Abstract: Ovariectomized (OVX) rats are accepted models for screening potential therapeutic agents for osteoporosis. A low-mineral diet accelerates bone loss in OVX rats. This study aimed to determine changes in bone quality caused by ovariectomy or by combined ovariectomy and a mineral-deficient diet. Forty-eight female Wistar rats, aged 19 weeks, were distributed into 3 groups (n = 16 per group): Group 1: (control) subjected to a sham operation and administered a normal diet, Group 2: OVX rats fed a normal diet, and Group 3: OVX rats fed a low-mineral diet. The rats were sacrificed after 4, 8, 16, and 24 weeks. The density distribution and volume of the femur mid-shaft were evaluated to assess bone quality using micro-computed tomography and polarized microscopy. Bone strength was evaluated using a 3-point bending test. Bone density distribution, bone volume, and strength were the lowest for Group 3 and the highest for Group 1 at all times. Using a polarized microscope, we found that the tubular structures of the Haversian and Volkmann’s canals were decreased in Group 3 compared with those of Groups 1 and 2. Positive correlations were noted between fracture load and bone density, fracture load and bone volume, and bone volume and bone density in Group 3, and between bone density and volume, and bone volume and fracture load in Groups 2 and 3. We conclude that estrogen deficiency (ovariectomy) and mineral deficiency had separate as well as combined effects on bone quality.

Key words: Micro-computed tomography, Bone quality, Bone density, Bone strength, Ovariectomized rat
The aim of this study was to determine changes in bone quality caused either by ovariectomy or by combined ovariectomy and mineral-deficient diet as assessed by micro-CT, polarized microscopy, and the 3-point bending test. Bone quality was based on bone microstructure, volume, density distribution, and strength.

Materials and Methods

Animals

Forty-eight female Wistar rats (Sankyo Labo Service Co., Tokyo, Japan), aged 19 weeks, were used and were housed in individual metal cages at a room temperature (23 ± 1 °C) and 50 ± 1% humidity, with ad libitum access to food and water. The experimental protocol was approved by an animal experiment ethics committee (approval no. AP09-MD011). All experiments were conducted according to the Guidelines for the Treatment of Animals, Nihon University, Tokyo, Japan.

Preparation of osteoporotic rats

The 48 rats were randomly distributed into 3 groups (n = 16 per group). Group 1 (control) included sham-operated rats, who were provided with a normal diet (Table 1, American Institute of Nutrition (AIN)-93M, Nosan Co., Yokohama, Japan). Group 2 consisted of OVX rats on a normal diet, and Group 3 consisted of OVX rats on a low-mineral diet (Table 2, AIN-93M with reduced mineral content; Nosan Co.).

Rats were anesthetized with isoflurane. In the sham-operated group, the bilateral ovaries were raised and then returned to their original positions. The abdominal area was sterilized with 75% ethanol before surgery. The bilateral ovaries were raised and completely excised. The uterus and adipose tissues were placed back into the abdomen, and the incision was sutured closed. All the 3 groups of rats were on the designated diets at the age of 20 weeks. The low-mineral diet of Group 3 involved the replacement of calcium (Ca) composition (1.25%), phosphorus (P) composition (8.75×10⁻¹%), magnesium (Mg) composition (0.84×10⁻¹%), zinc (Zn) composition (5.78×10⁻³%), and fluorine (F) composition (2.22×10⁻⁴%) with sucrose as 3.5% of the mineral composition compounded with AIN-93M.

Measurement of body weight

Body weights were taken 1 week before surgery (age, 19 weeks), at surgery (20 weeks), and 1 week (21 weeks), 4 (24 weeks), 8 (28 weeks), 16 (36 weeks), and 24 weeks (44 weeks) post-surgery. Animals were then euthanized with carbon dioxide, and their bilateral femora were removed (n = 4, for each period and group).

