Effects of Osthole on Postmenopausal Osteoporosis Using Ovariectomized Rats; Comparison to the Effects of Estradiol

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Osthole (7-methoxy-8[3-methylpent 2-enyl]coumarin), a plant coumarin compound, is extracted from a Chinese herb Cnidium monnieri (L.) CUSS which has been used since ancient times in China as a tonic and aphrodisiac. It was reported that castrated mouse received subcutaneous injections of an alcoholic extract of Cnidium monnieri (L.) CUSS once a day for 21 d, the copulation period appeared, suggesting that this alcoholic extract (including osthole) may have the estrogen-related activities. It has also been found that both total coumarins and osthole, extracted from Cnidium monnieri (L.) CUSS, have the positive effects on bone loss due to ovariectomy in rats, where cancellous bone histomorphometric variables in tibiae were preserved. However, even in animal experiments, little is known about the effects of osthole on other skeletal sites or other indices of bone metabolism such as biochemical markers of bone turnover, bone mechanical testing, etc.

Ovariectomy induced bone loss in rats and postmenopausal bone loss in humans share many similar characteristics, and similar skeletal response to therapy with 17β-estradiol. These similarities are strong evidence that the ovariectomized (OVX) rat bone loss model is suitable for studying the prevention and treatment of postmenopausal bone loss. Furthermore, the femoral neck of OVX rats, is a more clinically relevant sample site than other skeletal sites (i.e., proximal tibia) for preclinical testing of new therapeutic agents for the prevention and treatment of osteopenia.

The purpose of this study was to examine whether osthole has positive effects on bone loss due to OVX using the femoral neck of OVX rats; and, if so, whether osthole functions at the tissue level in a manner similar to 17β-estradiol.

MATERIALS AND METHODS

Experimental Protocols Twenty-four 3-month-old virgin female Wistar rats (220 ± 12 g, Charles River Inc. Tokyo, Japan) were used for the experiment. The animals were kept for 5 d before the onset of the experiment to acclimatize to our laboratory conditions (the room temperature was 25 °C for a 12 h/12 h light/dark cycle), then 18 rats were OVX and 6 rats were sham-operated under anesthesia with intraperitoneal (IP) injection of sodium pentobarbital at a dose of 30 mg/kg body weight. After operation, rats were kept in separate cages and fed standard diet before being sacrificed. To prevent hyperphagia associated with ovariectomy, the OVX rats were pair-fed to the mean intake of those in the sham group. All rats were allowed free access to drinking ion-exchanged distilled water for the duration of the whole experiment.

All rats were untreated for 2 weeks after surgery and divided into four groups (6 rats per group). The first group was sham-operated upon and received solvent vehicle (97% corn oil and 3% ethanol, 1.0 ml/kg). Groups 2 to 4 were OVX. Group 2 received solvent vehicle (same as the sham rats), Group 3 received 17β-estradiol (Sigma Chemical Company, St. Louis, MO, U.S.A.) 30 µg/kg and Group 4 received osthole (Wako Pure Chemical Industries Ltd., Tokyo, Japan) 9.0 mg/kg, 5 d/week for 4 weeks, respectively. 17β-estradiol or osthole was dissolved in a small volume of absolute ethanol and mixed with sesame oil to a proportion of 97% corn oil and 3% ethanol. Solvent vehicle and 17β-estradiol were given by subcutaneous injections, osthole was given by oral gavage from 2 weeks post-operation, respectively.

A 24-h fasting urine sample was collected by placing each rat in an individual metabolic cage 24 h before sacrifice. At sacrifice, blood was collected by cardiac puncture. Serum and urine samples were stored at −80 °C for biochemical analyses. The femurs and left tibia were also dissected out and cleaned of all soft tissue, right femur was placed in 10% phosphate-buffered formalin for 24 h for bone mass; left femur and tibia were filled in Ringer solution and stored at −80 °C for mechanical testing. The thymus, spleen and...
uterus were removed and weighed immediately. The success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns.

