Bone fragility increases the risk of fracture. For example, osteoporosis is a common disease characterized by a decreased bone mineral density (BMD) and a microarchitectural deterioration of the bone structure (1). It is a chronic condition chiefly affecting postmenopausal women in whom the skeleton loses a significant percentage of its mineralized mass and mechanical resiliency, thereby becoming prone to fracture (2).

In general, bone health status is evaluated using the bone metabolic marker or special measurement instruments such as Dual-energy X-ray Absorptiometry (DXA), called the gold standard of the bone mass measurement. However, the bone metabolic marker is associated with an invasive risk and a cost burden. Moreover, DXA brings with it a risk of exposure to X-rays, and special facilities are necessary to produce X-rays. Thus, bone evaluation is associated with several restrictions. Recently, attempts to achieve earlier detection of diseases such as human immunodeficiency virus (HIV), cardiovascular disease, and cancer using salivary samples have been successful (3). Gathering saliva samples is far simpler than gathering urine or blood samples.

The most common type of osteoporosis is the postmenopausal bone loss associated with ovarian hormone deficiency and dietary low calcium intake (4–6). Estrogen controls the balance between bone formation and resorption (7, 8). Notably, when the circulating estrogen level decreases, calcium in the bones rapidly decreases, and calcium loss via urinary excretion increases (9–11). Moreover, we previously demonstrated that a deficiency in calcium intake induces further increases in urinary calcium excretion in ovariectomized rats compared with sham-operated rats (12). In those cases, the amount of calcium excreted in the urine may increase temporarily and then immediately decrease (13). This phenomenon is due to calcium homeostasis, which demonstrates that calcium in vivo plays an important role in physiological

The Relationship between Salivary Calcium Concentration and Differences in Bone Mineral Density Level in Female Rats

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Summary It has not yet been examined whether salivary calcium levels reflect changes in bone mass. The purpose of this study was to investigate the relationship between salivary calcium concentration and differences in bone mineral density due to estrogen deficiency and/or different calcium intake levels in female rats. In Experiment 1, the animals (n=14) were divided into an ovariectomized group (OVX) (n=8, 0.6% calcium diet) and a sham-operated group (Sham) (n=6, 0.6% calcium diet). The bone mineral density (BMD) levels of the tibia and lumbar spine were significantly lower in the OVX group than in the Sham group (p<0.001 and p<0.01, respectively), whereas there was no significant difference in the salivary calcium concentration between the two groups. In Experiment 2, after an ovariectomy operation, the animals (n=42) were randomized into five groups that received 0.01%, 0.1%, 0.6%, 1.2%, and 2.4% calcium diets (n=10, 10, 6, 8, and 8, respectively). The BMD levels of the tibia and lumbar spine were significantly lower in the 0.01% or 0.1% calcium diet intake groups than in the 0.6%, 1.2%, 2.4% calcium diet intake groups (all p<0.001), whereas there were no differences in the salivary calcium concentration among the groups. In conclusion, the salivary calcium level did not change during periods of decreasing BMD and bone strength induced by estrogen deficiency and/or calcium intake restrictions in female rats.

Key Words salivary calcium, bone loss, different calcium intake, estrogen deficiency

Bone fragility increases the risk of fracture. For example, osteoporosis is a common disease characterized by a decreased bone mineral density (BMD) and a microarchitectural deterioration of the bone structure (1). It is a chronic condition chiefly affecting postmenopausal women in whom the skeleton loses a significant percentage of its mineralized mass and mechanical resiliency, thereby becoming prone to fracture (2).

In general, bone health status is evaluated using the bone metabolic marker or special measurement instruments such as Dual-energy X-ray Absorptiometry (DXA), called the gold standard of the bone mass measurement. However, the bone metabolic marker is associated with an invasive risk and a cost burden. Moreover, DXA brings with it a risk of exposure to X-rays, and special facilities are necessary to produce X-rays. Thus, bone evaluation is associated with several restrictions. Recently, attempts to achieve earlier detection of diseases such as human immunodeficiency virus (HIV), cardiovascular disease, and cancer using salivary samples have been successful (3). Gathering saliva samples is far simpler than gathering urine or blood samples.

