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Puerariae radix prevents bone loss in castrated male mice

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Abstract

Puerariae radix (PR) is one of the earliest and most important crude herbs used in Chinese medicine for various medicinal purposes. PR contains a high amount of isoflavonoids, such as daidzein and genistein, which are known to prevent bone loss induced by estrogen deficiency. We have demonstrated that PR not only completely prevents bone loss but also significantly increases the bone mass at high doses in ovariectomized mice without exhibiting estrogenic action in the uterus. In this study, we examined whether PR exhibits effects on bone loss in androgen-deficient male mice similar to estrogen-deficient female mice. Male mice were orchidectomized (ORX) and fed a diet containing low, middle, and high doses (5%, 10%, and 20% of diet, respectively) of PR or normal diet with subcutaneous administration of 17β-estradiol (E_2, 0.03 μg/d; Sigma, St Louis, Mo) for 4 weeks. In ORX mice, the seminal vesicle weight decreased markedly, and it was not affected by the administration of any doses of PR and E_2. The bone mineral density (BMD) of the whole femur was significantly decreased by ORX, and the decrease in BMD was completely prevented by intake of the diet with the low dose of PR. Intake of the diet with the middle dose of PR further normalized BMD in ORX mice. Furthermore, the high dose of PR administration (PR20) significantly increased BMD in ORX mice, and the potency was similar to that of E_2. Morphometric analysis of the femoral metaphysis showed that intake of the diet with the low dose of PR completely prevented the decrease in bone volume/tissue volume and trabecular number and restored the increase in trabecular separation in ORX mice. In addition, intake of the diet with the high dose of PR further increased bone volume/tissue volume and trabecular number and decreased trabecular separation in ORX mice. These results propose the possibility that estrogenic Chinese herbs such as PR can be one of the candidates for the treatment or prevention of osteoporosis in elderly men with hypogonadism.

1. Introduction

Osteoporosis is one of the most common disorders associated with aging and is a substantial public health problem in elderly women and men [1]. A recent epidemiological study revealed that the lifetime risk of an atraumatic fracture is approximately 25% in an average 60-year-old man [2]. It has been reported that some of the male patients with osteoporosis have decreased gonadal function [3]. Therefore, the role of androgens in the bone metabolism has been traditionally noticed. However, Khosla et al [4] and Slemenda et al [5] recently reported that bone loss in elderly men is more significantly correlated with declining estrogen levels than with androgen levels. Furthermore, a study was designed to examine the relative contributions of androgen vs estrogen in regulating bone turnover in normal elderly men, which demonstrated that estrogen was the dominant sex steroid regulating bone resorption [6]. These results suggest that estrogen also plays an important role in regulating the aged male skeleton.

Nonsteroidal estrogenlike plant compounds called phytoestrogens are currently being investigated as alternatives to hormone therapy for the prevention and treatment of postmenopausal osteoporosis women [7-9]. Isoflavonoids are major classes of phytoestrogen and are derived mainly from soybeans and other leguminous plants. Daidzein and genistein are the major isoflavones found in these plants [10-12]. They have been shown to exert many biological effects in cell culture systems, animal models, and human beings and have received considerable attention for their potential role in preventing osteoporosis [13-15].

In previous studies, we have reported that genistein prevented bone loss caused by estrogen or androgen deficiency without substantial effects on the reproductive organs in both male and female osteoporotic animal models [16-22]. In the search for new naturally occurring anti-osteoporosis agents in plants, we found that *Puerariae radix*

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(PR), the root of a wild creeper plant *Pueraria lobata* (Willd) Ohwi, which is the same leguminous plant as soybean, possesses a high content of isoflavonoids such as daidzein and genistein. This plant, also known as "kudzu," is usually used as an ornamental ground cover and for green manure and fodder.

PR is one of the earliest and most important edible crude herbs used in Chinese medicine. This herb has been used primarily to treat the common cold, influenza, and wrist and shoulder stiffness or as an antidipsotropic agent in China, Japan, and Korea. For the last several decades, PR crude extract has been used for the treatment of hypertension and angina pectoris in China [23]. Recently, we have first showed that PR not only completely prevents bone loss, but also significantly increases the bone mass at high doses in estrogen-deficient mice without exhibiting estrogenic action in the uterus [24]. In this study, we investigated the effects of PR on the bone mass in androgen-deficient mice to examine whether PR exhibits effects similar to those in estrogen-deficient mice.

