

Long-Term Treatment of Puerariae Radix Extract Ameliorated Hyperparathyroidism Induced by Ovariectomy in Mature Female Rats

Xiao-Li Dong, Quan-Gui Gao, Sa-Sa Gu, Hao-Tian Feng, Man-Sau Wong and Liya Denney

Abstract—Postmenopausal osteoporosis is a disorder characterized by the progressive bone loss induced by estrogen deficiency in postmenopausal women. This imbalance affects calcium–phosphate metabolism and results in secondary hyperparathyroidism. Puerariae Radix (PR), the root of *P. lobata* (Wild.) Ohwi, is one of the earliest medicinal herbs employed in ancient China. PR contains a high quantity of isoflavones and their glycosides, which are regarded as phytoestrogen. Few investigations of PR are related to its osteoprotective effects. The present study is designed to administer PR water extract to ovariectomized (OVX) female rats, for the investigation of its possibly protective actions on bone and to delineate the potential mechanisms involved. Our results demonstrated that long-term treatment of PR could not significantly improve bone properties, whereas it greatly ameliorated the condition of secondary hyperparathyroidism induced by ovariectomy in those animals. PR might be useful as alternative regimen for protecting against postmenopausal bone loss.

Keywords—Hyperparathyroidism, Ovariectomy, Postmenopausal Osteoporosis, Puerariae Radix

I. INTRODUCTION

POSTMENOPAUSAL osteoporosis is an estrogen deficiency induced disorder of the bone characterized by the progressive loss of bone tissue in both natural and surgical menopause [1]. As a consequence of the lack of estrogens, bone turnover increases, leading to an imbalance between bone formation and bone resorption, favoring the latter [2], [3]. This imbalance affects calcium–phosphate metabolism and may increase serum parathyroid hormone (PTH) levels [4].

Increased parathyroid hormone secretion will then aggravate bone resorption and results in general skeletal demineralization [5]. In postmenopausal women, estrogen was found to reduce serum PTH levels, through a poorly understood mechanism [4], [6]. Consumption of dietary isoflavones has also been discovered to reverse the state of secondary hyperparathyroidism associated with estrogen withdrawal and hence lower the rate of bone turnover in postmenopausal women [7].

Xiao Li Dong, Quan Gui Gao and Sa Sa Gu are all with Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Kowloon, Hong Kong, PRC.

Hao Tian Feng is with Nestlé Research Centre Beijing, No 5 Dijin Road, Beijing 100095, PRC.

Man Sau Wong is with Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University and Shenzhen Research Institute of The Hong Kong Polytechnic University, State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen, 518057, PRC (Tel: +852-3400-8665; Fax: +852-2364-9932, e-mail: bcmswong@inet.polyu.edu.hk).

Liya Denney is with Nestlé Research Centre, Vers-chez-les-Blanc, 1000 Lausanne 26, Switzerland (Tel: +41-21-7858954; Fax: +41-21-7858544; e-mail: liya.denney@rdls.nestle.com).

Puerariae Radix (PR), the root of *P. lobata* (Wild.) Ohwi, is one of the earliest medicinal herbs employed in ancient China. It is also known as Gegen, Yegen or Kudzu vine root [8]. Kudzu root was first documented as a medicinal herb in the Divine Husbandman's Classic of Chinese Materia Medica (Shen Nong Ben Cao Jing) during the western Han Dynasty (206 BC – 8 AD) for the relief of fever, diarrhea and emesis [9]. According to the Pharmacopoeia of the People's Republic of China (PPRC), dried Puerariae Radix is indicated for the treatment of fever, acute dysentery, diarrhea, thirst, diabetes and hypertension [10].