The most common type of osteoporosis is the postmenopausal bone loss associated with ovarian hormone deficiency and dietary low calcium intake (4–6). Estrogen controls the balance between bone formation and resorption (7, 8). Notably, when the circulating estrogen level decreases, calcium in the bones rapidly decreases, and calcium loss via urinary excretion increases (9–11). Moreover, we previously demonstrated that a deficiency in calcium intake induces further increases in urinary calcium excretion in ovariectomized rats compared with sham-operated rats (12). In those cases, the amount of calcium excreted in the urine may increase temporarily and then immediately decrease (13). This phenomenon is due to calcium homeostasis, which demonstrates that calcium in vivo plays an important role in physiological

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functions, and that the calcium level in blood should be kept constant \(3, 8\). A mineral reabsorption system also exists in the functioning of the salivary gland \(1, 4\). If the BMD level is different due to estrogen deficiency and/or different calcium intake in vivo, salivary calcium concentration is likely to be altered by the acceleration or deceleration of the mineral reabsorption system in the salivary glands. Thus, it is assumed that the amount of salivary calcium secretion would be a response to rapid bone loss. However, to our knowledge, the effect of bone loss due to estrogen deficiency and low calcium diet on the salivary calcium level has not yet been examined.

Accordingly, we proposed the hypothesis that the salivary calcium level would reflect bone condition. The purpose of this study was to investigate the relationship between salivary calcium concentration and differences in bone mineral density due to estrogen deficiency and/or different calcium intake levels in female rats.

**METHODS**

**Animals and feeding protocol.** In Experiment 1, 6 wk-old Sprague-Dawley female rats \((n=14)\) were divided into a sham-operated (Sham) group and an ovariectomized (OVX) group \((n=6, 8, \text{ respectively})\). Each group was fed a 0.6% calcium diet. The experimental period started 1 wk after the ovariectomy operation and lasted for the next 64 d. In Experiment 2, 6 wk-old Sprague-Dawley female rats \((n=42)\), after an ovariectomy operation, were randomized into five groups receiving 0.1%, 0.1%, 0.6%, 1.2%, and 2.4% calcium diets \((n=10, 10, 6, 8, \text{ and } 8, \text{ respectively})\). The experimental period started 1 wk after the ovariectomy operation and lasted for the next 64 d. In both studies, the numbers of animals differed among groups. In those experiments some conditions (e.g. 0.01% and 0.1% calcium intake groups) have been considered to have a higher death risk than the other groups. Thus, we added to the number of rats in those experimental groups in comparison to the other groups. Moreover, the reasons for these differences were that we excluded several animals because the animals died during the experiment. The animals were individually housed in cages under regular light/dark conditions (light on 8:00–20:00 h) and allowed access to food and distilled water ad libitum. Food consumption and body weight gain were measured every second day. The room temperature was maintained at 23±1°C, and the humidity range was 50±5%. Animal care and experimental procedures were approved by the Animal Experimental Committee of the University of Tsukuba.

**Collection of urine, saliva, bone, and blood.** Two and 3 d before the dissection, each rat was individually housed in a metabolic cage, and 24-h urine samples were collected using separators in both studies. Urine samples were collected under acidic conditions using 2 mL 2 N hydrochloric acid. At the end of the experimental period, the rats were deprived of food overnight. The rats were intraperitoneally injected with 0.5 mg/kg of pilocarpine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) to stimulate salivary secretion, and saliva collection was started 5 min after pilocarpine injection. Saliva was collected from the oral cavity by micropipette 15 min after the injection and was placed in microcentrifuge tubes. After the collection of saliva, under ether anesthesia, the rats were killed by exsanguination from the abdominal aorta. Then, samples of the lumbar spine, femur and tibia were isolated, and the muscle and connective tissue were carefully removed. Blood samples were obtained from the abdominal aorta.

