Radiological and histologic studies of the mandibular cortex of ovariectomized monkeys

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Objective. The objective was to study the radiological and histologic changes in the mandibular cortices of ovariectomized monkeys.

Study design. Twelve female, adult, Cynomolgus monkeys (Macaca fascicularis) were used. Under anesthesia, 1 group was bilaterally ovariectomized (OVX), and the other (control group) underwent sham surgery. Seventy-six weeks after surgery, the monkeys were humanely killed, their mandibles were excised, and their mandibular inferior cortices (MIC) and adjacent cortices were examined histologically and with panoramic radiographs and micro computed tomography.

Results. Striped shadows were seen on the endosteal side of the OVX cortices on panoramic radiographs. Histologic observation revealed many enlarged pores with eroded surfaces and calcein labeling (indicating osteon remodeling) in the OVX cortices.

Conclusions. In the MIC and adjacent cortices of OVX monkeys, enlarged Haversian canals were seen and there were indications of a high rate of bone turnover. The enlarged Haversian canals resulted in striped shadows and unclear endosteal margins on radiographic images. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;111:372-380)

Osteoporotic fractures are a worldwide health burden and result in lost physical mobility, increased medical costs, and poor quality of life. Mortality risk is also increased.1 In aging societies, such as Japan, the prevalence of osteoporosis exceeds 50% in women in their late 70s, and the prevalence for spine fractures ranges from 30% to 40% for women in their 70s.2,3 The prevention of osteoporosis is, therefore, of great importance in maintaining quality of life in the elderly and reducing medical expenditures for the treatments of fractures.

To screen for early signs of osteoporosis in elderly patients, dual energy X-ray absorptiometry (DXA) of vertebral bone mineral density (BMD) is commonly used. BMD measurements from both the spine and hip are recommended for a confident diagnosis of osteoporosis.4,5 One report recommends BMD testing for women older than 65 years, as well as for men older than 70.6 Recently, features of the mandibular inferior cortex (MIC) on panoramic radiographs have been demonstrated to be associated with BMD measurements. Two groups of investigators have found that people with osteoporosis have altered radiological findings with respect to their mandibular cortex morphology. One group suggests that erosive changes in the endosteal margin of the MIC is a reliable diagnostic indicator of osteoporosis.7,9 Because of the inconsistency of intraobserver and interobserver repeatability in detecting erosion, the other group (in its largest and most recent study) proposes that thinning of the MIC is better than cortical erosion in detecting osteoporosis.10-12 To date, no study has reported on the microstructural and histologic changes (caused by estrogen deficiency)
in the human MIC. To understand why radiographs show erosive changes and/or thinning, it is important to clarify our scientific understanding of these changes in the inferior cortices at the microstructural level. This improved understanding may allow us to establish further diagnostic methods based on radiographic observation of patients’ mandibles.

Because nonhuman primates have menopausal, anatomical, and physiological processes similar to humans, they are commonly used in research and have increased our knowledge of these processes. Because the type of investigation and histologic examination performed in this study would not be possible with human subjects, the microstructural and histologic changes in the mandibular cortices of ovariectomized monkey mandibles were investigated.

MATERIALS AND METHODS

Monkeys and experimental design

In our study, we used the mandibles of 12 adult, female wild-type Cynomolgus monkeys (Macaca fascicularis) that had been used to investigate the effects of estrogen on long bones and vertebrae. The monkeys were estimated to be older than 9 years, and no monkey was killed for our study. All procedures were in accordance with National Institutes of Health guidelines and institutional guidelines at Shin Nippon Biomedical Laboratories (Kagoshima, Japan). All experiments were reviewed by the Niigata University Committee for the Guidelines for Animal Experimentation and were performed according to the conditions and recommendations proposed by this committee. The monkeys were housed in individual metal cages (630 W x 700 D x 770 H mm³), at a constant temperature of 26 ± 4°C, a humidity of 50% ± 10% and a 12-hour-light (08:00-20:00)/12-hour-dark cycle. The monkeys were randomly divided into 2 groups of 6. Under surgical anesthesia (intramuscular chloral hydrate, 6 mg/kg body weight), the test monkeys (ovariectomized [OVX] monkeys) were bilaterally ovariectomized, and the controls (sham-operated monkeys) underwent sham surgery. Sham-operated monkeys had both ovaries exteriorized and reinserted intact to their original positions. To prevent possible infection, every day for 1 week after surgery each animal was injected intramuscularly with 0.5 mL ampicillin trihydrate (Kela Laboratoria NV, Hooogstraten, Belgium).