Micro-CT

Femoral micro CT images were acquired by setting the measurement range at the mid-point of the full femoral length, which was measured using calipers (4.0 mm × 4.0 mm × 2.0 mm) (Fig. 1). The micro-CT (R-mCT; Rigaku Co., Tokyo, Japan) imaging conditions were as follows: tube voltage, 90 kV; tube current, 88 μA; magnification, ×6.7; measurement time, 17 s; resolution, 30 μm; slice thickness, 240 μm; and slice spacing, 240 μm. Micro-CT images of the femur and of phantoms were acquired and used to check the CT value.

1) Measurement of bone density

The micro-CT values of the bones in the recorded images were calibrated using air CT (500 mg/cm³) and target CT (800 mg/cm³) values. Digital images were converted to 16-bit gray scale TIFF format using the Atlas TIFF Convertor software (Rigaku Co., Tokyo, Japan), and were observed using TRI/3D-Bon BMD software (TRI/3D; Ratoc System Engineering Co., Tokyo, Japan). For bone density measurements, a hydroxyapatite calibration curve was prepared from images of phantoms (hydroxyapatite contents:

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<tr>
<th>Table 1. Composition of normal diet (AIN-93M) fed to Groups 1 and 2.</th>
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<th>Table 2. Compositions of the 3.5% mineral content diet (AIN-93M) fed to Groups 1 and 2 and the reduced mineral content of 3.5% low-mineral diet fed to Group 3.</th>
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200 mg/cm\(^3\), 300 mg/cm\(^3\), 400 mg/cm\(^3\), 500 mg/cm\(^3\), 600 mg/cm\(^3\), 700 mg/cm\(^3\), 800 mg/cm\(^3\), and 1550 mg/cm\(^3\)), and the femur was measured using the TRI/3D trabecular structure analysis routine (auto-detection mode) using the CT values.

2) Observation of 3 Dimensional color map

Bone situation (inferred from bone density values) was determined from a 3D color map showing bone density distributions obtained by micro-CT, represented in pseudocolors (red, orange, yellow, green, light blue, and purple). The 3D image analysis was conducted using TRI/3D image analysis software from bone density values. The conditions of the 3D color map were as follows: 1 pixel, 30 \(\mu\)m; range, 300\quad\text{to}\quad1,300\text{ mg/cm}^3.

3) Measurement of bone volume

An area in the right femoral mid-shaft (2 mm in length, 4 mm in diameter) for each femur was selected for micro-CT analysis. Using the TRI/3D menu, the minimum and maximum bone density values at 300 and 1,300 mg/cm\(^3\) were chosen to represent the cortical and trabecular densities, respectively.

Measurement of bone strength

The right femur was placed in physiological saline and stored at 8 \(^\circ\)C for 24 h. The bone was dried with a sterile cloth at the time of the test. Bone strength was measured using an Instron type testing machine system (TCM500CR, Minebea Co., Kanagawa, Japan). The femur was supported using a 3-point bending jig with a between-fulcrum distance of 10 mm, and a bending force was loaded on the middle point of the bone length at 5.0 mm/min. The bone strength and a fracture load-deflection curve were calculated from the data.

Polarized microscopy

The left femur was cut vertically at the midpoint of the long axis using a diamond disc. The bone tissue was dehydrated with a 70–100% ethanol series and 100% acetone, and embedded in resin (Osteoresin Embedding Kit, Wako Pure Chemical Industries, Tokyo, Japan). The specimens were cut into thin sections (about 30 \(\mu\)m) perpendicular to the long axis of the femur using a diamond disc (Isomet: Buehler Ltd, Illinois, USA), and unstained non-decalcified femur diaphyseal region specimens were obtained. Non-decalcified specimens of bone are complex tissues containing both inorganic and organic phases. Polarized microscopy measures polarization and interference due to birefringence\(^{17}\). Polarized microscopy was performed using an Optiphoto2-POL system (Nikon Co., Tokyo, Japan) with gypsum plate methods to analyze changes in the exterior and interior of the femur.

Statistical analysis

The means and standard deviations of body weight, bone density, bone volume, bone strength, and deflection value were calculated for each group and compared using one-way analysis of variance and Tukey’s multiple comparison test with IBM SPSS software (SPSS Inc., Illinois, USA). Furthermore, bone density, volume, and strength were evaluated between the 3 groups by Pearson’s correlation. Values of \(P < 0.05\) were considered statistically significant.

