EFFECTS OF PHYSICAL EXERCISE ON FEMORAL BONE MINERAL DENSITY AND
OSTEOCYTE MICROMORPHOLOGY IN YOUNG OVARIECTOMIZED (OVX) RATS

TOSHIKAZU KAWAKAMI1,2, SADAFUMI TAKISE1,3 and HIROSHI KAWATA2)

Abstract

To investigate the effects of exercise on femoral regional bone mineral density and osteocyte micromorphology in young ovariectomized (OVX) rats, animals were divided into an ovariectomized group (OVX group), an ovariectomized + exercise group (OVX + exercise group), and a sham surgery group (SHAM group). Femoral bone mineral density was significantly lower in the OVX and OVX + exercise groups than in the SHAM group. A large difference in bone mineral density in the distal femoral epiphysis was observed between the OVX and OVX + exercise groups. In the OVX group, bone lacunae showed less immunostaining for DMP-1 (dentin matrix protein-1) and osteocyte processes were fewer than in the SHAM group. In the OVX + exercise group, osteocyte processes were thicker (0.44 to 0.66 μm) than in the OVX group (0.22 to 0.26 μm). These results indicate that exercise induced structural changes in the femur, including formation of osteocyte processes and strengthening of the communication network between osteocytes.

key word: ovariectomized (OVX) rat, Physical exercise (treadmill running), bone mineral density, osteocytes, SEM

I. Introduction

The female hormones estrogen and progesterone are secreted by the ovaries and act to maintain periodic ovulation and regulate menstruation.1) If amenorrhea is prolonged, not only does restoration of menstruation become difficult, but secretion of estrogen from the ovaries is suppressed. Moreover, low estrogen levels induce high-turnover bone metabolism and a decrease in bone mineral density.

Studies in which ovariectomy (OVX) was performed to produce estrogen deficiency and the effects of physical training on bone mass were examined have found that estrogen acts to regulate bone metabolism.2) With estrogen deficiency, osteoblast differentiation is stimulated, and matrix decomposition and bone resorption progress. Moreover, although osteoblast function is promoted and metabolic turnover is high in ovariectomized animals, bone resorption exceeds bone formation, leading to a decrease in bone mass.5,6) In addition, osteocyte apoptosis has been reported to increase markedly in ovariectomized rats.7,8)

Exercise has been found to affect bone in numerous studies of physical training in humans and animals. Dynamic exercise is superior to static exercise in increasing bone mass, and the greater the intensity of the training, the greater the increase in bone mass. In women with menstrual abnormalities and ovariectomized animals, exercise is thought to contribute to improvement in the microstructure of bone tissue and to maintain bone mass by strengthening bone made brittle by a decrease in bone mass.2-4) In examining the effect of exercise, however, these studies analyzed only bone mass. Osteocytes are thought to be responsible for transmitting the signals involved in exercise-induced bone remodeling. However, although osteocytes are the most numerous cells in bone tissue, little is known about their morphology. This is due largely to the fact that these cells are located in a special environment, i.e., they are embedded in hard tissue.
The present study examined the effects of exercise on femoral bone density in ovariectomized rats. In addition, it examined osteocyte morphology in these animals using the EDTA-KOH method.

II. Methods

We used 15 female Sprague–Dawley rats (Japan SLC, Japan) in the study. The animals were divided into an ovariectomized group (OVX group, n=5), an OVX + exercise group (OVX + exercise group, n=5) and a sham-surgery group (SHAM group, n=5). Ovariectomy was performed when the animals were 9 weeks old. From 9 to 10 weeks of age, the animals were allowed a recovery period, and from 10 to 13 weeks of age, they were used in the 4-week study.

A treadmill for small animals (Natsume Seisakusho, Japan) was used to train the OVX + exercise group during the 4-week period when the animals were 10 to 13 weeks old. The animals were run at a speed of 20 m/min for 30 min/day, 6 days/week. Established training conditions for effective prevention of bone mass reduction in rats and mice are running at an intensity of 24 m/min for 25 min.⁹,¹⁰ During preliminary experiments, we attempted spontaneous training of rats at 24 m/min; however, it was difficult for them to run at this speed and we ultimately established training conditions emphasizing running time rather than speed.