**Saliva, serum, and urinary calcium concentration analysis.** The salivary calcium was diluted by a factor of 100 in nitric acid and determined using Inductively Coupled Plasma Atomic Emission Spectroscopy (Calcium measurement limitation=1 parts per billion: ICPS-8100, Shimadzu, Kyoto, Japan). The measurement of the salivary calcium concentration in the examiner was calculated by Intraclass Correlation Co-efficients \((0.992, p<0.001)\). Serum was separated by centrifugation at 2,500 rpm for 15 min at 4°C and stored at −80°C. Serum calcium concentration was measured by the Calcium E-test Wako (Wako Pure Chemical Industries, Ltd.) according to the manufacturer’s protocol. Urinary calcium excretion \((\text{mg/d})\) was measured using ICPS-8100 according to the manufacturer’s protocol.

**Breaking force and breaking energy of femoral diaphysis.** The breaking force and energy of the femoral diaphysis was assessed by three-point bending using the method of a previous study (distance between the fulcrum and the 1 cm, plunger speed 100 mm/min, full scale 50 kg, chart speed 120 cm/min) \((4)\). The breaking force indicated the loading weight (gravitational acceleration) that would cause bone breakage. Moreover, the breaking energy that indicated the workload that would cause the bone to break (moved workload at the power of 1 dyn) was exerted.

**Bone mineral content and bone mineral density of tibia and lumbar spine.** The bone mineral content (BMC) and BMD of the tibia or lumbar spine were analyzed by dual-energy X-ray absorptiometry (DXA: Aloka DCS-600R, Hitachi Aloka Medical, Ltd., Tokyo, Japan) as previously reported \((15)\). Briefly, the BMC of the lumbar spine was analyzed at the site of L3–L6. The BMC of the tibia at the proximal metaphysis site contains mainly cancellous bone. The tibia was divided into five divisions, and the first division was considered to be the proximal metaphysis site. The BMC of the tibia at the diaphysis site contains mainly cortical bone. The second to third divisions were considered to be the diaphysis site.

**Bone turnover markers analysis.** Serum bone-type alkaline phosphatase (bone-type Alp) activity was measured as previously reported \((16)\). Serum total alkaline phosphatase activity was determined at 37°C, and serum heat-stable alkaline phosphatase was determined at 56°C. Because the bone-type Alp deactivated at 56°C, it was calculated by serum total alkaline phosphatase activity minus heat-stable alkaline phosphatase activity. Tartrate-resistant acid phosphatase (TRAP) activities were measured as previously reported \((16)\).

**Statistical analysis.** All data are expressed as the mean±standard error of the mean (SE) and were analyzed with SPSS (version 12.0 J; SPSS Inc, Chicago, IL).
An independent t-test was used to test for statistically significant differences between groups in Experiment 1. One-way analysis of variance (ANOVA) was used to test for statistically significant differences among groups in Experiment 2. If significant differences were detected among groups, these were further evaluated by the post hoc Scheffe-test. Association between BMD and salivary calcium excretion was determined using the Pearson correlation test. The significance level for major effects was set at $p<0.05$.

**RESULTS**

**Experiment 1**

**Body weight, food intake, and food efficiency.** The final body weight and food intake were significantly higher in the OVX group than in the Sham group ($p<0.001$) (Table 1).

**Bone strength, BMC, and BMD.** No significant difference between Sham and OVX groups in the breaking force of the femoral diaphysis was observed. The breaking energy of the femoral diaphysis was significantly lower in the OVX group than in the Sham group ($p<0.001$). The BMC and BMD of the proximal tibia in the OVX group were significantly lower than these in the Sham group (all $p<0.001$). The BMC and BMD of diaphysial tibia in the OVX group were higher than these in the Sham group ($p<0.001$). The BMC and BMD of L3–L6 lumbar spine in the OVX group were lower than these in the Sham group ($p<0.001$). The BMC and BMD of the L3–L6 lumbar spine in the OVX group were lower than these in the Sham group ($p<0.005$) (Table 2).

These findings indicate that the bone was fragile due to the estrogen deficiency induced by OVX, although bone volume at diaphysial parts was increased by body weight.

