Protective Effect of Puerariae Radix on Ovariectomy-induced Bone Loss in Rats

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Abstract — To examine the bone-protective effects of Puerariae Radix (PR) in an estrogen-deficient animal model, we treated ovariectomized rats with PR at a dose of 10.0 mg/kg 3 times a week for 16 weeks. Our results showed that PR increased mineral content and density of the trabecular bone at the neck of the left femur. Moreover, biochemical data indicated that PR had a positive effect on bone turnover. No endometrial hyperplasia was detected in the PR group. The present data suggest that PR should be considered for use in the treatment of bone loss in women with postmenopausal osteoporosis.

Keywords — Puerariae Radix, Osteoporosis, Bone-protective effect

Introduction

Osteoporosis is characterized by a reduction in bone mass with possible alterations in bone architecture and an increased risk of bone fractures (Kani et al., 1999; Raisz, 2005; Riggs, 1991; Rodan and Martin, 2000). The pathogenesis of postmenopausal osteoporosis is manifested by increased bone turnover with an increase in bone resorption by osteoclasts, resulting in decreased bone mass and a drop in ovarian estrogen levels. Postmenopausal osteoporosis is also associated with an increase in the production of proinflammatory cytokines including interleukin-1β (IL-1β) and interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α) and growth factors within the bone microenvironment (Kelly, 1996; Riggs et al., 2002). These proinflammatory cytokines contribute to osteoclastogenesis by increasing bone resorption (Pacifici, 1998; Weitzmann and Pacifici, 2006). Several studies have shown that estrogen replacement therapy (ERT) prevents bone resorption, resulting in the maintenance of skeletal mass and a reduction in the risk of fracture in post-menopausal women (Compston, 2005; Ribot and Tremolieres, 2006; Roggia et al., 2001). However, long-term hormone replacement therapy (HRT) with estrogen is associated with an increased risk of cancer in estrogen target tissues, including the endometrium and mammary glands (Bernstein, 2006; Creasman, 2005). Various pharmacological interventions are aimed at inhibiting bone turnover and preventing osteoporosis (Bahlous et al., 2006; Garnero et al., 1996; Garnero and Delmas, 1999). Until recently, calcium supplementation with vitamin D and HRT with estrogen, selective estrogen receptor modulators, calcitonin, raloxifene, amino bisphosphonates, teriparatide, parathyroid hormone, strontium ranelate, growth hormone and insulin-like growth factor-1 was the mainstay in the treatment of menopause-associated osteoporosis, resulting in the prevention or slowing of bone loss (Bilezikian, 2005; Compston, 2000; Hallworth, 1998; Poole and Reeve, 2005). However, recent attention has focused on the phytoestrogens as possible alternatives, or at least adjuncts, to HRT (Reinwald and Weaver, 2006; Usui, 2006; Zhang et al., 2004). Phytoestrogens, including flavonoids, coumestans and lignans, are plant-derived substances with estrogenic activity that structurally resemble 17β-estradiol (E2; Dang...
and Lowik, 2005).

Puerariae Radix (PR), commonly known as kalkon in Korea, is widely used in oriental medicine as an antipyretic and analgesic for treatment of the common cold, shoulder stiffness, issues in the occipital region and diaphoresis. PR is also used as an antiemetic and antidipsotropic agent. PR was previously shown to have antioxidant activity (Kim et al., 2004) and cardioprotective (Lee et al., 2002) effects. In addition, PR contains antioxidant enzymes that are known to affect lipid profiles in ethanol-treated rats (Lee, 2004; Lee et al., 2001), has been shown to ameliorate the effect of diabetes (Wang et al., 2004) and alleviate the effects of alcohol-induced disruption of hippocampal functions (Jang MH et al., 2003). PR has also been shown to modulate key early events in atherosclerosis (Sieveling, 2006), have protective effects on oxidative stress induced by hydrogen peroxide and streptozotocin (Kang KA et al., 2005), promote the immune response (Ma et al., 2002) and reduce the effects of lipolysis in differentiated 3T3-L1 adipocytes (Hong et al., 2002). Several recent studies have shown that PR can not only treat and prevent osteoporosis in elderly men with hypogonadism (Wang et al., 2005), but can also prevent bone loss in ovariectomized mice (Wang et al., 2003). Furthermore, PR promotes differentiation and mineralization in human osteoblast-like SaOS-2 cells (Huh et al., 2006) and PR contains high levels of isoflavones such as genistein and daidzein, which prevent bone loss in ovariectomized rats (Picherit et al., 2000; Ye et al., 2003; Lee et al., 2004).