The animal room where the rats were housed in individual cages was maintained at an ambient temperature of 24°C, and the running training was performed under the same environmental conditions. The room was lit from 08:00 to 20:00 and dark from 20:00 to 08:00. Animals were fed Rodent Laboratory Diet EQ (Japan SLC, Japan), with all groups receiving 30 g/day. Water was provided ad libitum. The rats were weighed once weekly using a small-animal scale (AND, HL-200) from the start of the study to its completion.

This study was carried out with the approval of Osaka University of Health and Sport Sciences Animal Ethics Committee and according to the “Basic guidelines for conducting animal studies in the field of physiology”, established by the Physiological Society of Japan and the “Public Health Service Policy on Humane Care and Use of Laboratory Animals” established by the National Institutes of Health.

1 Specimen collection

After completion of the study, the rats were laparotomized and then thoracotomized under pentobarbital sodium anesthesia, and antegrade perfusion with physiologic saline was performed from the left ventricle. The femur was then resected and immediately immersion-fixed in a 10% formalin fixative. Some of the tissue was immersion-fixed in a 2.5% glutaraldehyde-phosphoric acid buffer (pH 7.2, 4°C).

2 Measurement of bone mineral density

Femoral bone mineral density was determined by dual-energy X-ray absorption (DXA: QDR-4500, Hologic Inc., USA). The anterior aspect of the bone was scanned from the distal epiphysis to the proximal epiphysis in ultra-high resolution mode for small animal resected bone. To ensure that the scanning direction was consistent, the center of the test bone was placed on a straight line that had been drawn on the outside of the bottom of a styrene case and positioned so that the entire surface of the femur would be scanned. The images obtained by scanning were used to calculate the total area of the bone (cm²) bone mineral content (g) and bone mineral density (bone mineral content per unit area, g/cm²). Moreover, the femur was divided at equal intervals into 7 regions along the long axis of the bone, and bone mineral density was analyzed according to region (Fig. 1) based on the bone mineral density data obtained.

3 Observation by light microscopy

After being measured for bone mineral density, the fixed femur specimens were cut along the long axis of the bone using a sharp single-edged razor and postfixed using a 10% formalin fixative. The fixed specimens were washed with tap water and then decalcified for 30 days using an 8% EDTA-sucrose decalcifying solution (pH 7.2). The decalcified specimens
Femoral bone mineral density was determined by dual-energy X-ray absorption (DXA: QDR-4500, Hologic, USA). The anterior aspect of the bone was scanned from the distal epiphysis to the proximal epiphysis in ultra-high resolution mode for small animal resected bone. To ensure that the scanning direction was consistent, the center of the test bone was placed on a straight line that had been drawn on the outside of the bottom of a styrene case and positioned so that the entire surface of the femur would be scanned. The images obtained by scanning were used to calculate the total area of the bone (cm²), bone mineral content (g) and bone mineral density (bone mineral content per unit area, g/cm²). Moreover, the femur was divided at equal intervals into 7 regions along the long axis of the bone, and bone mineral density was analyzed according to region based on the bone mineral density data obtained.

Figure 1. Measurement of femoral bone mineral density (DXA: QDR-4500, Hologic, USA)

were then washed with tap water, dehydrated with ethanol, and embedded in paraffin, and the embedded specimens were sectioned into 4-μm-thick sections using a sliding microtome (Yamato Koki model TU-213, Japan). The specimens were subsequently stained with hematoxylin-eosin (HE-stain) and immunohistochemically stained.