**Salivary calcium, salivary flow rate, serum calcium, and urinary calcium.** There was no significant difference in the salivary calcium concentration between the Sham and OVX groups (Fig. 1A). There were no correlations between the salivary calcium concentration and BMD.

| Table 1. Final body weight, body weight gain, food intake, food efficiency (Exp. 1). |
|-----------------------------------|---|---|
|                                   | Sham | OVX |
| Final body weight (g)             | 277.0±10.8 | 351.2±11.3*** |
| Body weight gain (g/d)            | 2.1±0.17  | 3.3±0.19*** |
| Food intake (g/d)                 | 16.4±0.62 | 18.4±0.62*  |
| Food efficiency¹                  | 0.13±0.01 | 0.18±0.01*** |

Values are expressed as mean±SE. 
Sham, sham operated group ($n=6$); OVX, ovariectomy group ($n=8$). ¹Food efficiency is calculated by Body weight gain (g/d)/Food intake (g/d).

| Table 2. Bone strength, BMC of tibia and lumbar spine, and BMD of tibia (Exp. 1). |
|-----------------------------------|---|---|
|                                   | Sham | OVX |
| Breaking force ($\times 10^6$ dyn) | 21.1±0.9 | 22.0±0.7 |
| Breaking energy ($\times 10^6$ erg) | 15.4±1.1 | 10.0±0.7*** |
| BMC of proximal tibia (mg)        | 89.3±1.9 | 82.9±1.0*** |
| BMD of proximal tibia (mg/cm²)    | 167.1±3.0 | 142.7±3.6*** |
| BMC of diaphysial tibia (mg)      | 86.0±2.0 | 95.4±1.6*** |
| BMD of diaphysial tibia (mg/cm²)  | 125.3±2.0 | 129.3±0.9 |
| BMC of L3–L6 lumbar spine (mg)    | 499.4±15.0 | 470.3±15.6* |
| BMD of L3–L6 lumbar spine (mg/cm²)| 202.1±3.4 | 188.8±5.5 |

Values are expressed as mean±SE.  
Sham, sham operated group ($n=6$); OVX, ovariectomy group ($n=8$). ¹$p<0.05$, ²$p<0.001$ vs Sham.
of the lumbar spine in the Sham and OVX groups (Fig. 1B). There were no correlations between salivary calcium concentration and dietary calcium intake in Sham group ($r = -0.320, p = 0.368$).

There was no significant difference in the salivary flow rate between the Sham and OVX groups. Differences in serum calcium and urinary calcium concentrations between the Sham and OVX groups were not detected (Table 3).

**Bone metabolic markers.** Significant differences in the bone-type Alp and TRAP levels between the Sham and OVX groups were not shown (Table 3). Moreover, correlations between the salivary calcium concentration and dietary calcium intake in Sham group ($r = 0.185, p = 0.564$), TRAP levels ($r = 0.000, p = 0.999$) in the Sham and OVX groups were not observed.

**Experiment 2**

**Body weight, food intake, and food efficiency.** There was no significant difference in the body weight or food intake among any of the groups (Table 4).

**Bone strength, BMC, and BMD.** The breaking force of the femoral diaphysis was significantly lower in the 0.01% and 0.1% calcium diet intake groups than in the 0.6%, 1.2%, and 2.4% calcium diet intake groups (all $p<0.001$) and the breaking energy of the femoral diaphysis was significantly lower in the 0.01% and 0.1% calcium diet intake groups than in the 1.2% and 2.4% calcium diet intake groups (0.01%, or 0.1 vs 1.2%; $p<0.001$ respectively) (0.01%, or 0.1 vs 2.4%; $p<0.01$ respectively). Both the BMC and BMD at all sites were significantly lower in the 0.01% and 0.1% calcium diet intake groups compared with those of 0.6%, 1.2%, 2.4% calcium diet intake groups (all $p<0.001$) (Table 5).

These results indicate that the bone was made more fragile by a shortage in the amount of calcium intake in an estrogen-deficient state.