2. Materials and methods

2.1. Animal and intervention

Eight-week-old male mice of the ddY strain were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The mice were individually housed in 24 × 15 × 15-cm^3 cages under a 12:12-hour light/dark cycle at 22°C ± 1°C. The mice were divided into 6 groups of 8 mice each as follows: sham-operated (sham) + control diet, orchidectomized (ORX) + control diet, ORX + 5% PR diet (PR5), ORX + 10% PR diet (PR10), ORX + 20% PR diet (PR20), and ORX + 17β-estradiol (E_2, Sigma, St. Louis, Mo) fed control diet. E_2 (0.03 μg/d) was injected into the mice subcutaneously using a miniosmotic pump (Alza Corp, Palo Alto, Calif). The mice with the control diet were fed a powdered casein-based diet (modification of AIN-93G diet with corn oil instead of soybean oil). Crude PR powder (PR, 100 mesh) was purchased from Nihon Funmatsu Pharmaceutical Co (Osaka, Japan). The dry-powered PR was added to the diet at 5%, 10%, and 20% (wt/wt) instead of cornstarch because PR is rich in carbohydrate. The carbohydrate of PR is known as kudzu starch in Japanese foods, and it accounts for 80% of dried PR. The amount of daidzein and genistein in PR was quantified as 8.03 mg/g and 1.01 mg/g in our previous study [24]. The contents of calcium and phosphorus were 1.31 g and 59.7 mg per 100 g of PR, respectively. The mice were fed 4.0 g of the diet each day, and the daily intakes of the overall total of isoflavones in PR5, PR10, and PR20 groups were speculated as 1.8, 3.6, and 7.2 mg, respectively. The mice had free access to water. Four weeks after the experiment, the mice were killed, and the weight of the seminal vesicle was measured. The femurs were removed to analyze bone mineral density (BMD) and structure. All procedures were performed in accordance with the National Institute of Health and Nutrition Guidelines for the Care and Use of Laboratory Animals.

2.2. Radiographic analysis

Radiographic analysis of the femurs was performed by a soft x-ray system (model SRO-M50, SOFRON, Tokyo, Japan). Bone mineral content and BMD of the femur were determined using dual-energy x-ray absorptiometry (model DCS-600R, Aloka, Tokyo, Japan). BMD was calculated by bone mineral content of the measured area. The scanned area of the femur was equally divided into 3 regions (proximal, middle, and distal femur) to assess the regional differences in the femur.

2.3. Microcomputed tomography analysis

The femoral cancellous microarchitecture of the distal metaphysis was analyzed by the microcomputed tomography system (NX-CP-C80H1019, Nittetsu, Tokyo, Japan). Using 2-dimensional computed tomography, 9.43-μm thickness of one cross-sectional slice from each femur was scanned at the metaphysis, 1.06 mm from the distal lamina epiphysialis, to quantify morphometric parameters, such as trabecular

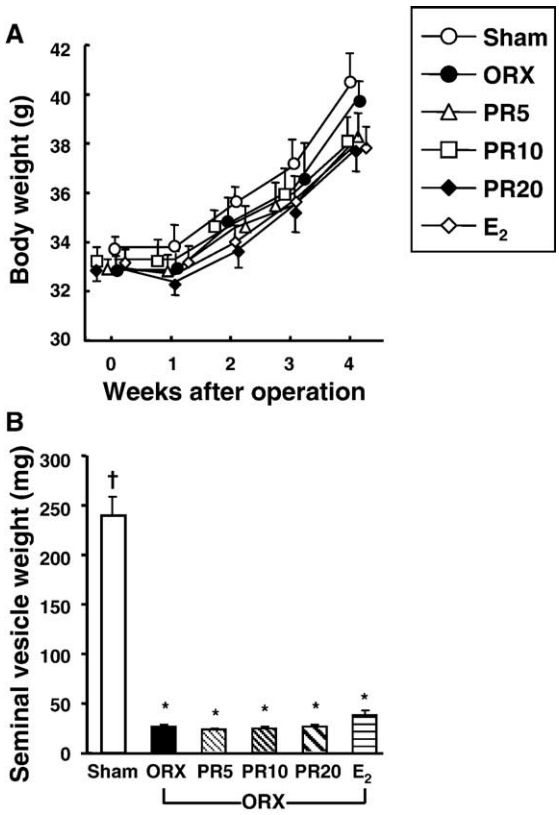

Fig. 1. Body weight and seminal vesicle weight in sham-operated mice (sham), ORX mice, ORX mice fed 5% PR diet (PR5), 10% PR diet (PR10), or 20% PR diet (PR20), or treated with 0.03 μg/d of E_2 subcutaneously. A, Body weight was measured at 0, 1, 2, 3, and 4 weeks after the operation in sham (○), ORX (●), ORX mice in PR5 (△), PR10 (□), PR20 (♦), or E_2 (◇) groups. B, Seminal vesicle weight was measured 4 weeks after the operation. Mean values not sharing a common marker were significantly different ($P < .05$). Data are expressed as the means ± SEM of 8 animals.