All animals were fed Teklad Global 25% Protein Primate Diet R (Harlan-Sprague-Dawley, Inc., Indianapolis, IN). Water was supplied using automatic water dispensers. Monkeys were injected intravenously with calcein (8 mg/kg; calcein: Dojin, Tokyo, Japan) 7 days before being humanely killed. Seventy-six weeks after surgery, animals were anesthetized with ketamine (6 mg/kg body weight, intravenously [IV]) and pentobarbital (1 mL/1.4 kg body weight, IV) and humanely killed.

Lumbar vertebral BMD

Just before surgery and 76 weeks after surgery, the BMDs of the lumbar vertebrae (L2-L4) were measured using DXA (Lunar DPX Alpha; Madison, WI). Because the monkeys had large differences in their initial BMD values, we determined the percentage change in lumbar vertebral BMD from the baseline BMD (percentage change [%] = [BMD at end of experiment/BMD at start of experiment] *100).15

Mandibular cortical BMD

Peripheral quantitative computed tomography (pQCT: XCT Research SA, Stratec Medizintechnik GmbH, Pforzheim, Germany) was performed at 0.18 mA and 50 kV with a slice thickness of 0.83 mm; the voxel edge length was 0.2 mm. A coronal scout view was obtained, with the distal roots of the second molars used as landmarks (Fig. 1, A). A reference line was drawn connecting the distal root apices (Fig. 1, B), and the area under this line was manually selected as the region of interest (Fig. 1, B square). The cortical BMD of the region of interest was determined with pQCT software (Contmode: 2, Peelmode: 2, Threshold: 394 mg/cm³).

Panoramic radiographs

Dental panoramic radiographs were obtained with an orthopantomograph 10S (Siemens, München, Germany) with an exposure of 15 seconds at 9.0 mA and 60 kV (Fig. 2, A). The MIC endosteal classes of the sham-operated and OVX monkeys were determined with the method used by Klemetti et al.7

Mandibular morphometry using micro computed tomography images

The right mandibular molar areas were scanned using micro computed tomography (microCT, Elescan; Nittetsu Elex, Osaka, Japan), with the following settings: voxel pitch, 54 μm; pixel size, 54 μm; projection number, 600; magnification, ×1.91; 80 kV; and 20 μA. A coronal image through the distal root of the second molar was used for the measurement of cortical thickness and pore volume (Fig. 3). The cortical thickness was calculated as the average of the values of 3 regularly spaced points on the buccal cortex (Fig. 3, arrows). To determine the pore volume, the total pore area on the section beneath the distal root of the right second molar (Fig. 3, square) was measured, and the pore volume was calculated; that is, (total pore area/total bone area) * 100 (Luzex F; Nireco, Tokyo, Japan).
Histologic observation and bone histomorphometry

The specimens were stained with Villanueva bone stain (Maruto, Tokyo, Japan) for 10 days, dehydrated with increasing concentrations of ethanol, and without decalcification embedded in methylmethacrylate (Wako, Osaka, Japan). The blocks were unilaterally ground down to the specimen surface. For histologic observation of the cortical bone in the second molar region, the block was mounted on a confocal, laser-scanning microscope (LSM-GB200; Olympus, Tokyo, Japan) and a fluorescence microscope (IMT-2, Olympus). After digitally saving the images, histomorphometrical analyses of the cortical areas were performed with an image-analysis system (Luzex F; Nireco, Tokyo, Japan). Bone histomorphometric parameters were as follows: the total number of pores, number of large pores (more than 30,000 μm²), number of pores with eroded surfaces, number of pores with calcein-labeled surfaces, and number of pores with both eroded and calcein-labeled surfaces.

