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ORIGINAL ARTICLE

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Endurance treadmill running training benefits the biomaterial quality of bone in growing male Wistar rats

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Abstract This study investigated the effects of endurance running training on the bones of growing rats. Thirty-two male Wistar rats (7 weeks old) were assigned to a sedentary control group (CON, $n = 10$), a continuous endurance running group (CEN, $n = 10$), or an intermittent endurance running group (IEN, $n = 12$). After an 8-week training period, both exercise groups had significantly less body weight (BW) gain but higher aerobic capacity, shown by increased muscle citrate synthase (CS) activity. Bone area (BA), areal bone mineral density (aBMD), and bone mineral content (BMC) were measured by dual-energy X-ray absorptiometry (DXA) in the total femur and sections of femora. Except for showing a significantly higher aBMD in total femora, the CON group was only slightly and nonsignificantly higher in other DXA measurements. In tissue weight measurements, the CON group showed a nonsignificantly higher tissue dry weight ($P = 0.146$), but a significantly lower tissue water content ratio (WCR, %) as compared to the exercise group. Despite having nonsignificantly lower long bone cross-sectional parameters, both exercise groups showed significantly better biomaterial properties, as measured by a three-point bending test. In extrinsic analysis, femora of the two exercise groups showed no difference in bending load and stiffness, but were significantly higher in post-yield bending energy and total ultimate bending energy ($P < 0.05$). Similar phenomena were revealed in tissue-level measurements; the CEN and IEN groups were significantly higher in ultimate toughness and post-yield toughness ($P < 0.05$). Higher post-yield energy shown by two exercise groups implied a change in bone matrix organization. In conclusion, this study demonstrated that two endurance treadmill training modes benefit bone, with subjects showing better tissue biomaterial properties without significantly increasing aBMD, BMC, or bone dimension. Further study would be valuable to investigate the effects of endurance running on other components of bone, such as organization of bone matrix and its relationship with bone biomaterial properties.

Key words treadmill running · biomechanics· intermittent endurance · continuous endurance · bone mineral density

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Introduction

Regular exercise or physical activity is critical for bone development and health. Many animal models and various training modes, including treadmill running, jumping, free fall landing, passive resistant training, etc., have been used to investigate the effects and mechanisms of exercise [1–4]. However, a limited number of animal studies have compared the effects of these different exercise modes just mentioned. Notomi et al. showed that running exercise did not benefit bone mineral density (BMD), bone mineral content (BMC), or bone size in growing rats, perhaps because of its lower impact on regional bones [2]. Additionally, 8 or 10 weeks of endurance treadmill training did not significantly influence the BMD, BMC, or cross-sectional parameters of the midshaft femur in growing male Wistar rats [5,6]. Healthy endurance trainees (runners, cross-country skiers, swimmers) among human subjects also showed no

differences in BMD and BMC when compare body weight (BW)-matched control groups, and even had lower BMDs and BMCs than the overweight sedentary group, which was approximately 28% higher in BW [7,8]. Conventionally, the extrinsic parameters of BMD, BMC, and size-related measurements have been widely agreed upon as indicators of bone strength. The results from endurance treadmill training models and human studies implied that endurance running could not benefit bone development as much as the other models mentioned above. From the point of view of Frost's mechanostat [9], this might be caused by the relatively low local impact of endurance training on bone, as compared to exercises with higher loading, such as jumping or free fall landing [3,4]. For rats, free fall landing from heights of 30 cm and 60 cm has been shown to cause a 4- to 7-fold BW ground reaction force (GRF) on each hindfoot [4], whereas running with a trotting gait causes only 0.7- to 0.8-fold BW GRF loaded on a single hindlimb [10]. Although higher running speeds would induce higher GRFs, that might lead to an increased BMD and BMC. Limited evidence is available about the effects of different running speeds on growing bone.