Results

Body weight

Figure 1 shows body weight measurements for the 3 groups at each observation time. In the week following the operation (ovariectomy or sham operation), the weight decreased slightly in all the groups, but it subsequently increased with time. From 8 to 24 weeks after the operation, the body weight for Groups 2 and Group 3.
Increased more than that of Group 1. Specifically, the body weights for the 3 groups at 19 weeks were as follows: Group 1, 195.25 ± 3.40 g; Group 2, 196.53 ± 1.73 g; Group 3, 194.58 ± 2.86 g. At 20 weeks, the values were as follows: Group 1, 194.48 ± 5.62 g; Group 2, 195.50 ± 1.68 g; Group 3, 191.38 ± 3.02 g; at 24 weeks, the values were as follows: Group 1, 252.13 ± 9.72 g; Group 2, 262.83 ± 9.23 g; Group 3, 278.93 ± 8.20 g. The body weight changes in each group were not significantly different for each observation period (4, 8, 16, and 24 weeks).

**Bone density**

Changes in bone density with time for each group are shown in Fig. 2. The bone density for Group 1 slightly decreased at 8 weeks and then slightly increased until 24 weeks. For Group 2, the density decreased with time and was significantly lower than that of Group 1. Specifically, the bone density for Group 1 slightly decreased at 8 weeks and then slightly increased until 24 weeks. For Group 2, the density decreased with time and was significantly lower than that of Group 1. The changes in bone density for the 3 groups are shown in Fig. 2. Differences between each of the groups at any given time are represented by paired tests (*P < 0.05, **P < 0.01). Group 1: SHAM-normal diet (solid line), Group 2: O VX-normal diet (dashed line), Group 3: O VX-low-mineral diet (dashed/dotted line).

Figure 3. 3D pseudocolor maps of cross section (Z-axis plane) of the femoral mid-shift region for the 3 groups (Group 1, control; Group 2, ovariectomy; Group 3, ovariectomy + low-mineral diet) 24 weeks after surgery. Red and orange indicate high bone density, yellow and green indicate medium bone density, and light blue and purple indicate low bone density.
that for Group 1 at 16 and 24 weeks. For Group 3, the density also decreased with time, with a greater decrease observed from 4 through 16 weeks, and a smaller decrease from 16 through 24 weeks. The bone density was significantly lower for Group 3 compared with that of Groups 1 and 2. The bone density values for the 3 groups at 24 weeks were as follows: Group 1, 1228.38 ± 6.00 mg/cm^3; Group 2, 1200.45 ± 7.08 mg/cm^3; and Group 3, 1179.35 ± 6.56 mg/cm^3.

3 Dimensional color map

Figure 3 shows the 3D color maps for the 3 groups after 24 weeks. High, medium, and low bone densities are indicated, respectively, by red and orange, yellow and green, and light blue and purple. For Group 1, the density of the exterior of the cortical bone was high, and the density of the region adjacent to the marrow cavity was low. The values of Group 3 were similar. The bone densities for Group 3 were than that of Groups 1 and 2. In summary, the cortical bone thickness in the femoral cross section decreased as follows: Group 1 > Group 2 > Group 3.

Bone volume

The femoral mid-shaft cortical bone volumes for all the groups are shown in Fig. 4. The bone volume increased slightly from 4 through 24 weeks for Group 1; it decreased slightly from 4 through 16 weeks and further from 16 through 24 weeks for Groups 2 and 3. The bone volumes for Group 3 were significantly lower than those for Groups 1 or 2, and the bone volume for Group 2 was significantly lower than that for Group 1, especially after 16 and 24 weeks. The bone volumes at 24 weeks for the 3 groups were as follows: Group 1, 94.70 ± 2.80 cm^3; Group 2, 73.69 ± 2.27 cm^3; and Group 3, 62.23 ± 2.10 cm^3.