Kudzu root is a rich source of polyphenolic compounds, including isoflavones, isoflavonoid glycosides, coumarins, puerarols, but-2-enolides and their derivatives. Isoflavones and their glycosides are the major bioactive constituents [11]. Animal and cellular studies have provided support for the traditional use of kudzu root on cardiovascular, cerebrovascular and endocrine systems, including diabetes and its complications [10]. In addition, some animal studies have proved the osteoprotective actions of Puerariae Radix (PR) in both ovariectomized female [8] and castrated mice [9]. These findings suggest estrogenic Chinese herb of PR could be one of the candidates for the treatment of osteoporosis in postmenopausal women and elderly men with hypogonadism.

The mechanisms of PR to exert osteoprotective effects, however, are far from clear and require further investigation. Hence, the present study is designed to administer PR water extract to ovariectomized (OVX) female rats, for the investigation of its possibly protective actions on bone and to delineate potential mechanism involved.

II. METHODOLOGY

A. Puerariae Radix (PR) water extract

PR water extract was purchased from Huangshan Tianjian Sci-Tech Ltd (Anhui Province, China). The content of total isoflavones of the extract was 18.7%, in which, the contents of two main isoflavones, Puerarin and Daidzin were 9.7% and 2.3% respectively.

B. Animal experimental design

Forty-eight 6 month-old SD female Sprague Dawley rats were randomly assigned to 4 groups. They were then subjected to bilateral ovariectomy (OVX) or sham-operation. After operation, the rats were allowed to rest for two weeks and fed with diets containing medium calcium content (MCD; 0.6%Ca, 0.65%P; Teklad, USA) before treatment.

For E_2 group, 17 β -estradiol (E_2) was injected intraperitoneally to OVX rats every week and fed daily with MCD diet. For PR group, Puerariae Radix (PR) water extract mixed with modified MCD (Supplement Basal Mix; Teklad, USA) was administered daily to OVX rats. Total treatment was 12 weeks.

Grouping is shown as follow:

- Sham: Sham + MCD diet
- OVX: OVX + MCD diet
- E₂: OVX + MCD diet + E₂ (200 ug/kg, weekly injection)
- PR: OVX + Supplement Basal Mix diet + PR water extract (300 mg/kg/d)

C. Treatment by diets mixed with/without PR water extract

The diet containing PR (Table I) was prepared by mixing modified MCD powder diet (Supplement Basal Mix Diets) with PR water extract, according to the dosage of herb at 300mg/kg/d and nutrition composition balance. Before treatment, sham or OVX rats were fed with MCD to adapt for one week. They were then administered with MCD or PR treatment diet at the dosage of 15g/rat/day. From week 6, the diet intake of all rats was adjusted to 13g/rat/day, and quantity of PR in treatment diet formula was kept constant (Table I). Total treatment period was 12 weeks.

D. Sample collection

On week 12, rats were placed into metabolic cages for 5 days, including 2-day adaptation and 3-day sample collection. Urine and feces were collected separately daily for 3 days. After the last day of collection, rats were sacrificed. Blood was withdrawn from abdominal aorta under light ether anesthesia. Serum was prepared and stored at -80°C for biochemical determinations. The tibias and femurs were collected, cleaned of all soft tissue, wrapped together with intact lumbar vertebra in saline-soaked towels, and stored at -20°C for further analysis.

E. Biochemical marker measurements

Serum and urine calcium (Ca) and phosphorus (P) levels were detected by clinical autoanalyzer. Osteocalcin (OCN), the major non-collagenous protein of bone matrix, is produced by osteoblasts, and is a commonly measured biomarker of bone formation. CTx is an abbreviation for urinary or serum collagen type 1 cross-linked C-telopeptide (CTx). CTx are sensitive markers of bone resorption in osteolytic diseases such as osteoporosis and osteoarthritis.

Serum OCN and urinary CTx were measured using commercially available immunoassay methods (Immunodiagnostic Systems Ltd, Boldon, UK). Serum levels of intact parathyroid hormone (PTH 1-84) were determined using rat bioactive intact PTH ELISA assay (Immutopics, Inc., San Clemente, CA). Serum 1,25(OH)₂D₃ was extracted with two separate extraction columns and measured by competitive enzyme immunoassay (Immunodiagnostik AG, Bensheim).