Besides densitometry parameters, a growing number of reports suggest that bone with better quality was not necessarily associated with an absolutely higher BMD and/or BMC [11–14]. Relative (intrinsic) parameters at the tissue level, rather than absolutely extrinsic bone parameters, seem to be more closely related to real bone material properties. In the study comparing resistance and running training mentioned above [2], biomechanical properties were not measured at the tissue level. Indeed, moderate endurance training increased bone stress and bending toughness without increasing BMD, BMC, and bone size, suggesting that this exercise regimen could benefit bone by strengthening tissue properties without increasing size and/or bone mineral accumulation [5]. Furthermore, long-term intensive endurance training in dogs even caused a 10% BMD decrement, but no difference between exercise and non-exercise groups in the indentation stress required to fracture cancellous bone [11].

None of the previous studies compared the effects of different endurance running intensities to clarify their effects on growing bones. In the current study, we adopted either a high-intensity intermittent or a moderate continuous endurance training protocol to investigate the effects of different running intensities (speed) on BMD, BMC, and biomechanical properties of bones.

Materials and methods

Animals

Thirty-two male Wistar rats (3 weeks of age) were purchased and bred in the Laboratory Animal Center in National Taiwan University and kept under controlled conditions, which included a room temperature of 22° ± 1°C and a 12:12 h light–dark cycle. All the animals were fed with Purina Laboratory Rodent Diet (PMI, St. Louis, MO, USA, 0.95% calcium) and distilled water ad libitum. The BW of each animal was measured weekly. The exercise training programs were initiated when the rats reached an age of 7 weeks. During the training periods, all animals were healthy and without infection. The procedures of animal experiment throughout this study followed the APS's "Guiding Principles in the Care and Use of Animals."

Experimental design

Animals were BW matched and assigned to three groups: a continuous endurance running exercise group (CEN, $n = 10$), an intermittent endurance running exercise group (IEN, $n = 12$), and a sedentary control group (CON, $n = 10$).

Exercise training protocol

The training protocol for animals of the CEN group was developed in our previous study [5], which was modified from the ~70% $\dot{V}O_2$ max running protocol for male Wistar rats [15]. Animals in the CEN group underwent endurance running training on the treadmill 5 days per week for 8 weeks. For the CEN rats, the training program was started at a speed of 12 m/min on a level treadmill for 20 min/day and progressively increased to 22 m/min for 60 min/day during the 8-week period. Animals in the IEN group were trained with the same total distance, but the course was intermittent. The daily training repetitions (rep/day) were increased weekly from 1 to 9 rep/day, intensity (running speed, m/min) was progressively increased weekly from 12 to 30 m/min, and resting time between repetitions was raised from 1 to 2 min. Because rats are more active in darkness, the front portion of the treadmill lanes was covered with a thick paper to darken this area. At the rear of the lanes, an electric grid provided a stimulus for running. An electric stimulus (30 V, 0.5 A) was manually turned on for less than 2 s when the animals stayed on the electric grid for longer than 10 s. During the initial 2 weeks, the rats in two training groups could easily complete the daily training regimen because of the relatively low exercise intensity. Most of the animals were compliant with the increase in training intensity. One animal in the IEN group was withdrawn because of its inability to keep up with the increasing running intensity. Three days after the end of the 8-week training program, all the animals (15 weeks old) were killed under deep anesthetization by intraperitoneal (i.p.) injection of sodium pentobarbital (65 $mg \cdot kg^{-1}$) and decapitation.

Muscle sample collection and citrate synthase activity assay

To determine the effects of these two different training protocols on aerobic metabolism, we measured the citrate synthase (CS), a key enzyme in the citric acid cycle, in four

different muscles of the animals. Soleus (SOL, predominantly type I fibers), extensor digitorum longus (EDL, predominantly type II fibers), left heart muscle, and diaphragm were harvested, weighed, frozen with liquid nitrogen, and stored at –80°C for further enzyme activity assay. Fresh homogenate solution of each muscle was made in an ice-cold (0°–4°C) sucrose-EDTA-Tris (SET) buffer [0.01 mole/l Tris-HCL, 0.25 mole/l sucrose, and 0.002 mole/l ethylenediaminetetraacetic acid (EDTA); pH = 7.4] and centrifuged at 10 000 *g* for 15 min [16]. The enzyme activity of citrate synthase was measured according to a previous report [17].