Bone strength

The values for cortical bone strengths (fracture load) are shown...
in Fig. 5. For Group 2, bone strength decreased with time and was significantly lower than that for Group 1 at 24 weeks. For Group 3, bone strength also decreased with time, with a greater decrease observed from 4 through 16 weeks, and a smaller decrease observed from 16 through 24 weeks. For Group 3, bone strength was significantly lower for Group 1 from 4 through 24 weeks and was significantly lower for Group 2 from 8 through 24 weeks. The bone strengths at 24 weeks for the 3 groups were as follows: Group 1, 165.0 ± 5.9 N; Group 2, 130.6 ± 7.1 N; and Group 3, 102.2 ± 8.1 N. Typical fracture load-deflection curves for the 3 groups are shown in Fig. 6. The yield and final breaking points were higher for Group 1 than for Group 2 throughout the observation period, but the fracture load-deflection curves were similar. In contrast, the yield and final breaking points for Group 3 were lower than those of Groups 1 and 2 after 8, 16, and 24 weeks. In addition, changes in the fracture load-deflection curve
were observed for Group 3. The deflection levels (in mm) at the
time fracture after 4, 8, 16, and 24 weeks, respectively, were as
follows:

Group 1: 0.59 ± 0.04; 0.56 ± 0.03; 0.55 ± 0.04; and 0.56 ± 0.05
Group 2: 0.54 ± 0.07; 0.52 ± 0.05; 0.53 ± 0.05; and 0.49 ± 0.04
Group 3: 0.50 ± 0.04; 0.50 ± 0.07; 0.50 ± 0.05; and 0.46 ± 0.05

There was no significant correlation between the deflection
level and time period among the 3 groups.

Correlation among the bone density, volume, and strength

Results of the correlation analysis between the bone strength
and density, between the bone density and volume, and between
the bone volume and strength are shown in Figs. 7, 8, and 9,
respectively. No correlation was noted between the bone strength
and density for Groups 1 or 2, whereas a positive correlation (r = 0.692, \( P < 0.05 \)) was noted for Group 3 (Fig. 7). No correlation
was noted for Group 1, whereas the highest positive correlation
(r = 0.807, \( P < 0.01 \)) was noted for Group 2, and a positive
correlation (r = 0.699, \( P < 0.05 \)) was also noted for Group 3 (Fig.
8). No correlation was noted between the bone volume and strength
for Groups 1 or 2, whereas a positive correlation (r = 0.628, \( P < 0.05 \)) was noted for Group 3 (Fig. 9). Positive correlations were
noted between all parameters for Group 3.
Polarized microscopy

The femoral mid-shaft was observed at 24 weeks (Fig. 10). For Group 1, strong polarization was noted at low magnification (Fig. 10 A), at which the characteristic orientation of collagen fibers was observed. At higher magnification (Fig. 10 Aa), tubular structures of the Haversian and Volkmann’s canals and osteocytes were noted on both the exterior and the interior of the femur, and the boundary between the bone and marrow cavity was clear. For Group 2, strong polarization was noted at a low magnification (Fig. 10 B), similarly to Group 1, and the characteristic orientation of collagen fibers was observed. At a higher magnification, the tubular structures of the Haversian and Volkmann’s canals and osteocytes were noted on the exterior and interior (Fig. 10 Bb), but the bone was concave on the interior adjacent to the marrow cavity due to bone resorption. For Group 3, strong polarization was noted at low magnification (Fig. 10 C), and the characteristic orientation of collagen fibers was observed. At a higher magnification, osteocytes were present on the exterior and interior (Fig. 10 Cc) of the femur, but the number of tubular structures of the Haversian and Volkmann’s canals was decreased compared with those for Groups 1 and 2. Bone resorption, including collagen fibers in the interior, was more marked than that for Group 2, and the boundary between the bone and marrow cavity was unclear.

Discussion

The present study using rats demonstrates the effects of estrogen deficiency (ovariectomy) alone or combined with mineral deficiency on bone quality (strength, volume, density, and microarchitecture) as assessed by 3D micro-CT, polarization microscopy, and the 3-point bending test. The periods of observation (4, 8, 16, and 24 weeks) allowed the monitoring of the early and late changes in bone quality.