Based on these studies, we investigated the preventive effects of PR on osteoporosis in ovariectomized rats as a postmenopausal bone loss model. Bone volume and thickness were monitored using a novel technique involving zoom-in microcomputed tomography (Micro-CT; Chun et al., 2006). We also screened for the influence of PR on uterine hypertrophy.

**Materials and Methods**

**Plant materials** – Puerariae Radix (PR) was supplied from the Dongjea Oriental Clinic (Ulsan, Korea). A voucher specimen (KHU-KSY-PR-001) was deposited at the herbarium of the Graduate School of East-West Medical Science, Kyung Hee University (Yongin, Korea). Specimens were identified and confirmed by Dr. Seong Cheol Lim (a specialist in plant classification).

**Extraction** – Dried plant material (3.0 kg) was extracted with distilled water in a reflux apparatus. The extract was filtered and then lyophilized with an aqueous extract yield of 270.0 g (9.0%, w/w).

**HPLC analysis** – A Shimadzu Instruments CO. LTD. (Tokyo, Japan) series HPLC system equipped with an auto sampler, a column oven, a binary pump and a degasser was used. A 20.0 ul volume of sample solution was directly injected on a YMC-PackODS-AM-303 column (4.6 × 250 mm) using an acetonitrile-water solvent system containing 0.1% acetic acid. Chromatography was conducted in a gradient mode using a 1.0 ml/min flow rate at 25 °C. The main isoflavones content from the PR extract were measured at 254 nm.

**Animals** – Nine-week-old virgin female Sprague-Dawley rats (n = 24) with a body weight of 172 ± 10.0 g were purchased from the Experimental Animal Research Center (Republic of Korea). The animals were divided into 4 groups based on diet and were randomly assigned to a sham-operated group (SHAM) or 3 ovariectomized (OVX) subgroups. The rats were housed together in cages at 20 ± 2 °C under a 12-h light/dark cycle, with free access to water for 7 days. Four weeks later, the rats were anesthetized with a combination of 1.5% isoflurane (Choong-Wae Pharma Co., Republic of Korea), 70% N2O, and 30% O2 using a Tabletop Research Anesthesia machine (SurgiVet, Waukesha, WI, USA). Bilateral ovariectomy was performed on the OVX animals via a dorsal midline incision, while the SHAM rats underwent the same surgery without ovariectomy. Immediately after surgery, the rats were given free access to a 5L79 diet (Dyets Inc., Bethlehem, PA, USA) containing 20% casein for 12 weeks. At 12 weeks after surgery, bone loss was confirmed and the trabecular bone in the femoral neck was compared between the SHAM and OVX groups using Micro-CT. One week later, the rats were assigned to the following treatments: SHAM and OVX groups, OVX + alendronate (1.75 mg/kg ALE p.o., 3 times a week) and OVX + PR (10.0 mg/kg PR p.o., 3 times a week), and were fed a 5L79 diet for an additional 12 weeks. The PR and ALE (Takara, Singa, Japan) were each dissolved in distilled water prior to administration. Body weight and food intake were measured once a week during the experimental period. Local mineralization in the trabecular neck of the left femur was compared using Micro-CT images calibrated for bone mineral content assessment after 4, 16, and 28 weeks. The experiments were performed in accordance with the animal care guidelines of the Korean Academy of Medical Sciences and the National Institutes of Health. Special care was taken to minimize suffering and the number of animals used. At 28 weeks after surgery, the rats were anesthetized using a chloral hydrate solution (0.3 ml/kg, Sigma-Aldrich), and...
exsanguination was performed by cardiac puncture followed by cervical dislocation. The blood was immediately placed on ice prior to serum isolation by centrifugation. The samples were then promptly frozen at −80 °C until inspection for the expression of markers related to bone turnover.