Blood samples

Blood samples were collected after decapitation, centrifuged at 1500 *g* for 20 min, and suspended sera were stored under –70°C for future serum markers assay. Serum total alkaline phosphatase (ALP), calcium, and phosphorus were determined with an ELAN analyzer (Eppendorf, Hamburg, Germany). Bone formation and resorption activities were measured through serum carboxy-terminal propeptide of type I procollagen (PICP) and carboxy-terminal cross-linked telopeptide of type I collagen (ICTP), respectively, by standard radioimmunoassay (RIA) assay kits (Orion Diagnostica, Espoo, Finland). The ratio between PICP and ICTP (PICP/ICTP ratio) served as an index of relative bone formation activity.

Bone sample collection and measurement

After isolating the muscle tissues, femora of all animals were harvested and cleaned of the soft tissues. Bone tissues were then weighed as wet weight (WW), wrapped in 0.9% sodium chloride-soaked gauze and aluminum foil, and stored at –20°C for future densitometry and biomechanical testing. After the completion of biomechanical testing, bone tissues were immersed in a solvent of 2 volumes chloroform combined with 1 volume methanol for 1 week and dried at 80°C for 24 h. Then, the fat-free dry weights (DW) were measured [18]. Tissue water content ratio (WCR) was calculated by the formula as follows: WCR (%) = [(WW – DW) / WW] × 100%.

Densitometric analysis

The bone area (BA, cm^2), areal bone mineral density (aBMD, mg/cm^2) and bone mineral content (BMC, mg) of femora were measured with a Norland XR-26 dual-energy X-ray absorptiometer (DXA; Fort Atkinson, WI, USA) according to our previous work [5]. The bone tissues of all animals were measured by means of the DXA small subject mode. Before measurement, bone tissues were thawed at room temperature. Each femur underwent densitometry analysis on the entire, proximal quarter, diaphysis, and distal quarter portions of long bones.

Biomechanical three-point bending testing

A three-point bending testing model was adopted for measuring the mechanical properties of bone tissues using a materials testing system (MTS-858; MTS System, Minneapolis, MN, USA). The span of the two support points was 20 mm, and the deformation rate was 1 mm/min. Load/deformation data were transported to a personal computer and acquired by Team 490 software (version 4.10; Nicolet Instrument Technologies, Madison, WI, USA). Original data were used to calculate yield load, ultimate load, stiffness, and the energy for pre-yield load, post-yield load, and ultimate load. After three-point bending testing, the failure sites of all bone specimens were photographed with a measurement standard according to our previous work [5]. Cross-sectional parameters, including cortical bone thickness and cross-sectional area of cortical bone (CSA), were measured from the photographs using the software Image Pro Plus 6.1 for Windows (Media Cybernetics, Silver Spring, MD, USA). The cross-sectional moment of inertia (CSMI) of the failure site was measured by the methods of Turner and Burr for irregular cross sections:

$$I = \sum_{i=1}^{n} (wh^3/12 + whd_i^2)$$

where I is CMSI, n is the number of pixels, w and h are the height and width of each pixel, and d_i is the distance from the center of the element of the area to a given axis on the cross section [19]. All these parameters were obtained using the software Image Pro Plus 6.1 for Windows (Media Cybernetics). Data of load displacement were transferred to a stress–strain curve using the following equations:

$$\sigma = FLc/4\mathrm{I}$$
$$\varepsilon = 12cd/L^2$$
$$E = F/d \cdot L^3/48\mathrm{I}$$

where σ is ultimate stress, ε is strain, c is the maximal distance from pixels to the line crossing the center of mass, F is the applied load (N), E is elastic modulus, d is the displacement (mm), and L is the span between the two support points of the bending fixture (mm). Yield stress, ultimate stress, elastic modulus, and the toughness for pre-yield, post-yield, and ultimate stress were measured. Yield load and yield stress were determined following the 0.2%-offset method [19]. A line 0.002-strain offset and parallel to the linear part of the stress–strain curve was constructed. The intersected point of this 0.002-strain offset line and the stress–strain curve was called the yield stress (Fig. 1). The original loading value of this point was called the yield load.