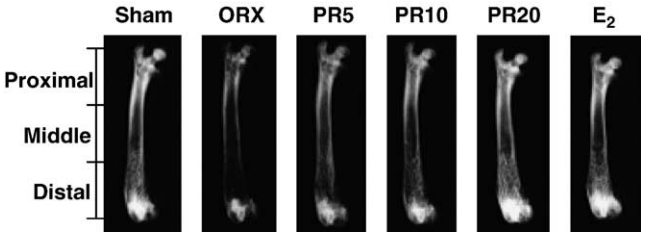

Fig. 2. Radiograms of the femurs collected from sham-operated mice (sham), ORX mice, ORX mice fed 5% PR diet (PR5), 10% PR diet (PR10), or 20% PR diet (PR20), or treated with 0.03 μg/d of E_2 subcutaneously. Femurs were collected 4 weeks after the operation and were used for x-ray analysis.

bone volume/tissue volume (BV/TV), trabecular number (TbNo), and trabecular separation (TbSp).

2.4. Statistical analysis

Data are presented as means ± SEM. The significance of the differences was determined by 1-way analysis of variance followed by Fisher protected least significant difference test (StatView 5.0, Abacus Concepts, Calabasas, Calif). Differences were considered significant at the level of $P < .05$.

3. Results

3.1. Body weight and seminal vesicle weight

Six groups of mice started with similar initial mean body weight (Fig. 1A). The mice in the all groups gained weight during the 4-week experimental period. The body weight was not significantly different in all the groups each week after surgery, because the animals were pair-fed, and the daily consumption of diet was not different among any group.

The seminal vesicle weight strikingly decreased in ORX mice, indicating that the mice were androgen-deficient. Intake of the diet containing 5% to 20% of PR for 4 weeks also did not affect seminal vesicle weight in the ORX mice group (Fig. 1B). Treatment with E_2 also did not affect seminal vesicle weight in ORX mice.