Immunohistochemical staining was performed according to the labeled streptavidin-biotinylated antibody method (LSAB method; Dako, Japan), using anti-DMP-1 peptide antibody (dentin matrix protein-1, Takara, Japan) and anti-rabbit TGF-β-1 polyclonal antibody (transforming growth factor-β-1, Santa Cruz Biotechnology, USA) as the primary antibodies.11 The primary antibodies were diluted 1000-fold with PBS and reacted for 12 h at 4°C. Biotin-labeled anti-rabbit IgG sheep serum was used as the secondary antibody and reacted for 30 min at room temperature. It was then reacted for 30 min at room temperature with peroxidase-labeled streptavidin and for 10 min with 3,3′-diaminobenzidine·H2O2 solution for peroxidase coloration. Following nuclear staining with hematoxylin, dehydration, clearing, and inclusion were performed. The specimen was then observed by light microscopy (BX51, Olympus Systems Microscope, NY Supersystem, Japan) and photographed.

4 Observation by scanning electron microscopy (SEM)

Specimens were prepared for SEM observation of osteocytes by fixing in a 2.5% glutaraldehyde-phosphate buffer solution (pH 7.2, 4°C) for 4 to 5 days, then decalcifying with an 8% EDTA-sucrose decalcification solution (pH 7.2) for 30 days. The specimens were then fractured along the horizontal axis under a binocular stereoscopic microscope, then fixed again in a 2.5% glutaraldehyde-phosphate buffer solution (pH 7.2, 4°C).

Specimens were then immersed for 8 min in a 5 N KOH-water solution that had been heated to 60°C to digest the collagen of the bone matrix and expose the osteocytes. The specimens were soaked overnight in 0.1 M cacodylic acid buffer, then conductive-stained using a 2% tannic acid–water solution and a 1% osmic acid–water solution. Specimens were then dehydrated with ethanol and then substituted with isoamyl acetate. Critical point drying and gold vapor deposition (SC 7610, TOPCON, Japan) were then performed. Osteocytes were then observed using SEM (DS-600, TOPCON, Japan) and photographed.12,13 Images obtained through SEM were input and saved on a computer (Windows PC). Osteocytes per section (area, 643,672 μm²) of cancellous bone trabeculae
were imaged over a total of five sections (total area, 321,836 $\mu m^2$). Images were then taken from five rats from each group (total of 25 sections; total area 1,609, 180 $\mu m^2$) and five osteocytes were randomly selected per section for image analysis on a bioimaging analysis system (Lumina Vision; Mitani Corp., Japan). Osteocyte size (diameter, $\mu m$) and cell process size (diameter, $\mu m$) were measured for each group.$^{12,13}$

5) Analysis

Statistical analysis of the differences between the O VX group, the OVX + exercise group, and the SHAM group with respect to femoral bone mineral density and bone mineral density by femoral region was performed by ANOVA (Fisher PLSD) using Macintosh Statview. Results are expressed as mean ± standard deviation. Ap-value of ≤ 0.05 was considered statistically significant.

III. Results

1) Comparison of body weight, intraperitoneal fat weight, and femoral bone mineral density

A between-groups comparison of body weight during the study period is shown in Table 1. Body weight at 1 week after ovariectomy was greater in the OVX and OVX + exercise groups as compared with the SHAM group, confirming that ovariectomy had been completed in the former groups. Body weight at study completion was significantly greater in the OVX (271 ± 21.68 g) and OVX + exercise (253.60 ± 15.24 g) groups as compared with the SHAM group (227.50 ± 5.07 g; OVX group, $p = 0.008$; OVX + exercise group, $p = 0.020$).

Intraperitoneal fat weight was significantly greater in the OVX group (13.76 ± 1.89 g) than in both the SHAM group (10.86 ± 1.39 g; $p = 0.016$) and the OVX + exercise group (9.78 ± 1.62 g) ($p = 0.002$, Table 2).