Rats in each of the 3 groups lost weight 1 week following ovariectomy, but gained weight thereafter, consistent with the findings of other investigators. For Groups 2 and 3, OVX-induced reduction of estrogen secretion influenced metabolism of various hormones, and lipid synthesis might have been particularly impaired, resulting in fat accumulation and body weight gain. In our study, a massive fat accumulation was observed when the abdomens of the OVX rats (Groups 2 and 3) were opened under general anesthesia.

Micro-CT is particularly useful for determining bone density and microstructural analysis of bone tissue and is widely applied in other fields. The analytical system employing micro-CT and TRI/3D used in this study is capable of 3D visualization of the trabecular structure, qualitative analysis by pseudocolor rendering, and quantitative analysis of bone volume, density, and trabeculae. In conventional micro-CT imaging, black and white images are evaluated because the color intensity representing X-ray penetration and non-penetration through materials is judged. Moreover, it is difficult to observe changes in bone quality with regard to the bone density presented in 3D color maps in conventional X-ray or pathological images, suggesting the usefulness of this analytical system including micro-CT. Furthermore, much useful information can be obtained by a single determination because this analytical system is capable of...
qualitative and quantitative analyses.

Although there have been many reports on OVX-induced bone density reduction and analysis of changes in the histology of bone cross sections, no study, to our knowledge, has evaluated bone quality based on the bone density in bone cross sections depicted by a pseudocolor scale. In the present study, changes in bone quality were evaluated by measuring femoral mid-shaft bone density and preparing 3D color maps. The mean bone density in the measurement range was acquired at 24 weeks. The cortical bone density was shown to be high on the exterior and low on interior for all 3 treatment groups with the lowest densities observed for Group 3 compared with those for Groups 1 and 2. Others have found that when bone density is reduced by OVX, reduced estrogen secretion strongly influences TNFα, IL-1β, and receptor activator of NF-kB ligand (RANKL) levels, leading to bone resorption by osteoclasts and induction of T-lymphocytes, which also cause bone resorption. The results of our present study support these findings. Moreover, since the blood mineral level was reduced in Group 3 because of reduced estrogen secretion and ingestion of a low-mineral diet, homeostasis might have been maintained by trabecular bone resorption resulting in the lower bone density compared with that for Group 2.

Ca²⁺ is one of the most important minerals for normal bone metabolism, and its intra- and extracellular levels are strictly controlled to maintain homeostasis. Bone is the largest Ca²⁺ storage organ, and it also regulates homeostasis itself. Since Group 3 lacked a sufficient supply of external minerals, the blood Ca²⁺ level might have been increased by trabecular resorption to maintain homeostasis. Further, we found here that bone volumes in Group 3 rats were influenced by reduced estrogen secretion and also by low-mineral diet-induced trabecular bone resorption in both cortical and cancellous bones. This resulted in more severe bone volume reduction than that of Group 2.

It has been reported that both bone formation and resorption are transiently promoted, and bone metabolic turnover was enhanced after OVX, and bone metabolism more rapidly responded in cancellous bone present in the marrow cavity than in cortical bone in long bones because of fast blood flow. Salomon et al. reported that bone resorption from the interior of the cortical bone is enhanced on the interior of the microtubular lumen of the Haversian and Volkmann’s canals, suggesting that the bone volume was reduced by resorption of cancellous bone trabeculae and the interior of the cortical bone. In our present study, the inner cortical bone of Group 3 rats was lower in density compared with Group 2. The concavity in the interior bone of Group 3 may be attributed to bone resorption. Furthermore, polarized microscopy revealed a rough structure in the resorbed inner cortical bone, decreasing the bone width for Group 3, compared with that for Group 2. A decrease in bone width was also reported by Salomon et al. and Thomas et al.