**Salivary calcium, salivary flow rate, serum calcium, and urinary calcium.** In Experiment 2, there were no significant differences in the salivary calcium concentrations

### Table 3. Serum BAP, serum TRAP, serum calcium, urine calcium, and salivary flow rate (Exp. 1).

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum bone-type Alp (mU)</td>
<td>32.8±3.42</td>
<td>38.2±4.59</td>
</tr>
<tr>
<td>Serum TRAP (mU)</td>
<td>10.8±0.95</td>
<td>11.6±0.84</td>
</tr>
<tr>
<td>Serum calcium (mg/dL)</td>
<td>8.9±0.33</td>
<td>8.3±0.31</td>
</tr>
<tr>
<td>Urinary calcium (mg/dL)</td>
<td>1.0±0.19</td>
<td>0.7±0.07</td>
</tr>
<tr>
<td>Salivary flow rate (mL/min)</td>
<td>0.22±0.07</td>
<td>0.13±0.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE.
Sham, sham operated group ($n=6$); OVX, ovariectomy group ($n=8$).

### Table 4. Final body weight, body weight gain, food intake, food efficiency, and salivary flow rate (Exp. 2).

<table>
<thead>
<tr>
<th></th>
<th>0.01%</th>
<th>0.1%</th>
<th>0.6%</th>
<th>1.2%</th>
<th>2.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>340.5±7.5</td>
<td>354.7±4.8</td>
<td>375.0±13.8</td>
<td>360.0±5.9</td>
<td>332.8±8.5</td>
</tr>
<tr>
<td>Food intake (g/d)</td>
<td>17.8±0.21</td>
<td>19.0±0.27</td>
<td>20.5±0.78</td>
<td>19.2±0.43</td>
<td>19.2±0.55</td>
</tr>
<tr>
<td>Food efficiency$^1$</td>
<td>0.19±0.00</td>
<td>0.19±0.00</td>
<td>0.20±0.00</td>
<td>0.20±0.00</td>
<td>0.18±0.01</td>
</tr>
</tbody>
</table>

Value are expressed as mean±SE.
Ovariectomized rats ($n=42$) were randomized into five groups, of 0.01%, 0.1%, 0.6%, 1.2%, and 2.4% calcium diets ($n=10, 10, 6, 8, and 8$, respectively).

$^a$p<0.01 vs 0.6%, $^{bb}$p<0.01 vs 1.2%.
$^1$Food efficiency was calculated by Body weight gain (g/d)/Food intake (g/d).

### Table 5. Bone strength, BMC of tibia and lumbar spine, and BMD of tibia (Exp. 2).

<table>
<thead>
<tr>
<th></th>
<th>0.01%</th>
<th>0.1%</th>
<th>0.6%</th>
<th>1.2%</th>
<th>2.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breaking force ($\times 10^6$ dyn)</td>
<td>9.0±1.2$^{aa, bb, cc}$</td>
<td>12.1±0.5$^{aa, bb, cc}$</td>
<td>23.8±1.6</td>
<td>24.2±1.2</td>
<td>23.2±0.8</td>
</tr>
<tr>
<td>Breaking energy ($\times 10^6$ erg)</td>
<td>5.5±1.0$^{bb, cc}$</td>
<td>6.1±0.4$^{bb, cc}$</td>
<td>11.7±1.1</td>
<td>13.8±2.0</td>
<td>11.7±0.8</td>
</tr>
<tr>
<td>BMC of proximal tibia (mg)</td>
<td>63.8±3.1$^{aa, bb, cc}$</td>
<td>68.4±1.3$^{aa, bb, cc}$</td>
<td>103.1±3.2</td>
<td>102.0±3.6</td>
<td>101.0±2.7</td>
</tr>
<tr>
<td>BMD of proximal tibia (mg/cm$^2$)</td>
<td>106.0±2.5$^{aa, bb, cc}$</td>
<td>113.5±1.4$^{aa, bb, cc}$</td>
<td>143.0±3.9</td>
<td>143.9±2.8</td>
<td>144.0±2.2</td>
</tr>
<tr>
<td>BMC of diaphyseal tibia (mg)</td>
<td>55.6±1.7$^{aa, bb, cc}$</td>
<td>57.7±0.9$^{aa, bb, cc}$</td>
<td>85.3±2.7</td>
<td>85.2±2.7</td>
<td>84.0±1.7</td>
</tr>
<tr>
<td>BMD of diaphyseal tibia (mg/cm$^2$)</td>
<td>94.1±3.3$^{aa, bb, cc}$</td>
<td>102.7±1.2$^{aa, bb, cc}$</td>
<td>133.4±2.1</td>
<td>131.7±1.9</td>
<td>133.2±2.0</td>
</tr>
<tr>
<td>BMC of L3-L6 lumbar spine (mg)</td>
<td>316.0±9.0$^{aa, bb, cc}$</td>
<td>320.4±1.6$^{aa, bb, cc}$</td>
<td>482.0±10.2</td>
<td>493.1±21.0</td>
<td>501.4±22.2</td>
</tr>
<tr>
<td>BMD of L3-L6 lumbar spine (mg/cm$^2$)</td>
<td>146.4±3.4$^{aa, bb, cc}$</td>
<td>150.9±1.5$^{aa, bb, cc}$</td>
<td>189.3±4.3</td>
<td>189.7±4.3</td>
<td>196.1±3.6</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE.
Ovariectomized rats ($n=42$) were randomized into five groups, of 0.01%, 0.1%, 0.6%, 1.2%, and 2.4% calcium diets ($n=10, 10, 6, 8, and 8$, respectively).