**Zoom-in microcomputed tomographic analysis (Micro-CT)** – Tomographic images were taken by Micro-CT according to a previously published method (Yin et al., 2006). Briefly, a Microfocus X-ray Source (L8121-01, Hamamatsu Corp., Hamamatsu City, Japan), a rotating subject holder, a CMOS flat-panel detector (C7943CP-02, Hamamatsu Corp.) and the parallel data processing system for Micro-CT were used. Scanning of the left femoral bones was performed in live rats at week 4, 16 and 28 using a tube voltage of 60.5 kV and a tube current of 170 μA. The rats were maintained in an anesthetized state (1.5% isoflurane, 70% N2O and 30% O2) for the duration of each measurement, approximately 22 min per rat. All images (512 × 512 × 512) were reconstructed from 450 projection data and pixel resolution was 15 μm. Bone morphometric parameters of the left femoral bone images were used to calculate trabecular thickness (Tb/Th; in μm) and bone volume over the total volume (BV/TV%).

**Histology** – Each uterus was immersed in a solution of 10% neutral buffered formalin for 48 h, processed in a tissue processor and embedded in paraffin using routine methods. Representative transverse sections (5.0 μm thick) were cut and stained with hematoxylin and eosin. Histopathological examination of the slides was performed using a light microscope.

**Serum biomarkers** – Serum biomarkers of bone formation were assessed at week 28. Serum alkaline phosphatase (ALP) activity was determined using a commercial ALP kit (Asan, Seoul, Republic of Korea). Osteocalcin (OC) was measured using an enzyme immunoassay (EIA) kit specific for rat OC (Biomedical Technology, Stoughton, IN, USA). The effects of PR and ALE treatments on bone resorption were evaluated using a RatLaps™ ELISA kit (Nordic Bioscience Diagnostics, Herlev, Denmark) to detect type I collagen C-terminus degradation fragments (CTX) generated by osteoclasts (Meli et al., 2004).

**Statistical analyses** – The means and SEM of the data were determined using the Statistical Analysis System (PRISM). Analysis of variance (ANOVA) was used to identify statistically significant (P < 0.05) differences among the groups. Tukey’s multiple comparison tests were used to confirm significant differences among the means (P < 0.05).

**Results and Discussion**

Phytoisoflavones exhibit a variety of biological activities and are widely used in foods, medicines and cosmetics. Several isoflavones have been isolated from PR. Based on the HPLC analysis, it was determined that PR contained, in the order of the amount detected, daidzin, daidzein, genistin and genistein isoflavones (Table 1).

Compared with the values in the SHAM group, the final body weights in the OVX group significantly increased after 28 weeks (Table 2). The body weights of the rats in the PR and ALE groups increased continuously during the study period. However, the mean final body weights of the animals in the PR and ALE groups were lower than those of the animals in the OVX group (5.79% and 2.41% lower, respectively). Moreover, this difference between the OVX and PR group was statistically significant.

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>mean</th>
<th>--- μg/g ---</th>
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<tbody>
<tr>
<td>Daidzin</td>
<td>184.62 ± 24.45</td>
<td></td>
</tr>
<tr>
<td>Genistin</td>
<td>247.84 ± 15.77</td>
<td></td>
</tr>
<tr>
<td>Daidzein</td>
<td>445.67 ± 3.98</td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>23.22 ± 1.55</td>
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**Table 2. Effects of Pueraariae Radix (PR) and alendronate (ALE) on food consumption, body weight and uterine weight in rats**