F. Analysis of Trabecular Structure using Micro-computed Tomography

Cone-beam X-ray micro-computed tomography (Micro CT), vivaCT 40 (Scanco Medical), was used to take Micro CT images of the femur end (FE), femur midshaft (MF), tibia head (TH) and tibia midshaft (MT) and intact lumbar vertebra (LV). The scanned regions of FE and TH were ~200 slices starting at 4.2 mm apart from femur end and 2.2 mm apart from tibia head.

The scanned bone contained both cortical and trabecular bone. Femur midshaft and tibia midshaft were located at approximately the middle of femur and tibia. Lumbar vertebra 2 (LV-2) was also selected for scanning. Next, three-dimensional images were set up based on the Micro CT images using three-dimensional image analysis software.

G. Statistical Analysis

Data from these experiments were reported as mean ± standard error of mean (SEM). All statistical analyses were performed using PRISM version 4.0 (GraphPad). Analysis of the effects of different treatment as grouping variables was performed by one-way analysis of variance (ANOVA). Differences in P value of less than 0.05 were considered statistically significant.

III. RESULTS

A. Body Weight and Uterus Index

We observed that PR treatment diet can decrease rats' appetite during feeding. Hence, by week 6, rats were fed with diets on the dosage of 15g/kg/d; from week 6 until the end of experiment, the feeding dosages were adjusted to 13g/kg/d, in order to balance diet intakes among different groups. Body weight in rats from all groups increased gradually during the whole treatment period. As previously reported, OVX rats had the greatest weight gain in comparison with sham rats (P<0.01 vs. OVX, table II). Injection of 17β-estradiol (E₂) did not significantly lower weight gain in OVX rats; however, PR water extract treatment significantly inhibited weight gain in OVX rats (P<0.001 vs. OVX, table II). In addition, as indicated by the uterus index, uterus weight of OVX rats was lower than sham rats, suggesting that the operation was successful (P<0.001 vs. OVX, table II). E₂ significantly increased uterus weight in OVX rats as expected (P<0.001 vs. OVX, Table 2). PR water extract also significantly stimulated uterus proliferation in PR treated OVX rats (P<0.05, vs. OVX, Table II).

B. Serum Calcium (Ca) and Phosphorus (P)

Serum Ca levels were found to be lower in vehicle treated OVX rats than that in sham rats (P<0.05, vs. OVX, table II). E₂ treatment seemed to increase serum Ca levels, though it was not statistically significant. PR treatment resulted in significant elevation of serum Ca levels in OVX rats (P<0.05, vs. OVX, Table II). Serum P levels did not differ between OVX and sham rats; E₂ treatment did not influence serum P levels; however, PR treatment significantly enhanced the serum P levels in OVX rats (P<0.01, vs. OVX, table II).

C. Urinary Calcium (Ca) and Phosphorus (P) excretion

OVX appeared to decrease urinary P and increase urinary Ca excretion in rats, but the changes did not reach statistical significance (Table II). E₂ treatment slightly decreased urinary Ca excretion but increased urinary P excretion. PR treatment did not exert any influence on urinary Ca excretion, but greatly suppressed urinary P excretion in OVX rats (P<0.05, vs. OVX, Table II).

D. Serum Calcitropic Hormones Levels

As expected, OVX rats appeared to have higher levels of serum PTH than sham rats, although the increase was not statistically significant (Fig. 1a). E_2 treatment appeared to suppress serum PTH levels in OVX rats and the alteration was also not statistically significant. In comparison, PR treatment significantly decreased the levels of serum PTH in OVX rats ($P < 0.01$, vs. OVX, Fig. 1a). Serum $1,25(OH)_2D_3$ levels decreased significantly in vehicle treated OVX rats when compared to those of Sham rats ($P < 0.05$, vs. OVX, Fig. 1b). Neither E_2 nor PR treatment significantly elevated the levels of serum $1,25(OH)_2D_3$ in OVX rats (Fig. 1b).