$^{aaa}$p<0.001 vs 0.6%, $^{bbb}$p<0.001 vs 1.2%,$^{ccc}$p<0.01,$^{cccc}$p<0.001 vs 2.4%.
among the groups (Fig. 2A). There were no significant correlations between the salivary calcium concentrations and BMD of the lumbar spine in the different calcium diets (Fig. 2B).

The serum calcium concentration was not significantly different among the groups. The urinary calcium concentration was significantly lower in the 0.01%, 0.1% calcium diet intake group than in the 1.2% and 2.4% calcium diet intake groups (all $p<0.001$), and it was significantly higher in the 2.4% calcium diet intake group than in the 0.6% calcium diet group ($p<0.05$). There were no significant differences in the salivary flow rate among the groups (Table 6).

These findings indicate that high or low calcium restriction does not influence salivary calcium concentration, though it leads to alteration of urinary calcium levels.

**Bone metabolic markers.** The bone-type Alp level was significantly lower in the 0.01% calcium diet intake group than in the 0.6% and 2.4% calcium diet intake groups (all $p<0.05$). Significant differences in the TRAP levels among all groups were not shown (Table 6). Moreover, there were no significant correlations between the salivary calcium concentration and bone-type Alp levels ($r=-0.067, p=0.686$), TRAP levels ($r=0.289, p=0.087$) in the OVX groups fed different calcium diets.

**DISCUSSION**

The purpose of this study was to examine whether the salivary calcium level would reflect the status of rapid bone loss induced by estrogen deficiency (Experiment 1) or calcium intake restrictions combined with estrogen deficiency (Experiment 2) in female rats. The major findings in the present study were that the salivary calcium level was not shown to be related with the decreases in BMD and bone strength induced by estrogen deficiency and calcium intake restrictions, and that the urinary calcium level was influenced by the calcium intake restrictions.

In the present study, the BMD levels of the proximal tibia and lumbar spine were found to decrease in OVX rats fed a 0.6% calcium diet compared with Sham rats fed a 0.6% calcium diet (Experiment 1). Moreover, the BMD levels of the tibia and lumbar spine were found to further decrease in OVX rats fed a 0.01% and 0.1% calcium diet compared with those fed a 0.6% calcium diet (Experiment 2).

These results were consistent with those of our previous studies (12). We also demonstrated that there were no differences among the 0.6%, 1.2%, and 2.4% calcium diet groups in BMD or bone strength in the OVX rats, suggesting that a higher-calcium diet may not maintain a higher level of bone status in an estrogen-deficient state. These results correspond to other human studies showing that increases in calcium intake have little

![Fig. 2. Salivary calcium concentration (A) and correlation between salivary calcium concentration and BMD of the L3–L6 lumbar spine (B). Values are expressed as mean±SE. Ovariectomized rats (n=42) were randomized into five groups, of 0.01% (●), 0.1% (■), 0.6% (△), 1.2% (○), and 2.4% (○) calcium diets (n=10, 10, 6, 8, and 8, respectively).](image)

Table 6. Serum BAP, serum TRAP, serum calcium, urine calcium, and salivary flow rate (Exp. 2).