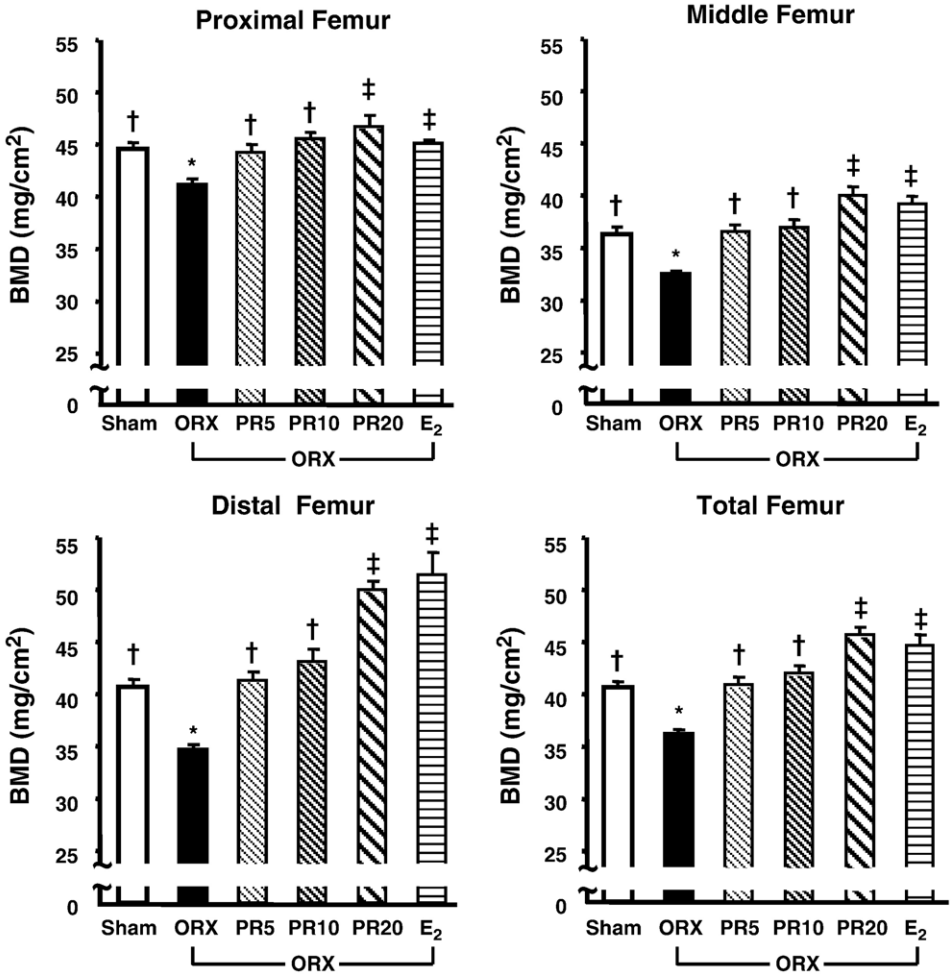

Fig. 3. BMD in sham-operated mice (sham), ORX mice, ORX mice fed 5% PR diet (PR5), 10% PR diet (PR10), or 20% PR diet (PR20), or treated with 0.03 μg/d of E_2 subcutaneously. The BMD of the proximal, middle, distal, and whole region of the femurs was measured 4 weeks after the surgery by dual-energy x-ray absorptiometry. Mean values not sharing a common marker were significantly different ($P < .05$). Data are expressed as the means ± SEM of 8 animals.

Table 1
Morphometric analysis of the trabecular bone in the distal femoral metaphysis in mice

	BV/TV (%)	TbNo (per mm)	TbSp (mm)
Sham	17.6 ± 2.1†	3.9 ± 0.3†	0.26 ± 0.03†
ORX	9.0 ± 1.6*	2.0 ± 0.3*	0.54 ± 0.08*
PR5	16.5 ± 1.3†	3.5 ± 0.2†	0.29 ± 0.04†
PR10	17.1 ± 0.8†	3.8 ± 0.1†	0.28 ± 0.03†
PR20	23.9 ± 2.4‡	4.5 ± 0.3‡	0.22 ± 0.02‡

Data are expressed as the means ± SEM of 4 animals. Femurs were collected 4 weeks after operation, and 2-dimensional morphometric parameters of trabecular bone were calculated by 2-dimensional computed tomography. Mean values not sharing a common marker were significantly different ($P < .05$). All differences were analyzed by multiple comparison with 1-way analysis of variance.

3.2. Bone mass analysis

To examine the effects of PR on bone loss induced by androgen deficiency, we fed ORX mice for 4 weeks with a diet containing 5% to 20% of PR, and the femurs were used for radiographic analysis. The x-ray radiograms of the femurs revealed that the mineralized bone mass significantly decreased, especially in the distal metaphysis in ORX mice. Intake of PR dose-dependently prevented the bone loss from the distal metaphysis of the femur in ORX mice (Fig. 2). Treatment with E_2 completely prevented the bone loss in ORX mice.

The effects of PR on bone mass in ORX mice were further determined by measuring the BMD. The whole femoral BMD was significantly reduced, by 10.9%, by ORX (Fig. 3), and the decrease in BMD caused by ORX was completely prevented by intake of the diet with 5% PR. Intake of 10% PR in the diet further increased BMD in ORX group compared with the 5% PR group, but the value was not significantly different. The high dose of PR administration (PR20) remarkably increased BMD by 26.1% and 12.4% compared with those in ORX and sham groups, respectively. The potency of PR20 is similar to that of E_2 in ORX mice.

To evaluate a site-specific effect of PR, we further analyzed the femoral BMD at the proximal, middle, and distal regions of the femur (Fig. 3). In the 3 regions of the femur, ORX significantly reduced the BMD in the distal region by 15.0%, compared with the proximal region by 8.7% and middle regions by 10.6%. At the distal regions of the femur, intake of 5% PR in the diet completely prevented bone loss in ORX group. Intake of 10% PR in the diet further increased BMD in ORX groups, but the value was not significant from 5% PR group. It is notable that the BMD in the PR20 group was obviously higher at all 3 regions compared with those in the sham-operated group, and this value was the same level as in the E_2 group.

3.3. Bone structural analysis

To define the effects of PR administration on trabecular bone, we analyzed the femoral cancellous microarchitecture of the distal metaphysis by the microcomputed tomography system. Morphometric analysis of the femoral metaphysis showed that BV/TV and TbNo were markedly decreased, whereas TbSp was dramatically increased in ORX mice compared with that in sham-operated mice (Table 1). However, administration with 5% PR in the diet completely prevented the decrease in BV/TV and TbNo and restored the increase in TbSp in ORX mice. On the other hand, intake of the 20% PR diet further increased BV/TV and TbNo and decreased TbSp in ORX mice compared with that in the sham-operated mice.