Statistical analysis

One-way analysis of variance (ANOVA) was used for statistical analysis. When the significant level was reached, post hoc comparison was made using Fisher's least squares difference (LSD) method. All data were expressed as the mean ± standard error (SEM), and differences were considered significant if $P < 0.05$.

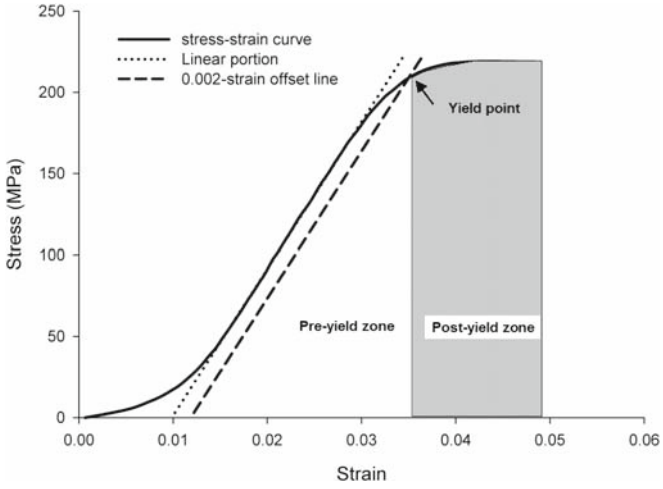

Fig. 1. Yield point determination using a 0.002 strain offset line parallel to the linear portion of the stress–strain curve. Area under the stress–strain curve in the *left-hand side* of yield point is called the pre-yield zone and represented the yield energy (toughness). The area under the stress–strain curve in the *right-hand side* of the yield point is called the post-yield zone and represents the post-yield energy (toughness)

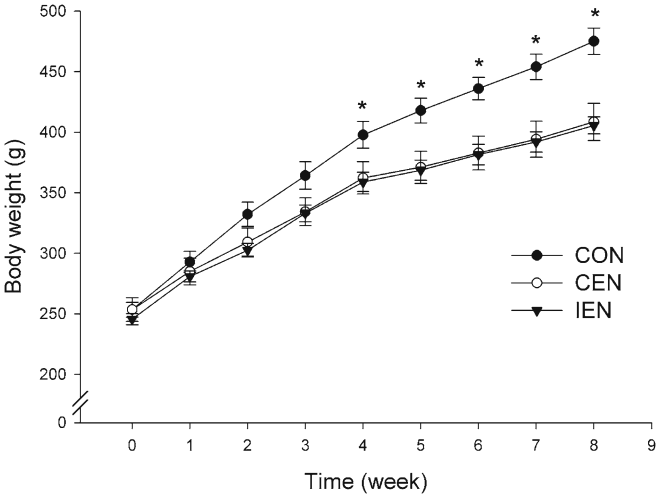

Fig. 2. Body weight change during training period; *, the control (*CON*) group was significantly higher than the continuous endurance running (*CEN*) and intermittent endurance running (*IEN*) groups

Results

Body weight (Fig. 2)

After an 8-week training period, both CEN and IEN groups showed less BW gain. After 4 weeks of training, both CEN and IEN groups were significantly lower in BW (Fig. 2).

Citrate synthase activity (Table 1)

In CS assay, the CEN and IEN groups showed significantly higher CS activities than the CON group in soleus muscle. Additionally, the CEN group showed significantly higher heart muscle CS activity as compared with the IEN and CON groups.

BA, aBMD, BMC, and tissue weight measurements (Table 2)

In densitometry analysis, only total aBMD was significantly higher in the CON group as compared to the CEN and IEN groups ($P = 0.040$). Measurements in other regions of the femur were slightly and nonsignificantly lower in the two exercise groups than in the CON group. Regarding tissue weight measurements, the CON group showed a nonsignificantly higher tissue dry weight ($P = 0.146$), but a significantly lower tissue WCR ($P = 0.019$).