Statistical analysis

The chi-square test was used to assess the differences in panoramic-radiograph, MIC endosteal classifications between sham-operated and OVX monkeys. Means and standard deviations (SDs) for each group for BMD and morphometric data were calculated and differences between groups assessed with the Mann-Whitney U test. Regression analyses were performed to assess the relationship between the MIC radiographic endosteal status and other parameters. For the purposes of this assessment, we assigned values of 3, 2, and 1 to C1, C2, and C3. For all tests, the level of statistical significance was set at P < .05. Statistical analyses were performed with statistical software (StatView J-4.5, Abacus Concepts, Inc., Hulinks, Inc., Tokyo, Japan).
RESULTS

Table I contains the results of panoramic-radiograph, MIC, endosteal-margin classifications. In sham-operated monkeys, there were 5, 1, and 0 monkeys with C1, C2 and C3, and for the OVX monkeys there were 1, 2, and 3. Sharp endosteal margins were visible in all but one of the sham-operated monkeys (Fig. 2, B); however, in almost all the OVX monkeys, striped shadows passed mesiodistally through the cortex, making the endosteal edges unclear (Fig. 2, C). A significant difference (P = .048) was found between sham and OVX groups with respect to their MIC endosteal classifications.

Table II contains the results of the testing for differences between groups for BMD and morphometric measurements. With respect to the percentage change in lumbar vertebral BMD, there was a significant loss in OVX monkeys compared with a slight gain in sham-operated monkeys. OVX monkeys also had significantly lower mandibular cortical BMDs than did the sham-operated monkeys. For microCT measurements, there was no difference in cortical bone thickness between groups, but pore volume was significantly higher in OVX compared with sham-operated monkeys. There was also no difference in number of pores, but there were more large pores and pores with eroded and/or calcein-labeled surfaces in OVX monkeys.

Table III contains the results of the regression analyses, with the radiographic MIC endosteal classification as the dependent variable. The percentage change in lumbar vertebral BMD, the mandibular cortical BMD, the pore volume measured in microCT images, the number of large pores, and the number of pores with eroded and/or calcein-labeled surfaces were significantly associated with the endosteal classification.

Fig. 3 contains microCT images of the cortices in sham-operated and OVX monkeys. More pores are visible in the cortex of the OVX monkey, which is in agreement with the results obtained with the microCT morphometry. Figs. 4 and 5 represent the histologic findings observed with confocal laser-scanning and flu-
orescence microscopy. In the longitudinal sections of the sham-operated cortex, several narrow canals pass mediodistally (Fig. 4, A [arrows] and B), whereas in OVX cortex, several enlarged canals are present (Fig. 4, D [arrows] and E). At high magnification, flattened osteoblastlike cells are present along the internal walls of the narrow canals in the sham-operated cortex (Fig. 4, C). In some of the OVX specimens, osteoclasts (indicating active bone resorption) were observed on the walls of the enlarged canals (Fig. 4, F).

**DISCUSSION**

OVX monkeys had significant losses of vertebral and mandibular-cortical BMDs compared with sham-operated monkeys, and this indicates that bone loss in both the lumbar vertebrae and mandibular cortex were successfully induced by ovariectomy. In the panoramic radiographs, the endosteal margins of the MICs were sharply defined for all but one of the sham-operated monkeys. On the other hand, 5 OVX monkeys had unclear MIC endosteal margins owing to striped shadows appearing in the MIC in response to estrogen deficiency. These findings are similar to those seen in human patients with osteoporosis. It is not clear why one of the OVX monkeys had a normal MIC classification. Perhaps this is because wild-type monkeys were used, or perhaps this represents normal biological variation as occurs in humans. Although less variation appears to be present in rats, monkeys are more similar to hu-