Femoral bone mineral density was significantly lower in the OVX (0.178 ± 0.005 g/cm$^2$) and OVX + exercise (0.182 ± 0.006 g/cm$^2$) groups than in the SHAM group (0.199 ± 0.006 g/cm$^2$; OVX group, $p = 0.004$; OVX + exercise group, $p = 0.004$). Although femoral bone mineral density tended to be higher in the OVX + exercise group than in the OVX group, the difference between these groups was nonsignificant (Table 3). Comparison of bone mineral density by femoral region showed bone mineral density in the proximal femoral epiphysis to be significantly lower in the OVX (0.174 ± 0.008 g/cm$^2$) and OVX + exercise (0.182 ± 0.006 g/cm$^2$) groups as compared with the SHAM group (0.199 ± 0.0005 g/cm$^2$; OVX group, $p = 0.003$; OVX + exercise group, $p = 0.038$). A large difference in bone mineral density in the distal femoral epiphysis was seen between the OVX and OVX + ex-

Table 1. Comparison of body weight in the SHAM, OVX, and OVX + exercise groups

<table>
<thead>
<tr>
<th></th>
<th>Pre-OVX (9week old)</th>
<th>Study Start (1 week post-OVX) (10week old)</th>
<th>Study Completion (13week old)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHAM group</td>
<td>(g)</td>
<td>196.44 ± 6.88</td>
<td>205.60 ± 3.44</td>
</tr>
<tr>
<td>OVX group</td>
<td>(g)</td>
<td>196.60 ± 8.71</td>
<td>228.40 ± 14.77</td>
</tr>
<tr>
<td>OVX + exercise</td>
<td>(g)</td>
<td>194.00 ± 9.00</td>
<td>218.00 ± 11.36</td>
</tr>
</tbody>
</table>

Mean±SD, *: $p<0.05$ vs SHAM group, **: $p<0.01$ vs SHAM group.

A between-groups comparison of body weight during the study period. Body weight at 1 week after ovariectomy was greater in the OVX and OVX + exercise groups as compared with the SHAM group, confirming that ovariectomy had been completed in the former groups. Body weight at study completion was significantly greater in the OVX (271 ± 21.68 g) and OVX + exercise (253.60 ± 15.24 g) groups as compared with the SHAM group (227.50 ± 5.07 g; OVX group, $p = 0.008$; OVX + exercise group, $p = 0.020$).
Table 2. Comparison of intraperitoneal fat weight in the SHAM, OVX, and OVX + exercise groups

<table>
<thead>
<tr>
<th></th>
<th>SHAM group</th>
<th>OVX group</th>
<th>OVX + exercise group</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=5</td>
<td>n=5</td>
<td>n=5</td>
<td></td>
</tr>
<tr>
<td>Intraperitoneal fat weight (g)</td>
<td>10.86±1.39</td>
<td>13.76±1.89 *</td>
<td>9.78±1.62 **</td>
</tr>
</tbody>
</table>

Mean±SD,
* : p<0.05 (vs SHAM group )
** : p<0.01 (vs OVX group )

Intraperitoneal fat weight was significantly greater in the OVX group (13.76 ± 1.89 g) than in both the SHAM group (10.86 ± 1.39 g, p = 0.016) and the OVX + exercise group (9.78 ± 1.62 g, p = 0.002).

Table 3. Comparison of femoral bone mineral density in the SHAM, OVX, and OVX + exercise groups

<table>
<thead>
<tr>
<th></th>
<th>SHAM group</th>
<th>OVX group</th>
<th>OVX + exercise group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area (cm²) (n=5)</td>
<td>1.506±0.047</td>
<td>1.530±0.055</td>
<td>1.552±0.040</td>
</tr>
<tr>
<td>BMC (g)</td>
<td>0.300±0.016</td>
<td>0.275±0.012 *</td>
<td>0.282±0.015 **</td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td>0.199±0.005</td>
<td>0.178±0.005 **</td>
<td>0.182±0.006 **</td>
</tr>
</tbody>
</table>

Mean±SD,
BMC : * : p<0.05 (vs SHAM group )
BMD : ** : p<0.01 (vs SHAM group )

Femoral bone mineral density was significantly lower in the OVX (0.178 ± 0.005 g/cm²) and OVX + exercise (0.182 ± 0.006 g/cm²) groups than in the SHAM group (0.199 ± 0.005 g/cm²). OVX group, p = 0.004; OVX + exercise group, p = 0.004. Although femoral bone mineral density tended to be higher in the OVX + exercise group than in the OVX group, the difference between these groups was nonsignificant.