<table>
<thead>
<tr>
<th>Measure</th>
<th>SHAM</th>
<th>OVX</th>
<th>PR</th>
<th>ALE</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>174 ± 11.3</td>
<td>163 ± 2.08</td>
<td>181 ± 11.1</td>
<td>169 ± 9.54</td>
<td>0.1867</td>
</tr>
<tr>
<td>Final</td>
<td>284 ± 6.66</td>
<td>346 ± 18.06</td>
<td>326 ± 14.06</td>
<td>337 ± 12.5</td>
<td>0.021</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>18.0 ± 1.49</td>
<td>20.0 ± 0.07</td>
<td>19.4 ± 0.64</td>
<td>18.4 ± 1.98</td>
<td>0.6026</td>
</tr>
<tr>
<td>Uterus (mg/g)</td>
<td>1.60 ± 0.10</td>
<td>0.30 ± 0.046</td>
<td>0.30 ± 0.01c</td>
<td>0.30 ± 0.01c</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
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The values represent the means ± SEM (n = 6). Values with a superscript are significantly different from those in the SHAM groups. (a, P < 0.05; b, P < 0.01; c, P < 0.001).
significant. The average food consumption of the PR-treated rats after 28 weeks was 2.07% less than that of the vehicle-treated OVX rats, which was also statistically significant. The mean food consumption of the PR-treated rats was very similar to that of the ALE-treated rats. As expected, the mean uterine weight among the ovariectomized rat groups was significantly lower than that of the SHAM group, indicating success of the surgery. The ALE group did not show a significant increase in uterine weight compared to the OVX group, but the uterine weight was still lower than that in the SHAM group (Table 2).

Tomographic images of the femoral neck revealed differences in trabecular architecture among the treatment groups. PR was effective in reversing the loss of certain trabecular structural parameters at the neck of the femur, including trabecular thickness, bone volume and connectivity (Fig. 1). Trabecular bone volume (Fig. 2A) and trabecular thickness (Fig. 2B) at the neck of the femur were measured 4, 16, and 28 weeks after surgery. At week 4, the BV/TV and Tb/Th were not significantly different in each group before ovariectomy. At week 16 in the ovariectomized animals, BV/TV and Tb/Th were significantly decreased. There was also a reduction in BV/TV (24.79%, 32.98% and 41.22%) and Tb/Th (30.33%, 25.40%, and 16.43%) among the OVX, PR and ALE groups, respectively, compared with the SHAM group indicating that osteoporosis was successfully induced (Table 3). At week 28, the PR- and ALE-treated rats exhibited an increase in BV/TV compared with the OVX
controls (67.57% and 50.24%, respectively). The ratios of Tb/Th in the PR and ALE groups were significantly increased (19.37% and 15.32%, respectively) compared to the OVX controls. Representative samples of the extension data collected at 28 weeks showed increases in trabecular bone volume and trabecular thickness, indicating that PR had restorative effects on the microarchitectural properties of the femur (Fig. 1).

Uterine structure in the SHAM, OVX, PR and ALE groups was assessed by histological analysis. The SHAM rats exhibited a smooth endometrial luminal epithelium, a compact stroma and multiple endometrial glands with a viscous glandular secretion (Fig. 3A and C). Leukocytic infiltration into the stroma and epithelium was also observed. At 28 weeks after surgery, a wide, uniform and unproliferated lumen was observed in the OVX control rats with a single layer of smooth, poorly developed endometrial luminal epithelial cells (Fig. 3D, E, and F). The PR- and ALE-treated rats exhibited decreased cell height in the endometrial luminal epithelium and endometrial glands, similar to the OVX rats (Fig. 3G, H, and I, J, K, and L).

At the end of the 28-week study, the serum levels of several bone biomarkers were measured as indicators of the protective effects of PR and ALE on osteoporosis in ovariectomized rats. Serum OC and ALP activities were both lower in the PR- and ALE-treated rats than in the OVX rats (31.48% and 33.86%, vs. 55.19% and 55.82%, respectively). Type I collagen was abundant in the organic bone matrix, where it is broken down by an osteoclast-derived acid protease to produce CTX fragments. Serum CTX concentrations in the PR- and ALE-treated rats were lower than those in the OVX rats (49.20% and 56.23%, respectively). Thus, treatment with PR decreased the bone turnover rate (Fig. 4).