<table>
<thead>
<tr>
<th></th>
<th>0.01%</th>
<th>0.1%</th>
<th>0.6%</th>
<th>1.2%</th>
<th>2.4%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum bone-type Alp (mU)</td>
<td>68.68±10.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>853.54±2.78</td>
<td>36.33±6.04</td>
<td>44.57±4.30</td>
<td>37.40±4.66</td>
</tr>
<tr>
<td>Serum TRAP (mU)</td>
<td>10.87±2.73</td>
<td>12.87±1.68</td>
<td>13.65±1.68</td>
<td>12.32±0.89</td>
<td>12.11±1.22</td>
</tr>
<tr>
<td>Serum calcium (mg/dL)</td>
<td>9.55±0.32</td>
<td>9.17±0.64</td>
<td>9.01±0.47</td>
<td>9.45±0.25</td>
<td>9.95±0.19</td>
</tr>
<tr>
<td>Urinary calcium (mg/dL)</td>
<td>0.32±0.22&lt;sup&gt;bb, ccc&lt;/sup&gt;</td>
<td>0.35±0.02&lt;sup&gt;bb, ccc&lt;/sup&gt;</td>
<td>0.73±0.07</td>
<td>1.17±0.01</td>
<td>1.79±0.17&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Salivary flow rate (mL/min)</td>
<td>0.20±0.01</td>
<td>0.20±0.08</td>
<td>0.16±0.01</td>
<td>0.15±0.02</td>
<td>0.23±0.10</td>
</tr>
</tbody>
</table>

Values are expressed as mean±SE.

Ovariectomized rats (n=42) were randomized into five groups, of 0.01%, 0.1%, 0.6%, 1.2%, and 2.4% calcium diets (n=10, 10, 6, 8, and 8, respectively).

<sup>a</sup> $p<0.05$ vs 0.6%, <sup>bb</sup>$p<0.001$ vs 1.2%, <sup>c</sup>$p<0.05$, <sup>ccc</sup>$p<0.001$ vs 2.4%.
Bone Mineral Density and Salivary Calcium Concentration

The salivary flow rate is believed to influence the salivary mineral resorption system (24). If the salivary flow rate becomes rapid, the salivary calcium concentration decreases. In addition, if the salivary flow rate becomes slower, the salivary calcium concentration increases. Thus, we measured the salivary flow rate to exclude the possibility that the salivary calcium concentration might be influenced by changes in the salivary flow rate. However, we confirmed that there was no difference in salivary flow rate between the OVX and Sham groups (Experiment 1) or among the OVX groups with differing calcium diets (Experiment 2). In the present study, we used an interperitoneal pilocarpine injection for the salivary secretion extract. When the salivary gland is functioning normally, pilocarpine activates the muscarinic receptor and promotes salivary water secretion (25). Although ovarian hormone deficiency may influence salivary gland function (26) and the symptoms of many xerostomia are present in postmenopausal women (27), estrogen deficiency induced by the OVX operation in the present study seems to have not influenced the adrenergic nervous stimulus of the salivary secretion function in the salivary gland.

The BMC and BMD at the diaphysial tibia in the OVX group were higher than those in the Sham groups, although those at the proximal tibia in the OVX group was low compared with the Sham group. These results may be related to differences in the body weight and the strain threshold for an osteogenic response of the two groups. A previous study reported that the activity of bone formation and the increase in cortical bone thickness caused by mechanical loading in the diaphysial part were greater than those in the proximal part (28). Moreover, it has been shown that threshold strain for activation of bone formation by mechanical loading in the diaphysial part differed from that in the proximal part in the long bone (29). Therefore, the BMC and BMD at the diaphysial tibia in the OVX group might have been increased by mechanical loading due to heavy body weight compared with the Sham group.