### Table II. BMD measurement and morphometric analysis results

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Sham</th>
<th>OVX</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumbar vertebrae</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% change of lumbar vertebral BMD</td>
<td>102 ± 4.2</td>
<td>88 ± 7.3</td>
<td>.0039†</td>
</tr>
<tr>
<td>Cortical BMD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mandibular cortical BMD, mg/cm³</td>
<td>1007 ± 160</td>
<td>645 ± 290</td>
<td>.010*</td>
</tr>
<tr>
<td>Morphometry by microCT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickness of cortical bone, mm</td>
<td>2.1 ± 0.12</td>
<td>2.2 ± 0.14</td>
<td>.26</td>
</tr>
<tr>
<td>Pore volume, %</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.4 ± 0.75</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Histomorphometry</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. pores</td>
<td>32 ± 2.3</td>
<td>31 ± 5.6</td>
<td>.75</td>
</tr>
<tr>
<td>No. large pores</td>
<td>1.3 ± 1.2</td>
<td>14 ± 7.3</td>
<td>.0051†</td>
</tr>
<tr>
<td>No. pores with eroded surface</td>
<td>0.2 ± 0.4</td>
<td>5.0 ± 3.1</td>
<td>.0039†</td>
</tr>
<tr>
<td>No. pores with labeled surface</td>
<td>0.8 ± 1.0</td>
<td>6.3 ± 1.9</td>
<td>.0039†</td>
</tr>
<tr>
<td>No. pores with eroded surface and labeled surface</td>
<td>0.2 ± 0.4</td>
<td>4.2 ± 2.3</td>
<td>.0039†</td>
</tr>
</tbody>
</table>

Values are presented as means ± SDs of the 6 monkeys per group.

BMD, bone mineral density; CT, computed tomography; OVX, ovariectomized.

*P < .05.
† P < .01.

### Table III. Result of single-regression analyses using MIC classification as the dependent variable

<table>
<thead>
<tr>
<th>MIC classification on dental panoramic radiographs</th>
<th>Standardized coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumbar vertebrae</td>
<td>–0.78</td>
<td>.003†</td>
</tr>
<tr>
<td>% change of lumbar vertebral BMD</td>
<td>–0.78</td>
<td>.003†</td>
</tr>
<tr>
<td>Mandible</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cortical BMD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mandibular cortical BMD, mg/cm³</td>
<td>0.60</td>
<td>.039*</td>
</tr>
<tr>
<td>Morphometry by microCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thickness of cortical bone, mm</td>
<td>0.19</td>
<td>.54</td>
</tr>
<tr>
<td>Pore volume, %</td>
<td>–0.82</td>
<td>.0011†</td>
</tr>
<tr>
<td>Histomorphometry</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total no. pores</td>
<td>0.32</td>
<td>.31</td>
</tr>
<tr>
<td>No. large pores</td>
<td>–0.84</td>
<td>.0007†</td>
</tr>
<tr>
<td>No. pores with eroded surface</td>
<td>–0.79</td>
<td>.0022†</td>
</tr>
<tr>
<td>No. pores with labeled surface</td>
<td>–0.75</td>
<td>.0054†</td>
</tr>
</tbody>
</table>

Number of monkeys = 6. The endosteal status, C1, C2, and C3 classifications were assigned 3, 2, and 1 values.

BMD, bone mineral density; CT, computed tomography; MIC, mandibular inferior cortices.

*P < .05.
† P < .01.
mans than are rats, and we assume that similar variation (as we found in monkeys) in MIC scoring may also occur in humans. One recognized limitation of using the Klemetti method when evaluating and classifying the status of the MIC endosteal margin is its reproducibility; however, some reports have suggested that this method has sufficient reproducibility to be effective as a diagnostic tool in general dental

![Fig. 4. Histologic longitudinal sections of the mandibular cortex. A, Confocal laser-scanning microscopic image of the cortex for a sham-operated monkey (original magnification ×2). Several narrow canals (arrows) pass mesiodistally through the cortex. Ma, marrow. B, Fluorescence microscopic image of the cortex for a sham-operated monkey (original magnification ×10). Several narrow canals are visible. The square region is magnified in C. C, Magnification of square in B (original magnification ×20). Flattened osteoblastlike cells (arrows) are visible along the internal walls of the thin canals. D, Confocal, laser-scanning, microscopic image of the cortex of an OVX monkey (original magnification ×2). Several enlarged canals (arrows) are visible in the cortex. Ma, marrow. E, Fluorescence, microscopic image of the cortex of an OVX monkey (original magnification ×10). The enlarged canal visible in the square is magnified in F. F, Magnification of square in E (original magnification ×20). In this specimen, osteoclasts (OC) indicating active bone resorption are visible on the wall of the enlarged canal.]