This study is the first to report the protective effects of PR against losses in bone strength using zoom-in Micro-CT and shows the reduction in endometrial hyperplasia in OVX rats. We predicted that PR-treated rats would exhibit increases in bone volume and thickness based on

### Table 3. Effects of Pueraiae Radix (PR) on the trabecular bone of the left femoral neck as measured by Micro-CT

<table>
<thead>
<tr>
<th>Parameters</th>
<th>BVTV (%)</th>
<th>Tb/Th (µm)</th>
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<tbody>
<tr>
<td>Groups</td>
<td>SHAM</td>
<td>OVX</td>
</tr>
<tr>
<td>4 weeks</td>
<td>35.94±2.51</td>
<td>39.40±2.51</td>
</tr>
<tr>
<td>16 weeks</td>
<td>32.50±2.18</td>
<td>24.44±1.60&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>28 weeks</td>
<td>26.60±2.75</td>
<td>17.87±1.50&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The values represent the means ± SEM (n = 6). Values with a superscript are significantly different from those in the SHAM (a, P < 0.05; b, P < 0.01; c, P < 0.001) or OVX (d, P < 0.05; e, P < 0.001) groups.
the fact that PR was previously shown to promote differentiation and mineralization in human osteoblast-like SaOS-2 cells and prevent bone loss both in castrated male mice and in ovariectomized mice (Jeong-Eun Huh et al., 2006; Xinxiang Wang et al., 2005; Xinxiang Wang et al., 2003). Ovariectomized rats have been widely used as a model of postmenopausal osteoporosis with estrogen insufficiency (Mosekilde, 1995). In a previous study, OVX rats showed significant increases in food intake and body weight compared to SHAM rats (Halaas et al., 1995). In addition, another study showed that PR decreased lipolysis in basal and isoproterenol-stimulated 3T3-L1 adipocytes (Hong et al., 2002). In the current study, animals in the OVX group had greater body weight gain than in PR-treated rats. Not only were the mean final body weights and food consumption in the PR and ALE groups significantly lower than those in the OVX controls by 5.79% and 7.31%, respectively.

Osteoporosis is a skeletal disorder characterized by compromised bone strength that predisposes individuals to an increased risk of hip and spine fractures. Bone strength is related to the overall quality of bone, which is determined by such characteristics as microarchitecture, geometry and material properties, all of which are affected by the rate of bone turnover. Therefore, measuring such microarchitectural parameters as percent bone volume, trabecular bone structure, trabecular thickness and trabecular separation may improve our ability to estimate bone strength (Kazakia and Majumdar, 2006; Kimura et al., 2002; Kurasawa, 2005; Teo et al., 2006; Yoneda et al., 1998). Our study is the first to demonstrate that PR prevents cancellous bone loss induced by estrogen deficiency in ovariectomized rats using zoom-in Micro-CT. Ovariectomized rats are a classic model of postmenopausal bone loss (Kalu, 1991; Thompson et al., 1995) as there are many similarities between ovariectomy-induced bone loss in rats and postmenopausal bone loss in