Serum metabolic markers (Table 3)

In the current study, serum calcium, phosphorus, ALP, PICP, and ICTP were measured to investigate bone metabolism after the whole training period. No significant difference was found among the three groups. Relative bone formation activity (PICP/ICTP ratio) was numerically higher in the IEN group ($P = 0.078$).

Biomechanical quality and cross-sectional measurements (Tables 4, 5)

Biomechanical properties of bone tissue were measured extrinsically and intrinsically. The CEN and IEN groups showed significantly higher post-yield energy and ultimate energy in the femoral three-point bending test as compared to the CON group (Table 4).

In tissue-level (intrinsic) measurements (see Table 5), both exercise groups were significantly higher in post-yield toughness and ultimate toughness when compared to the CON group (Fig. 3).

Cross-sectional measurements were performed on the breaking sites of long bone tissues after the three-point bending test. In various cross-sectional parameters, the two exercise groups showed smaller values, but these did not reach statistically significant levels (Table 5).

Discussion

Body weight and aerobic training effects

First, the results of the present study showed general exercise training effects on BW and aerobic capacity. Under ad libitum feeding, control rats showed a significantly higher BW gain than both CEN and IEN groups, which was similar to previous work [5,6,20–22]. Using CS activity as a marker of aerobic capacity, both exercise groups showed significantly higher CS activity in soleus muscle (predominantly type I fibers). In addition, the continuous endurance training program seemed to benefit the aerobic system more, as

Table 1. Citrate synthase activity of different muscles among three groups (activity: 1/mg/min)

	CON	CEN	IEN	*P* value
SOL	19.03 ± 0.87	24.65 ± 1.28*	24.10 ± 1.20*	0.003
EDL	4.85 ± 0.41	5.44 ± 0.86	4.67 ± 0.37	0.607
Heart muscle	69.50 ± 2.35	83.31 ± 3.45*	73.44 ± 1.95	0.003
Diaphragm	25.76 ± 1.25	27.37 ± 1.63	23.48 ± 0.90	0.108

Data presented as mean ± SEM
CON, control group; CEN, continuous endurance running group; intermittent endurance running group; SOL, soleus muscle; EDL, extensor digitorum longus
*Groups marked with an asterisk are significantly higher than the ones unmarked

Table 2. BA, aBMD, BMC, and tissue weight of femora

	CON	CEN	IEN	*P* value
Densitometry				
Total				
BA	3.18 ± 0.04	3.08 ± 0.08	3.12 ± 0.05	0.475
aBMD	138.3 ± 1.6*	131.3 ± 2.2	131.6 ± 2.2	0.040
BMC	440.6 ± 8.8	406.2 ± 17.2	411.6 ± 10.6	0.141
Proximal				
BA	1.00 ± 0.02	0.96 ± 0.03	0.99 ± 0.02	0.656
aBMD	138.0 ± 1.3	132.8 ± 1.5	135.6 ± 3.6	0.359
BMC	137.6 ± 4.0	127.9 ± 5.2	134.5 ± 5.6	0.406
Diaphysis				
BA	1.48 ± 0.03	1.37 ± 0.04	1.37 ± 0.05	0.087
aBMD	127.0 ± 2.4	120.7 ± 3.2	121.5 ± 2.5	0.236
BMC	188.5 ± 6.4	165.6 ± 7.7	166.9 ± 7.4	0.060
Distal				
BA	1.00 ± 0.02	0.91 ± 0.05	0.98 ± 0.03	0.149
aBMD	147.6 ± 2.5	144.2 ± 2.7	140.5 ± 1.7	0.115
BMC	148.2 ± 4.6	131.7 ± 9.0	137.2 ± 3.8	0.178
Tissue weight				
WW	1222.8 ± 30.1	1292.3 ± 42.9	1254.7 ± 26.0	0.360
DW	914.9 ± 17.2	844.7 ± 36.0	850.8 ± 24.3	0.146
WCR	24.7 ± 2.6	34.5 ± 2.3*	31.9 ± 2.1*	0.019