Exercise groups (difference, 0.010 g/cm²), although this difference was nonsignificant (Table 4).

2) Change in treadmill running workload in the OVX + exercise group during the study period.

The change in treadmill running workload each week during the study period is shown for the OVX + exercise group in Fig. 2. The treadmill running workload for the OVX + exercise group was 130,800 g m [grams multiplied by meters] / 30 min during study week 1, 140,760 g m [grams multiplied by meters] / 30 min during week 2, 147,480 g m [grams multiplied by meters] / 30 min during week 3, and 152,160 g m [grams multiplied by meters] / 30 min during week 4. Thus, the treadmill running workload increased as body weight increased with age.

3) Light microscopy appearance of osteocytes, bone lacunae, and osteoblasts in the distal femoral epiphysis (immunohistochemical staining).

Immunohistochemical staining showed numerous osteocytes embedded in bone lacunae in the SHAM group. Few bone lacunae were seen in the OVX or OVX + exercise groups, and those that were seen were smaller than those in the SHAM group. Many bone lacunae in the OVX + exercise group showed DMP-1 expression, while few did so in the OVX group (Fig. 3–A, B, C).

Immunohistochemical staining of osteoblasts showed TGF-β immunoreactivity to the endosteal surface and
Table 4. Comparison of bone mineral density by femoral region in the SHAM, OVX, and OVX + exercise groups

<table>
<thead>
<tr>
<th>Region</th>
<th>SHAM group (n=5)</th>
<th>OVX group (n=5)</th>
<th>OVX + exercise group (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>0.199±0.005</td>
<td>0.174±0.008</td>
<td>* * 0.182±0.006</td>
</tr>
<tr>
<td>R2</td>
<td>0.221±0.006</td>
<td>0.198±0.005</td>
<td>0.201±0.007</td>
</tr>
<tr>
<td>R3</td>
<td>0.190±0.007</td>
<td>0.174±0.009</td>
<td>0.181±0.009</td>
</tr>
<tr>
<td>R4</td>
<td>0.164±0.004</td>
<td>0.160±0.013</td>
<td>0.165±0.014</td>
</tr>
<tr>
<td>R5</td>
<td>0.147±0.004</td>
<td>0.139±0.007</td>
<td>0.143±0.008</td>
</tr>
<tr>
<td>R6</td>
<td>0.180±0.019</td>
<td>0.137±0.006</td>
<td>0.146±0.006</td>
</tr>
<tr>
<td>R7</td>
<td>0.255±0.008</td>
<td>0.218±0.011</td>
<td>0.229±0.010</td>
</tr>
</tbody>
</table>

Mean±SD, 
* : p<0.05 (vs SHAM group) 
** : p<0.01 (vs SHAM group) 
# : p<0.05 (vs OVX + EX group) 
## : p<0.01 (vs OVX + EX group) 

Comparison of bone mineral density by femoral region showed bone mineral density in the proximal femoral epiphysis to be significantly lower in the OVX (0.174 ± 0.008 g/cm²) and OVX + exercise (0.182 ± 0.006 g/cm²) groups as compared with the SHAM group (0.199 ± 0.0005 g/cm²). OVX group, p = 0.003; OVX + exercise, p = 0.038. A large difference in bone mineral density in the distal femoral epiphysis was seen between the OVX and OVX + exercise groups (difference, 0.010 g/cm²) although this difference was nonsignificant.

![Change in treadmill running workload in the OVX + exercise group](image)

Figure 2. Change in treadmill running workload in the OVX + exercise group

gm : grams multiplied by meters  

The change in treadmill running workload each week during the study period is shown for the OVX + exercise group. The treadmill running workload for the OVX + exercise group was 130,800 gm (grams multiplied by meters) 30 min during study week 1, 140,760 gm during study week 2, 147,480 gm during study week 3, and 152,160 gm during study week 4. Thus, the treadmill running workload increased as body weight increased with age.
4 SEM observation of osteocytes from the distal femoral epiphysis

Osteocytes in the SHAM group were 13.33 μm to 15.00 μm in diameter and were roughly egg-shaped, with a protrusion at the center of the cell body. Their processes formed a reticular tertiary structure in which the processes projected radially vertically and horizontally from the cell body, and the entire structure was tightly integrated. The processes had a thick base (0.71 μm in diameter) and thin tip (0.44 μm in diameter; Figs. 5-A, B, C).

