43.9±1.2</td>
<td>42.8±2.5</td>
<td>36.0±0.7**</td>
<td>27.2±1.3***††††</td>
</tr>
<tr>
<td>Cortical bone ratio (%)</td>
<td>35.6±1.0</td>
<td>35.4±1.1</td>
<td>35.4±0.7</td>
<td>36.2±1.3</td>
</tr>
<tr>
<td>Cortical bone thickness (µm)</td>
<td>441.9±14.1</td>
<td>429.6±9.0</td>
<td>396.3±9.7*</td>
<td>336.6±12.7***‡‡‡</td>
</tr>
<tr>
<td>Diaphyseal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical bone volume (mm³)</td>
<td>11.0±0.2</td>
<td>11.9±0.3</td>
<td>9.7±0.2**</td>
<td>8.3±0.2***‡‡‡‡</td>
</tr>
<tr>
<td>Total bone volume (mm³)</td>
<td>16.1±0.4</td>
<td>17.0±0.7</td>
<td>13.9±0.3**</td>
<td>11.8±0.3***‡‡‡‡</td>
</tr>
<tr>
<td>Cortical bone ratio (%)</td>
<td>68.5±0.8</td>
<td>70.5±1.4</td>
<td>70.0±1.2</td>
<td>70.2±0.6</td>
</tr>
<tr>
<td>Cortical bone thickness (µm)</td>
<td>664.3±9.8</td>
<td>711.2±13.0</td>
<td>636.5±14.5</td>
<td>587.9±10.1***‡‡‡</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE.

*p<0.05, **p<0.01, ***p<0.001 vs. Con group, †††p<0.001 vs. Ex group, ††p<0.01, †‡p<0.001 vs. R group.

Rats (n=28) were divided randomly into four experimental groups: Con (control group; n=7), Ex (exercise group; n=7), R (restriction group; n=7), REx (restriction and exercise group; n=7).
and REx group were significantly lower than those in the Con group (Fig. 3A and B).

Bone morphology, and histomorphometry to evaluate bone turnover: Trabecular bone at the proximal tibia

3D micro-CT images of the proximal tibia in the REx group showed bone loss (Fig. 4A and B). Trabecular bone volume (BV/TV) in the REx group was significantly lower than that in the Con group. However, detailed microstructure analysis of trabecular bone, namely the trabecular bone number, thickness, separation, SMI, and TBPF, were no different among the 4 groups (Table 2).

The mineral apposition rate (MAR) and bone formation rate (BFR/BS) in the REx group tended to be lower than those in the Con group (Table 2).

Bone morphology: Cortical bone at the proximal tibia and diaphysial tibia

Cortical bone volume, total bone volume, and cortical thickness at the proximal tibia in the REx group were significantly lower than those in the Con group (Table 3). 3D micro-CT images of diaphysial tibia in the REx group were thinner than those in the other groups (Fig. 4C). Cortical bone volume, all bone volume, and cortical thickness in the REx group were significantly lower than those in the Con group (Table 3).

Correlation between bone strength and cortical bone morphology

There was a significant positive correlation between the breaking force at the femurs and the cortical thickness at the diaphysial tibia, and between the breaking force at the femurs and the cortical bone volume at the diaphysial tibia (Fig. 5A and B). In addition, there was a significant positive correlation between the breaking energy at the femurs and the cortical thickness at the diaphysial tibia, and between the breaking energy at the femurs and the cortical bone volume at the diaphysial tibia (Fig. 5C and D).

DISCUSSION

In this study, we investigated the influence of food restriction on bone strength, BMD, and bone morphology in exercised growing male rats. The REx group showed low bone strength, and trabecular and cortical bone volume loss compared with the Con group. The REx group experienced a low food intake and high running distance, leading to lower energy availability than in the other groups. These findings indicate that low energy availability caused low bone strength and microarchitectural deterioration in exercised growing male rats.