Values are mean ± SEM
BA, bone area (cm^2); aBMD, areal bone mineral density (mg/cm^2); BMC, areal bone mineral content (mg); Area, bone area (cm^2); WW, tissue wet weight (mg); DW, tissue fat-free dry weight (mg); WCR, water content ratio (%)
*Groups marked with an asterisk are significantly higher than the ones unmarked

Table 3. Serum bone metabolic markers

	CON	CEN	IEN	*P* value
Calcium (mg/dl)	9.60 ± 0.07	9.72 ± 0.06	9.69 ± 0.08	0.582
Phosphate (mg/dl)	7.46 ± 0.20	7.75 ± 0.25	7.52 ± 0.20	0.528
ALP (U/l)	304.6 ± 17.0	291.1 ± 26.6	319.0 ± 11.3	0.628
PICP (µg/l)	20.3 ± 1.1	18.5 ± 0.7	20.8 ± 1.1	0.247
ICTP (µg/l)	7.58 ± 0.41	7.18 ± 0.52	6.35 ± 0.29	0.114
PICP/ICTP ratio	2.75 ± 0.23	2.67 ± 0.18	3.35 ± 0.25	0.078

Values are mean ± SEM
ALP, serum total alkaline phosphatase; PICP, serum carboxy-terminal propeptide of type I procollagen; ICTP, carboxy-terminal cross-linked telopeptide of type I collagen; PICP/ICTP ratio, served as relative bone formation activity

Table 4. Extrinsic biomechanical properties estimated by three-point bending test

	CON	CEN	IEN	*P* value
Yield load, N	182.8 ± 6.3	171.3 ± 6.5	175.7 ± 7.9	0.534
Ultimate load, N	187.7 ± 7.8	178.5 ± 7.1	180.4 ± 6.7	0.647
Yield energy, mJ	44.0 ± 3.0	44.0 ± 2.5	46.5 ± 3.2	0.786
Post-yield energy, mJ	5.4 ± 2.1	23.7 ± 4.2*	22.2 ± 5.7*	0.012
Energy to ultimate load, mJ	49.3 ± 3.9	67.7 ± 3.9*	68.6 ± 5.5*	0.010
Stiffness, N/mm	486.7 ± 23.8	441.5 ± 33.9	440.5 ± 17.4	0.337

Values are mean ± SEM
*Groups marked with an asterisk are significantly higher than the ones unmarked

Table 5. Intrinsic biomechanical properties estimated by three-point bending test and cross-sectional parameters

	CON	CEN	IEN	*P* value
Yield stress, MPa	211.3 ± 7.3	212.1 ± 10.3	231.3 ± 9.5	0.219
Ultimate stress, MPa	216.7 ± 8.1	224.0 ± 11.8	237.9 ± 8.8	0.254
Yield toughness, mJ/mm^3	2.61 ± 0.19	2.67 ± 0.15	2.96 ± 0.19	0.345
Post-yield toughness, mJ/ mm^3	0.31 ± 0.12	1.41 ± 0.25*	1.44 ± 0.37*	0.012
Ultimate toughness, mJ/mm^3	2.92 ± 0.22	4.08 ± 0.15*	4.40 ± 0.36*	0.002
Elastic modulus, GPa	10.9 ± 0.4	11.1 ± 1.0	12.0 ± 0.5	0.424
CSA of total cross section, mm^2	11.76 ± 0.26	11.25 ± 0.53	10.71 ± 0.28	0.148
CSA of cortical bone, mm^2	8.52 ± 0.20	8.06 ± 0.35	7.64 ± 0.19	0.063
CSA of medulla, mm^2	3.24 ± 0.24	3.19 ± 0.24	3.07 ± 0.14	0.833
Cortical bone thickness, mm	0.78 ± 0.03	0.76 ± 0.02	0.72 ± 0.01	0.129
CSMI, mm^4	7.44 ± 0.31	6.90 ± 0.55	6.20 ± 0.32	0.106

Values are mean ± SEM
CSA, tissue cross-sectional area; CSMI, cross-sectional moment of inertia
* Groups marked with an asterisk are significantly higher than the ones unmarked