In the OVX + exercise group, osteocytes were more sparsely distributed than in the SHAM group, and their cell bodies were smaller at between 8.88 μm and 10.00 μm in diameter. They were roughly square in shape, and flat. The cells had processes that projected vertically from the cell body and were thick at the base (0.66 μm) and thin at the tip (0.44 μm). The intercellular space between osteocytes was 7.67 μm (Figs. 6-A, B, C).

In the OVX group, the osteocytes were an intermediate size at 10.55 to 11.66 μm in diameter. The intercellular space between osteocytes was greater, at 8.67
Figure 5. Osteocyte appearance in the SHAM group

A (×2000), B (×5000), C (×7000)

Osteocytes in the SHAM group were 13.33 μm to 15.00 μm in diameter and were roughly egg-shaped, with a protrusion at the center of the cell body. Their processes formed a reticular tertiary structure in which the processes projected radially vertically and horizontally from the cell body, and the entire structure was tightly integrated. The processes had a thick base (0.71 μm in diameter) and thin tip (0.44 μm in diameter).

Figure 6. Osteocyte appearance in the OVX + exercise group

A (×2000), B (×5000), C (×10000)

In the OVX + exercise group, osteocytes were more sparsely distributed than in the SHAM group, and their cell bodies were smaller at between 8.88 μm and 10.00 μm in diameter. They were roughly square in shape, and flat. The cells had processes that projected vertically from the cell body and were thick at the base (0.66 μm) and thin at the tip (0.44 μm). The intercellular space between osteocytes was 7.67 μm.

μm. Objects thought to be very small osteocytes, 2.20 μm in diameter, were observed at the site corresponding to the center of the intercellular gap. Compared with those seen in the OVX + exercise group, cell processes were thinner (0.26 μm in diameter at the base and 0.22 μm at the tip) and formed a looser reticular structure (Figs. 7-A, B, C).

IV. Discussion

The present study demonstrated the efficacy of treadmill running in bone mass maintenance and bone strength in an animal model of bone mass reduction. These findings support those of reports that bone strength and mass are greater in running groups than OVX groups regardless of running speed, time, or distance. The main effects of treadmill running are suppression of bone mass reduction in cancellous bone and increase in cortical bone mass. Running-generated impact and load on bone are believed to suppress high metabolic turnover and to increase cortical bone mass through modeling.

Stimulation of the bone through exercise is indispensable to osteogenesis. The decrease in serum estrogen concentration, changes in the activity of enzymes involved in bone metabolism, shift to high-turnover bone metabolism, and decrease in bone mass seen in ovariectomized rats are characteristics that resemble bone metabolism immediately after the menopause.
Figure 7. Osteocyte appearance in the OVX group

In the OVX group, the osteocytes were an intermediate size at 10.55 μm to 11.66 μm in diameter. The intercellular space between osteocytes was greater, at 8.67 μm. Objects thought to be very small osteocytes, 2.30 μm in diameter, were observed at the site corresponding to the center of the intercellular gap. Compared with those seen in the OVX + exercise group, cell processes were thinner (0.26 μm in diameter at the base and 0.22 μm at the tip) and formed a looser reticular structure.

cise has been reported to inhibit bone mass decreases in ovariectomized rats. This suggests that exercise maintains bone mass by inhibiting the rate of metabolic bone turnover, which is enhanced immediately after ovariectomy.