Bone mass, structure, density, and matrix composition are all important factors for maintaining bone strength. Moreover, bone strength is associated with its mineral content as well as with collagen structure, level, and maturity of crosslinking. Bone consists of dense cortical bone present at the exterior, and cancellous bone formed by pillar-like structures at the interior. The morphology and bone matrix of these structures of trabeculae are maintained through regular remodeling by osteoclasts and osteoblasts. However, in our present study, bone remodeling may have been uncoupled due to reduced estrogen secretion by Groups 2 and 3, decreasing the bone density, volume, and strength for Group 2. These decreases may have been promoted by the low-mineral diet fed to Group 3. Jiang et al. determined that the femoral bone strength of OVX rats fed a low-Ca diet (0.1% Ca) for 16 weeks was 128.5 ± 9.73 N. Here, the bone strengths after 16 weeks were 130.6 ± 7.1 and 103.8 ± 11.6 N for Groups 2 and 3, respectively. For Group 3, the bone volume decreased with time, and the reduced bone width and mineral content may have reduced bone strength. Moreover, the reduced bone microstructure, density, volume, and strength may have affected the bone tissue for Group 3 as indicated by the fracture load-deflection curve. Our present findings, therefore, support the close relationship between the bone strength and bone density reported by a National Institutes of Health (USA) consensus panel and many others.

The fracture load-deflection curves were similar for the 3 groups after 4 and 8 weeks, but changed for Group 3 after 16 and 24 weeks. We speculate that the difference could have been due to the greater loss in minerals that might have affected collagen crosslinking and the collagen/mineral ratio causing the bone to lose stiffness, thereby greatly lowering the fracture load for Group 3.

Polarized microscopy is widely used in mineralogy and crystallography to measure polarization and birefringence, and polarization properties are important in identifying crystalline and molecular structures. Using the gypsum plate method, the microscope stage was rotated by 90 degrees to increase or decrease light. For crystal orientation, purple, green, blue, white, and orange interference colors were observed according to the phase based on polarization properties. In the gypsum plate method, the double refraction intensity and orientation of collagen fiber can be distinguished based on color, so that differences in the bone structure between the exterior and interior of the cortical bone can be observed. For Groups 2 and 3, the boundary between the inner side of the cortical bone and bone marrow was unclear due to variation of the orientations of collagen fibers. The 3D color maps, the results of the bone volume determinations, and the polarized microscope images.
suggest that the interior of the femur for Group 3 was resorbed. This might have been due to the resorption of the cancellous bone and of the interior of the femur in the region with rapid blood flow around the marrow cavity, which occurred to maintain homeostasis.

Positive correlations between bone density, volume, and strength were observed for Group 3, indicating that the experimental conditions of reduced estrogen secretion (ovariectomy) and the low-mineral diet reduced bone density, volume, and strength, thereby causing the observed changes in bone tissue. An NIH consensus panel reported that the microstructure, bone turnover, micro-damage, calcification, and collagen crosslinking of bone tissue are involved in maintaining bone quality \(^4\). Since our present study indicates that osteoclast activity became more dominant than that of osteoblasts, the microstructures on the interior of the cortical bone must have been resorbed. Similar resorption was observed for cancellous bone trabeculae \(^12\). In the femur, which is a long bone, bone turnover is fast because of fast blood flow in the marrow cavity \(^25\), suggesting that trabecular resorption was enhanced. The low-mineral diet might have induced trabecular resorption to maintain homeostasis of minerals supporting bone, resulting in bone deterioration due to calcification. Since the Ca\(^{2+}\) intake was insufficient for bone formation, absorption in the small intestine and excretion and resorption in the kidney were likely impaired, which further promoted bone resorption and reduced bone quality.

**Conclusions**

Reduced estrogen secretion and a low-mineral diet cause reduced bone strength, density, and quality, and might have caused susceptibility to fracture. These findings suggest the importance of daily ingestion of required minerals for the prevention of bone loss and deterioration of bone quality.

**Acknowledgments**

This study was supported in part by Grants-in-Aid for Young Scientists (B) (22791942), a Grant for Supporting Project for Strategic Research (S0801032) 2008–2012 from The Ministry of Education, Culture, Sports, Science and Technology, Japan, and a research grant (AR056208) from the National Institute of Arthritis and Musculoskeletal and Skin Disease/National Institutes of Health (NIAMS/NIH), USA.

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