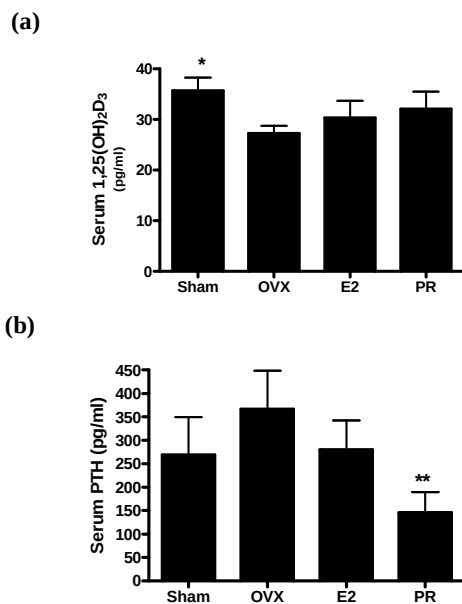


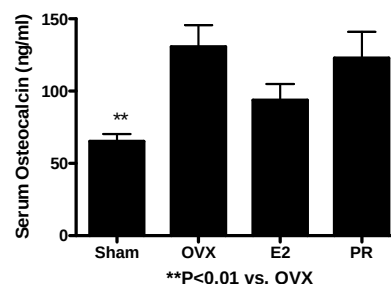
Fig. 1 Serum $1,25(OH)_2D_3$ (a) and PTH (b) levels in rats from different treatment groups. Rats were sacrificed at the end of experiments.

Blood was withdrawn from abdominal aorta under light ether anesthesia. Serum was prepared and levels of serum $1,25(OH)_2D_3$ and PTH were detected by commercial kits. Values are expressed as mean $\pm$ SEM, $n=9-10$. Sham, vehicle treated sham operated group; OVX, vehicle treated OVX rats; E_2 , Estrogen treated OVX rats; PR, PR water extract treated OVX rats. * $P < 0.05$, ** $P < 0.01$ vs. OVX group.

E. Bone Metabolism Markers

As stated, serum osteocalcin (OCN) and urinary collagen type 1 cross-linked C-telopeptide (CTx) have been chosen as the bone metabolism markers in the present experiment. Serum OCN was greatly increased in vehicle treated OVX rats in comparison with sham rats ($P < 0.01$ vs. OVX, Fig. 2a). E_2 treatment appeared to decrease the levels of serum OCN, although the change was not statistically significant (Fig. 2a). PR treatment did not alter serum OCN levels in OVX rats (Fig. 2a). Urinary CTx levels were expressed as a ratio to urinary Creatinine (Cr). Similarly, urinary CTx levels were found to be significantly increased in the vehicle treated OVX rats when compared with those of sham rats ($P < 0.05$ vs. OVX, Fig. 2b). Both E_2 and PR treatment have seemingly downregulated urinary CTx levels, but that was not statistically significant (Fig. 2b).

(a)



(b)

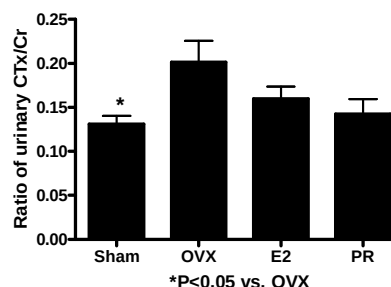


Fig. 2 Serum osteocalcin (OCN) and urinary C-telopeptide of type I collagen (CTx) in rats from different treatment groups. At the end of experiment, rats were put into metabolic cages for 5 days, including 2-day adaptation and 3-day sample collection. Urine was collected daily for 3 days. The third day urine sample in each rat was used for detection of urinary C-telopeptide of type I collagen (CTx). The final result of urinary CTx was corrected by urinary Creatinine (Cr) according to instructions of its detection kits. Rats were sacrificed at the end of experiments. Blood was withdrawn from abdominal aorta under light ether anesthesia. Serum was prepared and levels of serum osteocalcin (OCN) were measured by commercial kits. Values are expressed as mean $\pm$ SEM, $n=9-10$. Sham, vehicle treated sham operated group; OVX, vehicle treated OVX rats; E_2 , Estrogen treated OVX rats; PR, PR water extract treated OVX rats. * $P < 0.05$, ** $P < 0.01$ vs. OVX group.