We observed low BMD and cortical bone loss in the REx group compared with the Con group. Moreover, bone strength was significantly correlated with cortical bone volume and cortical thickness. These results showed that the bone likely become more fragile due to food restriction. In general, cortical thickness or volume is increased by mechanical loading (8, 26). Our results suggest that food restriction decreases bone strength due to cortical bone loss, although mechanical loading adds to bone strength.

There were no significant differences in histomorphometric parameters among the groups, in spite of trabecular bone volume (BV/TV) in the REx group being lower than that in the Con group. A previous study had shown that short-term caloric restriction caused alteration in bone turnover by inducing low bone formation, thereby causing trabecular bone loss (15). In contrast, long-term caloric restriction had not led to a drastic decrease in bone formation rate compared with an ad-libitum food intake group (27). Although the mechanism of the alteration in bone turnover by food restriction is unclear,
the bone turnover was likely to be altered against bone loss during 13-wk-experimental period in this study. Thus, it may be possible that the experimental period of this investigation was long-term, as in the previous study (15). More studies are required to investigate the influence of low energy availability on bone turnover according to time-course, because the significant difference in histomorphometric parameters among groups might be able to be observed only in the early stage of this experiment.

On the other hand, trabecular bone loss did not occur in the R group. The energy availability level in the REx group decreased more than that in the R group, because the energy consumption of the REx group was larger than that of the R group. Low energy availability involves the endocrine system and decreases androgen levels (15). Low androgen levels are known to affect bone metabolism and cause bone fragility (14). Actually, severe food restriction tends to remarkably lower bone mineral content in distal femurs, which mostly consist of trabecular bone in rats (28). Therefore, these data imply that low energy availability caused trabecular bone loss even though the REx group underwent exercise training.

Free testosterone and DHT levels were not statistically lower in the REx group, even though BMD decreased. Androgen production by the testis is mediated through the HPG-axis and exerts bone-protective effects (29). For example, androgen directly binds to androgen receptor on osteoclasts and blocks bone resorption in human, mouse, and avian osteoclasts in vitro (30). Actually, a previous study reported that low androgen levels led to decreased BMD in growing male mice (14). In contrast, long-term food restriction revealed that the testosterone level did not change, although short-term food restriction decreased the testosterone level in male rats (31). These studies are likely to suggest that the androgen secretion system adapts to food restriction. Thus, our results also may imply that the androgen production system has adapted to the low energy availability condition during long-term experimental period, because the experimental period was longer than that in the previous study.

The running distance in the REx group was higher than that in the Ex group. This result may have been elicited by the activity-stress paradigm in food-restrictive conditions, namely the phenomenon that the activity level is increased by a starvation state (32). This phenomenon has been considered as "activity-based or anxiety based anorexia" (33). Thus, the running distance in REx group was likely to be explainable in term of the activity-stress paradigm brought on by food restriction.

The femoral bone length was significantly decreased in the REx group compared with that in the other groups. A previous study reported that food restriction caused a decrease in the femoral length in growing male mice (15). Therefore, the result also suggested that low energy availability prevented skeletal growth in the growing male rats.

The growing male rats might be susceptible by low energy availability compared with mature male rats. In this study, low energy availability caused low BMD and microarchitectural deterioration in the growing male rats. In contrast, we revealed that the BMD and bone architecture had been little affected by low energy availability in mature male rats (19). Therefore, low energy availability is likely to greatly influence the bone tissue in growing male rats.

There are some limitations to this study. First, it is unclear how the energy availability level caused bone fragility, because the running distance and energy availability levels were different among the groups although food restriction leads to severe bone loss in exercised male rats. Second, it is unknown whether the bone fragility in the REx group was influenced by low energy availability, deficiency in another nutrient (e.g. calcium, phosphorus), or massive running exercise accompanied by high mechanical loading. Third, the energy expenditure in the Con and R groups in single cage was unclear, because these group were regarded as sedentary groups.

In summary, food restriction caused low bone strength and microarchitectural deterioration in exercised growing male rats. The results may suggest that the low energy availability condition caused bone fragility in males in the growing phase.

Conflict of interest
None.

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