**Serum and Urinary Biochemical Markers of Bone Turnover Assays** Urinary deoxypyridinoline (DPD) was measured by an ELISA kit (Metra Biosystems Inc., Mountain View, CA, U.S.A.) and creatinine was assayed using a Creatinine HR-II Test Wako kit (Wako Pure Chemical Industries Ltd., Tokyo, Japan). Serum osteocalcin (OC) was determined by a RIA kit using rat osteocalcin standard, goat-anti-rat osteocalcin antibody, \(^{125}\)I-rat osteocalcin and donkey-anti-goat second antibody (Biomedical Technologies Inc., Stoughton, MA, U.S.A.).

**Cancellous Bone Mass Measurements** The proximal femur was sawed off and transferred to Villaneuva stain for 3 d, then returned to 70% ethanol, defatted in acetone, and embedded undecalified in methyl methacrylate\(^{10}\) with the flexor side facing down. Before sectioning, each methyl methacrylate block with bone sample was ground carefully on the flexor side, parallel to the long axis of the femoral neck, until the whole marrow cavity of the proximal femur, including that of the femoral neck, was exposed to ensure uniform and consistent positioning. Bone sectioning was then started at approximately one third the depth of the femoral neck.\(^{11}\) These longitudinal sections of the proximal femur were cut with an AO Autocut/Jung 1150 microtome at 4 μm thickness. The sections were stained by the Masson–Goldner-trichrome method.

With Olympus BH-2 light microscope and analysis by the public domain NIH Image (1.62) program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). The percentage of cancellous bone mass (BV/TV) was measured from the entire cancellous spongiosa of the proximal femur in a standardized mid region (1.0×1.5 mm\(^2\)) of a zone area which begins 1 mm distal to the growth plate-metaphyseal junction and extending further distally to the junction of the femoral neck and greater trochanter\(^{12}\) at a magnification of 100×.

**Bone Mechanical Testing** Prior to mechanical testing, the left femurs and tibiae were slowly thawed and held at room temperature on the day of test, then connective tissues were carefully removed, the length of the femurs and tibiae were measured with a micrometer.

The mechanical strength of the femur and tibiae shafts were determined using a three-point bending test with a Bone Strength Tester Model TK-252C (Muromachi Kikai Co., Ltd., Tokyo, Japan). Under the conditions of 12 mm sample space, 8 mm/min speed, and 50.0 kg load range. The force and energy necessary for the break at the center of the femur or tibia shaft were measured.

The mechanical strength of the left femoral neck was determined using a compression test with the same instrument. In femur shaft mechanical test, all femoral specimens were broken into two pieces, the proximal part was used and embedded in resin (Ostron II, GC Co., Ltd., Tokyo, Japan) up to the lesser trochanter and a cylinder was shaped. The sample was inserted into a steel support in a vertical orientation. The mechanical resistance to failure was tested by applying a vertical load on the femoral head at a speed of 2 mm/min. From the load–deformation curve, maximal load (ultimate strength, N), stiffness (slope of the linear part of the curve representing elastic deformation, N/mm), and energy (area under the curve, N×mm) were obtained.

**Statistics** Data are expressed as the mean±standard error (S.E.) for each group. Statistical differences between groups were evaluated with two-tailed student’s \(t\)-test and one-way analysis of variance (ANOVA). \(p\) values <0.05 were considered to be significant.

**RESULTS**

The Effect of Ovariectomy and Treatment with Estradiol or Osthole on Body Weight As shown in Fig. 1, rats in all four experimental groups \((n=6\) rats/group) had similar initial body weights. At 2 weeks after operation when the administration of either estradiol or osthole was begun, there was a significant increase \((p<0.01)\) in the body weight of the OVX rats (including OVX+estradiol and OVX+osthle rats) compared to the sham rats. Estradiol significantly suppressed this rate of increase and returned body weight to the sham level at 4 weeks after treatment \((p<0.05)\). However, osthole had no effect on the increased body weight of the OVX rat. Since they were pair-fed, the differences in the body weights of the groups were therefore not due to differences in amount of food ingested.