Femoral bone mineral density in the OVX group was lower than in the SHAM group. One possible factor in this difference was an estrogen deficit or change in parathyroid hormone levels resulting from ovariectomy, although it is unclear when and where in the femur that bone resorption occurred. The mass and structure of bone change in response to mechanical conditions imposed by loading and exercise. Consequently, when bone mineral density was analyzed by dividing the femur into 7 dominant regions comprising cancellous and cortical bone based on the structural characteristics of the femur, changes in bone mineral density were seen in the distal femoral epiphysis. The distal femoral epiphysis around the knee joint is an area susceptible to loading or exercise-mediated stimulation even in rats, and the quadriceps femoris tendon inserts proximally to the patella and tibia. During treadmill running, the distal femoral epiphysis is strongly stimulated by muscle contraction and relaxation. Yuefeng et al. have described the importance to increasing bone mineral density of regulating the activity of osteocytes and osteoclasts through localized stress and strain on the bone and the deformation and compression of osteocytes and cell processes that result from changes in the flow of fluid within the bone. The lack of significant difference between the OVX and OVX+EX groups with regard to changes in regional bone mineral density in the present study can be explained by the fact that an increased effect of exercise on bone in OVX model rats is observed at longer periods in comparison with age-matched control animals under appropriate exercise conditions. This is particularly true in regions where impact is not directly transmitted to the bone, such as the femur or vertebral body. At short periods, the effect of exercise on mechanical bone strength is greater in the tibia than in the femur. Wheeler et al. conducted a study on bone characteristics under treadmill running conditions with three intensities (maximum oxygen uptake: 55%, 65%, and 75%) and three running times (30 min, 60 min and 90 min) and found that the tibia was more affected by exercise than the femur. These studies suggest that, under the exercise conditions in the present study, running at a speed of 20 m/min was ineffective at the present mechanical intensity for increasing bone mineral density. This is believed to be an optimal speed for bone mineral density maintenance but it may be necessary to establish a more effective exercise intensity. However, changes were observed in osteocyte micromorphology regarding network formation in the OVX+EX group, suggesting...
that bone formation may have been activated.

The microstructural changes seen in cells of the distal femoral epiphysis in the OVX + exercise group are summarized below. Of particular interest are the morphological observations of osteocytes that sense mechanical stimulation. This is because bone undergoes strain due to extrinsic and intrinsic mechanical stimulation, causing adaptation of the bone’s appearance and internal structure. Moreover, osteocytes that extend their processes into the bone lacunae are sensitive to this strain and are thought to play an important role in regulating bone remodeling. Osteocytes in the SHAM group exhibited an egg-shaped morphology, with a protrusion at the center of the cell body. Their processes formed a reticular structure, projecting radially vertically and horizontally from the cell body, and the entire structure was tightly integrated. The processes had a thick base (0.71 μm in diameter) and thin tip (0.44 μm in diameter). Fewer osteocytes were seen in the OVX + exercise group as compared with the SHAM group, and cell bodies of the osteocytes in the OVX + exercise group were squarish and flat. The cell processes of the osteocytes projected vertically from the cell body and were thick at the base (0.66 μm) and thin at the tip (0.44 μm). The gap between the osteocytes was 7.67 μm. In contrast, the gap between osteocytes in the OVX group was greater, at 8.67 μm. Objects thought to be very small osteocytes, 2.20 μm in diameter, were observed at the site corresponding to the center of the intercellular gap. This indicated that there was some relationship between the osteocytes and the decrease in bone mass.

The processes of the osteoblast-like osteocytes in the OVX + exercise group were arranged in the bone matrix in three dimensions. Growth of the vertical processes was pronounced, enabling osteocytes whose cell processes extended into the bone lacunae to sensitively detect a stimulus. The cells are highly responsive to mechanical stimuli, suggesting that this information may be conveyed to osteoblasts and osteoclasts. Immature, weak osteocytes that have a high rate of metabolic turnover may be unearthed by osteoclasts and localized to the bone surface and may again function as osteoblasts. Mechanical stimulation may have caused strain on the bone, and the osteocytes may have sensed the mechanical stimulation through the extension of cell processes of osteocytes in the bone lacunae or canaliculi or through compression of the cells. Alternatively, fluid filling the bone lacunae and canaliculi may flow as a result of the strain on the bone, with this flow dragging along the surface of the cell membranes of the osteocytes (shear stress). As a result, either of these stimuli may be more strongly propagated to osteocytes in the distal epiphysis, increasing the expression of substances such as connexin 43, a gap-junctional protein, or COX-2, the rate-limiting enzyme for prostaglandin synthesis. The OVX + exercise group appeared to respond to the stimulus applied to the knee joint by rapidly propagating information via the intercellular network to deep within the bone and conveying this information to other osteocytes and to osteoblasts and osteoclasts on the bone surface, facilitating local bone resorption or formation.