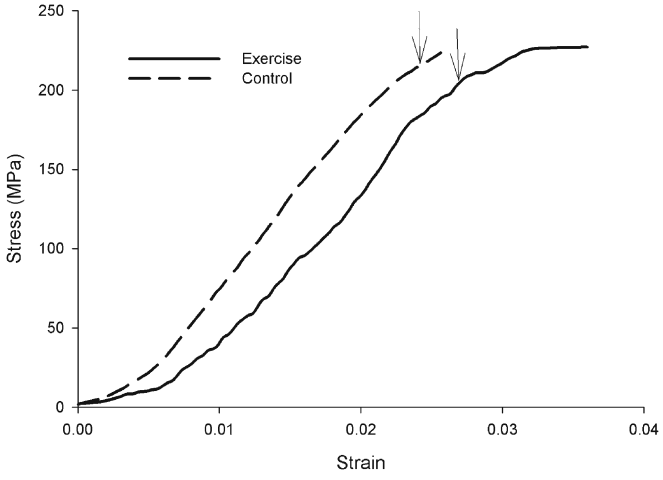

Fig. 3. Typical stress–strain curve of femora in the exercise and control groups. Endurance training showed significantly more post-yield strain as well as post-yield toughness. *Arrows* represent the yield points of femora

shown by additional higher CS activity in the CEN rat heart muscle.

Densitometric analysis

In densitometric measurement, the results of our study did not show any benefit from exercise training on aBMD; i.e., exercise groups showed equal or lower values in BA, aBMD, and BMC as compared with the control group (see Table 2). As the bone density produced by DXA was areal density, not volumetric density, the size of the samples interferes with the measured density. In other words, a larger bone tissue would show a higher aBMD than a smaller one with similar volumetric density. Given that the CON group was 6%–12% numerically higher in CSA and CSMI (see Table 5), the slightly higher total aBMD in the CON group (P = 0.04) might be partially the result of a greater sample size. Therefore, the two exercise groups might be equivalent to the CON group in real volumetric bone mineral density. Moreover, with 17% lower body weight, the bone density of the two exercise groups could be considered relatively higher. In previous studies, the effects of treadmill training on BMD and BMC in growing rats showed a gender difference and seemed to be BW related. Young female rats acclimated to treadmill running with accretion in BMD and BMC, which was accompanied by equal or higher BW gains as compared to control rats [23–26]. In growing male rats, treadmill-trained rats generally showed no change or lower BMD and BMC with concurrently lower BW gain [5,6,20–22], which is more similar to the BW change in humans after a period of intensive endurance training. Several previous studies adjusted food consumption to match the BW gain between exercise and control male rats to eliminate the interference from BW [27–29]. In these studies, BMD and/or BMC were significantly higher in exercise groups. However, it has been suggested that dietary restriction would not only cause a lower BMC but would also lead to additional adverse effects on tissue-level biomechanical properties [30,31]. Therefore, a sedentary control group fed ad libitum could be more natural than a BW-matched control group. In addition, it is widely agreed that many of the animal models were designed to mimic human behavior. From this point of view, the endurance training effects on young male rats are more similar to the effects shown in human subjects [7,8]. Therefore, it might be more appropriate to use male rats to investigate the impact of endurance training on growing bone.

Although epidemiological study suggests that fracture risk increases with a lower aBMD [32], fractures still occur in humans with normal aBMD, implying that aBMD is not the only critical component of bone strength [33]. On the other hand, the higher BMC of sedentary control rats might come primarily from their higher BW, as mentioned in previous reports [7,8,34–37]. Correlation analysis (data not shown) using Pearson's correlation showed that BA (r = 0.669), aBMD (r = 0.682), and BMC (r = 0.753) values were highly related to BW but not to intrinsic biomaterial parameters, suggesting that tissue-level biomechanical properties are independent of densitometric parameters. Interestingly, biomechanical analysis in the present study revealed that

both endurance training regimens showed benefits to bone development not related to increasing BMC or aBMD.