**Values for Cancellous Bone Mass (BV/TV) in the Femoral Neck** At 6 weeks after operation, ovariectomy caused a significant reduction (about 50%, sham vs. OVX; \(p<0.05\)) in the percent of cancellous bone mass at the femoral neck compared to the sham rats. Both estradiol (OVX+estradiol) and osthole (OVX+osthole) significantly protected the cancellous bone mass to levels of the sham rats \((p<0.05)\), and there was no significant difference between the treatment with these two drugs (Table 1).

**Changes in the Femur Length and Tibiae Length** During our experiment, no significant difference was found among the various groups, ovariectomy did not affect the femur and tibia length compared to the sham rats, estradiol...
Table 1. Percentage for Cancellous Bone Mass in the Femoral Neck

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cancellous bone mass (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n=6)</td>
<td>56.3±3.3(1)</td>
</tr>
<tr>
<td>OVX (n=6)</td>
<td>27.3±2.9(1)</td>
</tr>
<tr>
<td>OVX+estradiol (n=6)</td>
<td>49.6±4.2(1)</td>
</tr>
<tr>
<td>OVX+osthole (n=6)</td>
<td>51.1±5.8(1)</td>
</tr>
</tbody>
</table>

Data are expressed as mean±S.E. OVX: ovariectomized. Significant differences: a) vs. sham (p<0.05), b) vs. OVX (p<0.05).

Table 2. Changes in the Femurs Length and Tibiae Length

<table>
<thead>
<tr>
<th>Groups</th>
<th>Femur (mm)</th>
<th>Tibia (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n=6)</td>
<td>35.2±0.8</td>
<td>39.4±1.5</td>
</tr>
<tr>
<td>OVX (n=6)</td>
<td>36.6±0.7</td>
<td>39.8±1.6</td>
</tr>
<tr>
<td>OVX+estradiol (n=6)</td>
<td>35.9±1.1</td>
<td>39.1±1.1</td>
</tr>
<tr>
<td>OVX+osthole (n=6)</td>
<td>36.2±1.0</td>
<td>39.6±0.7</td>
</tr>
</tbody>
</table>

Data are expressed as mean±S.E. OVX: ovariectomized.

Table 3. Mechanical Parameters of the Femoral Neck

<table>
<thead>
<tr>
<th>Groups</th>
<th>Energy (mJ)</th>
<th>Stiffness (N/mm)</th>
<th>Maximal load (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham (n=6)</td>
<td>27.2±2.82</td>
<td>198.3±25.60</td>
<td>108.4±8.09(1)</td>
</tr>
<tr>
<td>OVX (n=6)</td>
<td>20.5±1.15</td>
<td>166.8±16.77</td>
<td>73.8±2.97(1)</td>
</tr>
<tr>
<td>OVX+estradiol (n=6)</td>
<td>22.7±3.63</td>
<td>168.1±23.90</td>
<td>107.9±10.69(1)</td>
</tr>
<tr>
<td>OVX+osthole (n=6)</td>
<td>24.3±3.27</td>
<td>179.7±26.30</td>
<td>106.8±5.94(1)</td>
</tr>
</tbody>
</table>

Data are expressed as mean±S.E. OVX: ovariectomized. Significant differences: a) vs. sham (p<0.01), b) vs. OVX (p<0.05).

(OVX+estradiol) and osthole (OVX+osthole) also did not influence the growth rates of these long bones (Table 2).

Mechanical Testing of Femoral Necks Ovariectomy caused a significant decrease of the maximal load of the femoral neck compared to the sham rats (p<0.01). Either treatment of estradiol or osthole remained the maximal load of the femoral neck to the levels of the sham rats, and no significant difference was found between the treatment with these two drugs. However, no significant effect was found on the stiffness and energy absorption of the femoral neck among various groups (Table 3). In addition, the mechanical parameters of the tibiae and femur shafts measured by three-point bending tests were not significantly different among various groups (data not shown).