F. Bone Properties by MicroCT

Five different bone sites were scanned and analyzed using microCT, which include metaphysis from rat tibia and femur (TH and FE), midshaft from rat tibia and femur (MT and MF), and intact lumbar vertebra 2 (LV-2). The most prominent changes by PR treatment occurred in the site of rat tibia metaphysis (TH), as shown in Table III. The bone properties in vehicle treated OVX rats were significantly different from those of sham rats in almost all the detected parameters except the parameter of total BMD and Tb.Th (Table III). Similarly, the improvement on bone properties in OVX rats upon E_2 treatment could be clearly demonstrated by an improvement of bone content parameters, bone structural parameters as well as structural mode at the site of TH ($P < 0.05$ vs. OVX, Table III). PR treatment appeared to improve bone properties in metaphysis of tibia head, however, the changes were not statistically significant, except for the parameter of Tb.N ($P < 0.05$, vs. OVX, Table III).

TABLE I
DIET COMPOSITION FOR MEDIUM CALCIUM (MCD) DIET AND PUERARIAE
RADIX (PR) TREATMENT DIET

	MCD Diet	PR Treatment Diet
Herb quantity	N/A	PR:300mg/kg/d
	MCD (Teklad, TD980005)	Supplement Basal Mix (Teklad)
		(g/kg)
Casein	110.0	110.2012
Egg White Solids	97.9	98.4909
DL-Methionine	3.0	3.0181
Sucrose	551.0903	548.9441
Corn Starch	100.0	100.6036
Corn Oil	50.0	50.3018
Cellulose	20.0	20.0201
Vitamin mix	10.0	10.0604
KH ₂ PO ₄	24.6	24.7485
CaCO ₃	14.74	14.829
KCl	5.6	5.6338
NaHCO ₃	4.62	4.6479
MgO	3.83	3.8531
NaCl	3.7	3.7223
Sodium Selenite	0.5	0.503
Ferric Citrate	0.21	0.2113
Manganese Carbonate	0.123	0.1237
Zinc Carbonate	0.056	0.0563
Chromium Sulfate	0.0193	0.0194
Cupric Carbonate	0.011	0.0111
Potassium Iodate	0.0004	0.0004

TABLE II
SERUM OR URINE CHEMISTRIES IN MATURE OVARECTOMIZED (OVX) FEMALE RATS

	Weight gain (g)	Uterus Index (mg/g)	Serum Ca (mg/dl)	Serum P (mg/dl)	Urinary Ca/Cr (mg/mg)	Urinary P/Cr (mg/mg)
Sham	23 ± 3**	0.05 ± 0.04***	9.62 ± 0.47*	4.66 ± 0.26	0.022 ± 0.005	2.35 ± 0.11
OVX	37 ± 3	0.12 ± 0.01	8.20 ± 0.43	4.38 ± 0.28	0.039 ± 0.013	1.93 ± 0.13
E ₂	34 ± 3	0.25 ± 0.02***	9.00 ± 0.82	4.33 ± 0.40	0.027 ± 0.009	2.00 ± 0.11
PR	8 ± 4***	0.21 ± 0.02*	9.49 ± 0.30*	6.28 ± 0.31**	0.041 ± 0.012	1.35 ± 0.09*