There are several limitations in the present study that should be acknowledged and addressed. First, the function of the salivary gland was not analyzed. Thus, it is not clear how estrogen deficiency and/or calcium restriction influence(s) the salivary gland function and extracellular calcium ion release system. Second, we could not evaluate whether other salivary mineral components would be influenced by OVX or the calcium-restricted diet. However, the main purpose of the present study was to examine whether the salivary calcium level would be related to decreases in bone mass, mainly decreases in calcium, induced by estrogen deficiency and calcium intake restriction. Third, we did not evaluate the calcium-regulating factor, namely 1,25(OH)2D and parathyroid hormone (PTH). Previous study reported that OVX and/or low calcium intake altered these factors in vivo (6, 22). However, the relationship between salivary calcium levels and calcium regulating factor was unclear, although it was considered that these factors would have been altered by OVX and/or calcium

Bone metabolic markers, namely the bone-type Alp and the TRAP levels, reflect the low BMD and a decrease in the bone strength caused by OVX or low calcium intake (12). In this study, the bone-type Alp and the TRAP levels reflected the BMD in the low calcium intake groups. On the other hand, the salivary calcium concentration did not correlate with the bone metabolic markers or the BMD. Therefore, the salivary calcium concentration is not likely to be related to the bone metabolic marker.

The influence of OVX on salivary calcium concentration during the periods of rapid bone loss, salivary calcium concentration might have been controlled by the salivary mineral resorption system in the salivary gland (21). More studies are required to investigate the influence of OVX on salivary calcium concentration before and after the operation, because it was unclear whether the estrogen deficiency would affect salivary calcium concentration.

Calcium loss via urinary excretion increases when calcium in bones rapidly decreases in an estrogen-deficient state (8, 10, 11). We confirmed the occurrence of urinary calcium excretion for the purpose of comparison with the salivary calcium concentration results.

In the results, the urinary calcium content accumulated over a day was significantly lower in the OVX groups fed 0.01% and 0.1% than in the OVX groups fed 1.2%, and 2.4% calcium diets (Experiment 2). A previous study reported that BMD levels and urinary calcium concentration were calcium intake-dependent, namely a 0.02–0.3% low calcium diet induced a low BMD and low urinary calcium concentration (12, 22), whereas a 1.0–3.0% high calcium intake induced high BMD and high urinary calcium concentration (6, 23). In this study, we gave a different calcium diet for the rats according to those previous studies to cause different BMD levels in each group. However, salivary calcium levels were not related to BMD of the lumbar spine, although urinary calcium concentration was altered by different calcium intakes. Thus, it was confirmed that the results for salivary calcium excretion were not consistent with the results for urinary calcium excretion. The dissection in the present study was performed 2 mo after the operation. The bone loss induced by OVX and the lower calcium diets would have been almost complete. To clarify the possible differences between saliva and urine in detail, time-course dissection is needed in a future study.

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Notably, in terms of both BMD and bone strength, there were no differences between the 0.01% and 0.1% calcium diet groups in OVX rats. The reason for the lack of differences was unclear, but it might be, partly, due to the increases in the efficiency of intestinal calcium absorption or renal calcium reabsorption induced by a lower calcium diet (20).

On the other hand, in the present study, no effects of estrogen deficiency and/or calcium diet restriction were seen on the salivary calcium level in female rats in which rapid bone loss occurred. Although it remains unclear why no differences were seen in salivary calcium concentration during the periods of rapid bone loss, salivary calcium concentration might have been controlled by the salivary mineral resorption system in the salivary gland (21). More studies are required to investigate the influence of OVX on salivary calcium concentration before and after the operation, because it was unclear whether the estrogen deficiency would affect salivary calcium concentration.

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intake restrictions.

In conclusion, the salivary calcium level did not change during periods of decreases in BMD and bone strength induced by estrogen deficiency and/or calcium intake restrictions in female rats. These data suggest that the salivary calcium level may not clearly reflect the status of rapid bone loss caused by estrogen deficiency and/or lower calcium intake.

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