Effects on Serum Osteocalcin (OC) Ovariectomy resulted in a significant increase in serum OC concentration compared to the sham rats (p<0.05). Estradiol significantly suppressed this increase to levels of the sham rats (p<0.05). No significant difference was found between levels of the OVX rats and OVX+osthole rats and osthole showed the significant increased serum OC levels compared to that seen in the OVX+estradiol rats (p<0.05) (Fig. 2).

Effects on Excretion of Urinary Deoxypyridinoline (DPD) Ovariectomy caused a significant increase in urinary DPD compared to the sham rats (p<0.05). Both OVX+estradiol rats and OVX+osthole rats all significantly decreased excretion of urinary DPD to levels observed in the sham rats (p<0.05), and there were no significant differences between these two drugs (Fig. 3).

Weights of the Uterus, Spleen and Thymus As expected, ovariectomy significantly caused atrophy of the uterus compared to the sham rats (p<0.05). Estradiol (OVX+estradiol) significantly increased uterine weight compared to OVX rats (p<0.01) but remained significantly lower than that of the sham rats (p<0.05). Osthole (OVX+osthole) did not affect the uterine weight and no significant difference was found compared to that of the OVX rats, but significant different from that of the OVX+estradiol rats (p<0.01). On
the other hand, ovariectomy significantly increased the thymus and spleen weights than that of the sham rats ($p<0.05$); Estradiol (OVX+estradiol) returned the thymus weight to the level of the sham rats, but remained the spleen weight significantly higher than that of the sham rats ($p<0.05$). Osthole (OVX+osthole) showed no effects on the thymus and spleen weights. Significant differences were found between treatment with these two drugs in the thymus and spleen weights, respectively (Table 4).

In addition, 9.0 mg/kg osthole had no effect on the behavior of sham rats (data not shown).

**DISCUSSION**

The present study suggests that the effects of 9.0 mg/kg p.o. osthole (5 d/week for 4 weeks), the dose selected on the basis of Li et al. and our preliminary experiment, protects bone against estrogen deficiency similarly to the effect of 30 μg/kg s.c. 17β-estradiol (5 d/week for 4 weeks). This is the usual dosage for 17β-estradiol treatment of osteopenia in estrogen deficient animals. In addition, a single dosage of osthole had no effect on osteopenia due to estrogen deficiency (data not shown).

OVX rats have been widely used as an animal model for the study of the prevention and treatment of postmenopausal osteoporosis. There are many observed similarities between ovariectomy-induced bone loss in rats and postmenopausal bone loss in humans. These include increased bone turnover with resorption exceeding formation, a significant loss of cancellous bone rather than cortical bone. The proximal femur in humans and rats share many histo-anatomic similarities and the femoral neck is clinically the most important site of fracture in humans. Therefore, the femoral neck of OVX rat, maybe a more clinically relevant sample site than other skeletal sites (i.e., proximal tibia). Furthermore, biochemical markers of bone turnover have been widely used as a research tool to measure effects of drugs on bone remodeling. Serum OC, a sensitive marker of bone formation, correlates with histomorphometric indice of bone formation. Urinary DPD, a specific marker of bone resorption, positively correlates with histomorphometric indice of bone resorption. These markers are useful to assess the protective treatment.

In this study, we used the mature rat model, in which rats are OVX at the age of 3 months. Significant cancellous bone loss in the femoral neck was seen together with the significant decrease in the maximal load, and as evidenced by the increase in the serum OC and urinary DPD levels, markers of bone turnover. These results support the former findings. However, when measuring the maximal load of the femoral neck in rats, several researchers did not find differences between OVX rats and sham rats. It is not clear yet if the reason for the possible discrepancy due to methodology or, for instance, differences in the sensitivities of assay and the species/strains or age of animals.