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practice, if it is used by an observer with sufficient experience and training.\textsuperscript{16,17}

With microCT, OVX monkeys had significantly larger pore volumes, and although there was no significant difference in the total number of pores between sham-operated and OVX monkeys, histologic examination indicated that the number of enlarged pores was significantly higher in OVX monkeys. This suggests that with estrogen deficiency, the pores in the MIC and adjacent cortices did not increase in number but en-

Fig. 5. Histologic transverse sections of the mandibular cortex. A, Confocal laser-scanning microscopic image of a mandibular transverse section from a sham-operated monkey (original magnification $\times$2). Ma, marrow. B, Fluorescence microscopic image of a mandibular transverse section from a sham-operated monkey (original magnification $\times$10). The square region was selected and magnified in C. Several small unlabeled pores are present. C, Magnification of square in B (original magnification $\times$20). Haversian canals (HC) are present in the osteons. D, Confocal, laser-scanning, microscopic image of a mandibular transverse section from an OVX monkey (original magnification $\times$2). Several enlarged pores (arrows) can be seen with calcein-labeling (\textit{short yellow lines}). Ma, marrow. E, Fluorescence microscopic image of a mandibular transverse section from an OVX Monkey (original magnification $\times$10). The square region is shown at high magnification in F. F, Magnification of square in E (original magnification $\times$20). An enlarged pore with eroded surfaces (ES), osteoid (OS), and calcein labeling (\textit{bright yellow}) is visible and indicates osteon remodeling.
larged in size. On the radiographic images, these enlarged canals (induced by estrogen deficiency) were visible as striped shadows inside the mandibular cortices, and resulted in the lack of clear endosteal margins.

In this study, animals were killed only 76 weeks after ovariectomy; therefore, if the pores had had more time to enlarge even further and reach the endosteal border of the cortex (a process that has been reported to occur in other bones),\textsuperscript{10} they might have led to semilunar defects and/or lacunar resorption on the endosteal margin.\textsuperscript{7} With the further increase in the number of striped shadows, the area of high density would become limited to only the inferior border, making the cortex appear thin, as previously reported.\textsuperscript{10}

In OVX monkeys, there were significantly more pores with eroded surfaces and/or calcine-labeled surfaces than in sham-operated monkeys. As a large number of pores with eroded and/or calcine-labeled surfaces is associated with osteon remodeling activity, these findings indicate active osteon remodeling, with ovariectomy having induced progressive bone turnover in the osteons of the mandibular cortex. Our study, therefore, found a relationship between osteon remodeling activity and dental radiographic findings (Table III) in that the status of the endosteal margin of the mandibular cortex was significantly associated with pore volume, the number of enlarged pores, and the number of pores with eroded and/or calcine-labeled surfaces. It appears that unclear endosteal margins on the mandibular cortex on panoramic radiographs represent enlarged Haversian canals.

Because these phenomena were found to occur inside the MIC and adjacent cortices of ovariecctomized monkeys (primates are similar to humans with respect to their hormonal system), this adds histologic and microstructural support to previous studies that have suggested that MIC radiographs may be valuable diagnostic tools for detecting systemic osteoporosis.\textsuperscript{7,9,19-24}

CONCLUSIONS

In the MIC and adjacent cortices of ovariecctomized monkeys, bone loss resulting from high bone turnover was found to have been induced by estrogen deficiency. Many enlarged Haversian canals with eroded and calcine-labeled surfaces in the cortex were visible, revealing the existence of osteon remodeling. Unclear endosteal margins seen on dental panoramic radiographs were associated with these enlarged Haversian canals and active bone turnover induced by the estrogen deficiency.

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REFERENCES


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