4. Discussion

Soy food and soybean isoflavones have received considerable attention as major diet phytoestrogen for their potential role in preventing osteoporosis [15]. However, there are few reports of PR, the same leguminous plant as soybean, affecting bone metabolism. The present study clearly demonstrates that PR, an important edible crude herb used in Chinese medicine, not only completely prevented bone loss, but also significantly increased the BMD at high dose in ORX mice. These results are consistent with our previous findings showing that PR protected bone loss and increased bone mass dose-dependently in ovariectomized (OVX) mice.

Clinical hypogonadism is a well-established cause of osteoporosis in men. It has been reported that an androgen deficiency caused by ORX results in bone loss as a result of stimulating bone resorption in experimental animals [25,26]. Erben et al [27] have reported that ORX reduces not only the serum levels of total and free testosterone, but also those of estradiol. They also showed that estradiol was the only significant predictor of histomorphometric indexes of bone formation and resorption in ORX animals. Miyaura et al [28] found that both male and female aromatase knockout mice exhibited marked bone loss, and bone mass was normalized by estrogen treatment. Aromatase knockout male mice show increased serum testosterone levels due to the lack of conversion of androgen to estrogen. Furthermore, it has been reported that a man with a mutated ERα gene exhibited delayed and low BMD [29]. Moreover, an aromatase-deficient man with no serum estrogen showed similar phenotypes in bone, similar to an ERα-deficient man, although the serum androgen level was adequate [30,31]. These findings indicate that estrogen aromatized from androgen plays an important role in bone metabolism in men. Because PR regulated bone metabolism in ORX mice in the same manner as that of E_2, it is possible that PR is effective in preventing bone loss caused by androgen deficiency. In fact, in this study, the whole femoral BMD was significantly reduced by ORX, and the decrease in BMD was completely prevented by intake of the diet with 5% PR (Fig. 3). ORX exactly reduced the BMD in the distal region, which is rich in trabecular bone, compared with the proximal and middle regions. The morphometric analysis in

the metaphysis of the femur showed that intake of the 5% PR diet completely prevented the decrease in BV/TV and TbNo and restored the increase in TbSp in ORX mice (Table 1). These results indicate that PR prevents bone loss by suppressing the increased trabecular bone resorption in ORX mice.

The PR used in this study contained 8.03 mg/g of daidzein and 1.01 mg/g of genistein, determined in our previous article [24]. In this experiment, a mouse was fed 4-g diet per day and was calculated to ingest about 1.8 mg/d (54 mg/kg body weight per day) of isoflavones in the diet with 5% PR. The amount of isoflavone intake was almost identical to that reported by Toda et al [32]. They demonstrated that oral administration of either glycoside of daidzein or genistein at 50 mg/kg body weight per day showed a preventive effect on bone loss in OVX rat.

The effective components of PR on bone appeared to be related to high contents of isoflavones, daidzein, and genistein. Intake of the diet with 20% PR significantly increased the whole femoral BMD in ORX mice, especially in the distal metaphysis compared with that in the sham-operated group (Fig. 3). The potency was similar to that of E_2. Morphometric analysis showed that PR at this high dose increased BV/TV and TbNo to levels higher than those in the sham-operated group (Table 1). Those results suggest that PR not only prevents bone loss, but also increases bone mass in the femurs of ORX mice. A mouse fed the diet with 20% PR ingested approximately 7.2 mg/d isoflavones. It is likely that this high amount of isoflavone can stimulate bone formation because we have recently recognized that ingestion of 4 mg/d isoflavones increases femoral BMD in normal mice [33]. Puerarin, another isoflavonoid in PR, did not affect bone mass in OVX mice (unpublished data). It is also possible that some components other than the isoflavonoids in PR may be attributable to the bone-forming activity of PR. Further studies are necessary to analyze the precise effects of PR on bone formation in ORX mice.

The mechanism of the effects of PR on bone is possibly that the isoflavones in PR affect the bone through estrogen receptors. The effects of PR on bone were similar to those of E_2 either in ORX or OVX animals. However, the administration of PR did not affect uterine weight in OVX mice in our previous article [24]. The different actions of PR from those of E_2 on the bone and uterus may be because of the selective effects of isoflavones on estrogen receptors. It has been already shown that daidzein and genistein have affinity for 2 estrogen receptor subtypes, ERα and ERβ, and they show higher affinity for ERβ than that for ERα [34]. It is possible that isoflavones in PR as natural selective estrogen receptor modulators activate estrogen signaling pathway in a tissue-specific manner.

In conclusion, we first showed that PR, an important edible crude herb used in Chinese medicine, not only completely prevents bone loss, but also significantly increases the bone mass at high doses in ORX mice. These results propose the possibility that estrogenic Chinese herbs such as PR can be one of the candidates for the treatment or prevention of osteoporosis in elderly men with hypogonadism.

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