The beneficial effects of estrrogen treatment are well documented by many researchers for both OVX rats and postmenopausal women. Ovariectomy induced bone loss in the rats and postmenopausal bone loss share many similar skeletal response to therapy with 17β-estradiol. The precise molecular mechanism of estradiol in the treatment of postmenopausal osteoporosis has not well elucidated, but the major therapeutic effect is to inhibit resorption. According to previous evidence, estrogen replacement therapy protects against bone loss caused by estrogen deficiency. As shown by quantitative bone histomorphometry, conventional doses of estrogen decreases both bone formation and resorption. These decreases are reflected in reductions in serum OC concentrations and in urinary DPD levels. 17β-estradiol administration also had varying effects on organ weights. The present study supports these observations. Alternatively, some findings suggest that 17β-estradiol may have an indirect effect on osteoclasts by controlling the production of various cytokines in osteoclasts and monocytes. These cytokines have been shown to play an important role in osteoclastic bone resorption and osteoclastogenesis.

Osthole, a plant coumarin compound, is extracted from a Chinese herb *Cnidium monnieri* (L.) CUSS. It was reported that castrated mouse received subcutaneous injections of an alcoholic extract of *Cnidium monnieri* (L.) CUSS once a day for 21 d, thecopulation period appeared, this suggests that this alcoholic extract (including osthole) may have the estrogen-related activities. The positive effects of total coumarins and osthole, extracted from *Cnidium monnieri* (L.) CUSS, on bone loss due to ovariectomy have been reported, where cancellous bone histomorphometric variables in tibiae were preserved.

In the present study, we have demonstrated that the effects of osthole protect against the bone loss owing to estrogen deficiency in the femoral neck using OVX rats. Similar to estradiol, osthole significantly protected cancellous bone loss (as shown in Table 1) together with significantly increased the maximal load (as shown in Table 3) in the femoral neck of OVX rats.

A comparison of treatment with osthole to estradiol shows many differences. One difference between osthole and estradiol was their effects on body and organs weight (as shown in Fig. 1 and Table 4). As reported in the past, estradiol significantly suppressed the increased body weight of OVX rats and returned it to the sham levels, significantly increased uterine and decreased thymus and spleen weight compared to OVX rats. Though alcoholic extract of *Cnidium monnieri* (L.) CUSS has been reported to produce uterotrophic effects in mouse, but we found that osthole had no effect on either body weight or organs weight of OVX rats. These discrepancies might be caused by compounds other than osthole. This lack of uterotrophic activity could be beneficial in reducing the risk of endometrial, breast or ovarian cancer associated with estrogen treatment. Another difference between osthole and estradiol was their effects on the bone formation marker-serum OC (as shown in Fig. 2) measured in this experiment. Estradiol significantly decreased serum OC level to sham levels while osthole did not alter serum OC level. Because serum OC levels most likely reflect newly synthesized protein as well as that released from bone matrix during resorption, it is possible that osthole may primarily affect loss of OC from bone matrix without significantly affecting synthesis of new protein. The different effects of osthole and 17β-estradiol indicate that their mechanisms of action may differ in relation to their skeletal effects and also suggest that osthole does not work through the estrogen pathway, fully. Furthermore, osthole is a coumarin compound, and may
therefore affect the skeletal system and osteopenia by influencing the vitamin K pathway. Further study should be conducted to better clarify the dose-dependent and other effects of this drug.

In conclusion, the findings of the present study indicate that osthole, similar to estradiol, could be just as effective as 17β-estradiol administration in suppressing bone loss due to ovariectomy and that the main effects of osthole would be the protection of bone resorption. However, it has a reduced uterotrophic effect and a different effect on bone formation compared with estradiol. Thus the administration of osthole, instead of estradiol, maybe a useful treatment for bone loss due to estrogen deficiency.

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REFERENCES