A mechanism by which osteocytes and osteoblasts work together to dissolve and discharge calcium was described by Belanger et al. as a form of osteocytic osteolysis. The authors asserted that there are two possible means of regulating the efflux and influx of calcium to and from bone tissue, osteocytes/osteoblasts, and osteoclasts. They stated that the structure of the cellular network enables the intracellular transport of ions by the osteocyte/osteoblast system that this system may play a major role in mobilizing calcium toward the bone. Neuman has asserted that because the calcium-shedding capacity of osteocytes accounts for only 0.1% of total calcium efflux and an increase in blood calcium concentration is seen before osteoclasts increase, osteoclasts account for only several percentage points of the rapid calcium mobilization from bone while the remainder derives from the osteocyte/osteoblast system. However, although it is clear that estrogen affects osteocyte regulation, the mechanism of this effect is unclear. In the ovariectomized rats in this investigation, the intercellular
spaces between osteocytes were large, and objects thought to be very small osteocytes were observed at sites corresponding to the center of these spaces.

Estradiol replacement in ovariectomized rats enhances osteoclast formation and induces osteocyte apoptosis.\(^{21}\) Therefore, estradiol deficiency may facilitate the resorption of osteocytes that have undergone apoptosis caused by osteoclasts. This apoptosis is inhibited by bisphosphonates and calcitonin. In addition, intermittent administration of PTH prevents osteocyte apoptosis.\(^{23}\) In the OVX + exercise group in this study, the immature, weak osteocytes that appeared to be nearest the bone matrix surface immediately after the osteoblasts were embedded were seen to express DMP-1, indicating abundant synthesis and secretion of bone matrix protein. DMP-1 is a matrix protein produced in large quantities by osteocytes.\(^{11,21}\) It binds with collagen and functions as the nucleus of hydroxyapatite. Together with the calcification of bone tissue and expression of TGF-\(\beta\) during development, this suggests that exercise is important for the activation of osteoblasts. The number of bone lacunae was greater in the SHAM group than in the OVX group, and osteocytes in the OVX + exercise group formed an intracellular network with relatively uniform intercellular spaces. This network bundle has been reported to have actin fibers that grow into the cells.\(^{25}\) In the present OVX group, objects thought to be very small osteocytes were observed at the sites corresponding to the center of the intercellular spaces. This reflects a decrease in direct communication between the osteocytes and bone matrix and indicates reduced transport of material such as ions and proteins between neighboring cells and reduced transport of substances from osteocytes to osteoblasts. However, the reduced osteocyte numbers and the fact that the some of the connections between the cells and the network joining them have been observed to be severed indicate that 1) the intracellular calcium concentration may respond to the formation of the osteocyte cytoskeleton and intercellular network, i.e., actin protein polymerization, and 2) calpain, the nick enzyme for other proteins, may act in response to changes in other conditions.\(^{26,27}\) Long, thin cell processes were observed to extend radially from osteocytes in the OVX + exercise group in this study, indicating that mechanical stimulation resulted in large osteocyte morphology in this study. The actin fiber bundles that developed may have maintained osteocyte morphology and rapidly transmitted to the osteocytes the information produced in response to mechanical stimulation. Osteocytes play an important role in mineral homeostasis in the body, and as Pritchard noted, the death of osteocytes signifies the death of the bone.\(^{28}\) OVX rat experiments indicate that changes in osteocyte function are important for maintenance of bone tissue during the developmental period. However, the present study clarified that the effects of mechanical stimulation on alterations in osteocyte morphology and network formation by osteocyte processes are also essential for bone mass regulation.

**AUTHOR DISCLOSURES**

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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**References**