Fig. 3. Transverse sections of rat uterus. SHAM rats exhibited a smooth endometrial luminal epithelium (LE) with a compact stroma and normal endometrial glands (GE). (A) × 100 magnification. (B and C) × 400 magnification. OVX rats exhibited a marked reduction in the endometrial LE with poorly developed GE. (D) × 100 magnification. (E and F) × 400 magnification. The PR-treated rats had a slightly enlarged endometrial LE with GE similar to those of the OVX rats. (G) × 100 magnification. (H and I) × 400 magnification. The ALE-treated rats exhibited a marked reduction in the height of the LE and endometrial glands. (J) × 100 magnification. (K and L) × 400 magnification.
humans, including increased bone turnover with resorption exceeding formation and a significant decrease in cancellous bone in the vertebrate and proximal femur compared to cortical bone. Several studies have shown statistically significant trabecular bone loss in the femoral neck of ovariectomized rats at 1 month post-surgery, with a peak at 3 months (Li et al., 1997). Our study demonstrates the time-course of bone architectural changes in a rat model of osteoporosis as determined in vivo by Micro-CT measurements of the femoral neck over a period of 28 weeks. As expected, the OVX rats exhibited osteoporosis within 12 weeks of surgery, with large decreases in bone volume ratio and trabecular thickness-associated morphological parameters. Micro-CT analysis confirmed that PR effectively increased BV/TV and Tb/Th. An increase in BV/TV of 67.57% was observed at week 28 in the PR-treated group compared to the OVX group, while an increase of 50.24% was observed in the ALE-treated OVX rats. Bone thickness in the PR group increased by 19.37% at week 28 compared with that in the OVX group. Observation of the tomographical images of the trabecular bone in the femoral neck of OVX rats by Micro-CT indicated that PR has a potent effect on bone architecture.

Currently, we cannot examine the trabecular number, connectivity and separation of bone by Micro-CT; thus, further refinement of the technique is required. We calculated several bone parameters from the Micro-CT images taken at 4, 16 and 28 weeks after surgery. The area within each femur that was examined was left to the discretion of the observer; therefore, the exact position of the region of interest may vary among the measurements. However, the effect of the spatial mismatch did not seem to be significant since uniform bone loss was observed in all femurs. Registration of the longitudinally acquired Micro-CT images may be used to compensate for the effect of spatial mismatches in future studies.

Biochemical markers of bone turnover have been widely used as a research tool to measure the effects of various drugs on bone remodeling (Bahlous et al., 2006; Kurasawa, 2005; Withold, 1996). A correlation between OC and ALP serum levels, two sensitive markers of bone formation, and CTX, a measure of bone resorption, with microarchitecture has been reported in studies of postmenopausal women and laboratory animals (Yin et al., 2006). In the present study, PR decreased serum ALP activity as well as OC and CTX concentrations after 28 weeks. Such results suggest that PR prevents OVX-induced increase in bone turnover in rats.

Estrogen therapy is the treatment of choice for the prevention of bone loss in postmenopausal women, but its role in postmenopausal osteoporosis increases the risk of endometrial cancer. Recent research has focused on finding a therapy with positive skeletal effects without negative effects on reproductive tissue, like those caused by some selective estrogen receptor modulators (SERMs;

![Fig. 4. Effects of Puerariae Radix (PR) on biochemical indicators of bone turnover in ovariectomized rats. Serum osteocalcin concentrations (A) and serum alkaline phosphatase activities (B) were used as markers of bone formation. The type I collagen C-telopeptide concentration (C) was used as a marker of bone resorption. The values represent the means ± SEM (n = 6). Values with a superscript are significantly different from those in the SHAM (a, P < 0.05; b, P < 0.01; c, P < 0.001) or OVX (d, P < 0.05; e, P < 0.01) groups.](image-url)
Jordan et al., 2001). A previous study reported that ethinyl estradiol produced significant increases in uterine weight, uterine diameter, endometrial thickness, endometrial epithelial height and the number of endometrial glands (Ke et al., 1997). In our results, PR-treated animals did not produce these increases in uterine characteristics, even though structural similarities between the 2 groups were observed. In this study, the PR-treated OVX rats had decreased body and uterine weights, as well as endometrial hyperplasia; thus, PR may reduce the risk of breast or ovarian cancer associated with ERT/HRT.

In conclusion, our results indicate that PR increases trabecular bone microarchitecture by decreasing bone turnover without producing an increase in the weight of the uterus. Therefore, PR may have a beneficial role in preventing the loss of trabecular bone induced by ovariectomy in rats. Additional in vivo studies should provide further evidence that PR is a promising alternative to current therapeutic agents for the management of bone loss and the prevention of uterine cancer in women with postmenopausal osteoporosis. However, further research is needed to identify the mechanisms that mediate the action of PR.

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