Biomaterial and geometric properties

With equal or smaller bone sizes and densitometric parameters as compared to the control group, both exercise groups showed similar bone strength and even showed a higher energy absorption capacity before breaking (see Tables 4, 5). Aside from BMD and bone dimension, tissue-level parameters, such as collagen organization and its integration with inorganic bone mineral, also contribute to bone strength [13]. Collagen molecules are aligned within the fiber in a quarter-staggered end-overlap fashion [13]. In such an arrangement, holes within the fiber provide spaces for nucleation of the apatite crystals, which grow parallel to the collagen fibrils. As compared to lamellar bone, woven bone with unorganized collagen fibrils probably has higher mineral content but lower mechanical properties [38]. Therefore, the collagen fiber organization is important for tissue mechanical properties; moreover, highly organized collagen fibrils could limit the size of crystals and control their orientation, which might partly explain why absolute aBMD and BMC did not increase and even slightly decreased in the current exercise groups.

Regarding the role of collagen on biomaterial properties of bone tissue, several studies have demonstrated that tissue mass and stiffness are mainly determined by the mineral phase [39,40], whereas the collagen matrix contributes mainly to bone toughness [14,41]. In the present study, significantly different post-yield behavior in the tissues of the exercise groups suggests a different organization of collagen (Fig. 3). Previous studies have suggested that bone tissue with higher post-yield energy absorption would be facilitated by a highly organized collagen fiber orientation [12,42]. Although we did not measure the organization of collagen in the present study, it has been reported that long-term intensive endurance training in dogs caused a 10% BMD decrement, but showed no difference in the indentation stress to fracture cancellous bone as compared to a non-training group, which might be a result of the high organization of collagen fibrils shown in the exercise group [11]. On the other hand, significantly higher WCR in swimming groups (see Table 2) also implies changes in bone matrix composition, as well as in biomaterial properties. At the tissue level, water is distributed within collagen fibers and plays a role in stabilizing collagen fiber as well as in enhancing tissue toughness [43]. Organization of collagen would affect water content in bone; for example, the glycation-induced cross-links of collagen would increase with age, which might decrease the interaction between water and collagen seen in connective tissues [44,45].

As described in the Introduction, the relatively low ground reaction force (GRF) of moderate endurance running implied that bone could not benefit from this type of exercise, as no obvious BMC and/or aBMD increase was found. To increase GRF, we designed an intermittent endurance training protocol with 36% higher running speed (30 m/min vs. 22 m/min). However, the IEN group still did not show any differences in densitometric data from the CEN group, suggesting that growing bones respond to endurance training differently from the way in which they respond to simple local mechanical loading modes [2–4]. It seems that endurance training tends to benefit bone via strengthening relative tissue-level biomaterial properties, whereas the local mechanical loading exercise increases bone size and bone mass. Further investigations would be valuable in clarifying the mechanisms through which bone tissues and their matrix organization adapt to endurance training.

Bone metabolism

One might argue that endurance training induced higher levels of serum calcium and bone resorption-related markers that might represent an impact on bone metabolism [46–48], which then prohibits the bone mineralization. From the aspect of exercise physiology, calcium in our skeletal systems provides a source for buffering exercise-induced low pH, and elevated levels of serum calcium will return to baseline levels after an exercise bout [49]. In the aspects of bone turnover, a transient higher bone resorption induced by a bout of moderate exercise is more likely to represent a status of bone under remodeling, not just degrading. In the present study, 3 days after 8 weeks of training, serum markers showed no statistical difference between the CEN and CON groups, and the IEN group even showed a numerical higher ($P = 0.078$) relative bone formation activity (PICP/ICTP ratio). This result implied that bone turnover of the two endurance running groups had already acclimated or was acclimating to the exercise stimulus, and the long-term effects of endurance training seemed to favor bone formation (see Table 3).

In summary, in the current study, both endurance running modes enhanced tissue biomaterial properties, not through increasing bone mass or bone size, but through tissue-level properties, probably the result of a well-organized bone matrix. Further studies would be valuable in clarifying the details of exercise-induced changes in bone matrix (collagen content, collagen orientation, bone mineral component, etc.) and the interaction among these parameters.

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