Sham, vehicle treated sham operated group; OVX, vehicle treated OVX rats; E₂, Estrogen treated OVX rats; PR, PR water extract treated OVX rats. Uterus Index, uterus dry weight to rat body weight ratio; Ca, calcium; P, phosphorus; Ca/Cr, urinary Ca to creatinine ratio; P/Cr, urinary P to creatinine ratio. Values are mean ± SEM, n=12. *p<0.05, **p<0.01, ***p<0.001 vs. OVX group

TABLE III
BONE STRUCTURAL AND BONE CONTENT PARAMETERS IN TIBIA METAPHYSIS OF MATURE OVARECTOMIZED (OVX) FEMALE RATS

	Bone Structural Parameters			Bone Content Parameters		
	Tb.N	Tb.Th	Tb.Sp	BS/BV	Conn-D	Total BMD
Sham	4.76 ± 0.12	0.091 ± 0.03	0.182 ± 0.008 ***	25.50 ± 1.24 ***	96.81 ± 5.77 ***	541.5 ± 14.0 *
OVX	1.20 ± 0.13	0.080 ± 0.002	0.944 ± 0.089	35.16 ± 0.70	4.01 ± 1.11	423.8 ± 6.6
E ₂	1.70 ± 0.16 *	0.080 ± 0.001	0.648 ± 0.065 *	34.45 ± 0.55	10.89 ± 1.98	429.5 ± 7.0
PR	1.72 ± 0.16 *	0.083 ± 0.003	0.612 ± 0.069	34.07 ± 1.08	8.75 ± 2.37	421.0 ± 7.8

Sham, vehicle treated sham operated group; OVX, vehicle treated OVX rats; E₂, Estrogen treated OVX rats; PR, PR water extract treated OVX rats. Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; BS/BV, bone surface to bone volume ratio; Conn-Dens., connectivity density; Total BMD, total bone mineral density; Tb.BMD, bone mineral density in trabecular bone; BV/TV, bone volume to tissue volume ratio. Values are mean ± SEM, n=6-12. *p<0.05, ***p<0.001 vs. OVX group.

IV. DISCUSSION

Our study has indicated that PR exerted estrogen-like actions on body weight and uterus weight in OVX animals. It was known that OVX rats usually have better appetite and higher body weight than normal rats, even while their diet intakes are similar; E₂ supplementation might reverse such status [12], [13]. In the present study, PR treatment significantly controlled the body weight, prevented the abnormal increment of body weight in OVX rats. Simultaneously, the observation by which PR stimulate uterus index also suggested that PR might exert estrogen-like actions in uterus.

In addition, PR altered serum Ca and phosphorus levels in OVX rats. It was found in our study that long-term PR treatment significantly restored serum Ca levels of OVX rats to a relatively normal level as that in sham rats. It also elevated serum phosphorus levels in those OVX animals. As previously reported, estrogen supplementation could help to increase serum Ca and phosphorus levels in OVX rats [13]. Hence, we might speculate that such actions of PR mimic actions of estrogen in OVX animals.

It is known that, hyperparathyroidism induced by estrogen deficiency during menopause is a major factor involved in accelerating bone loss and developing osteoporosis [14]. Both E₂ and phytoestrogens have been proven to reduce PTH production in postmenopausal women [4], [6], [7]. In the present study, PR treatment resulted in significant decline of serum PTH levels in the OVX rats, such action of PR is even more apparent than E₂. Although no direct report has been found regarding the fact that PR regulates serum PTH levels, Pueraria mirifica (PM), a herb that belongs to the same genus of PR, has been found to downregulate serum PTH levels upon chronic treatment in aged menopausal monkeys [16]. In addition, PM, reported as phytoestrogens, was found to be able to ameliorate bone loss caused by estrogen deficiency [15]. Our results suggested that the action of PR on PTH regulation might also relate to its estrogen-like properties.

Urinary phosphorus (P) excretion was suppressed and serum Ca and serum P were upregulated in OVX rats in response to PR treatment. Such changes could be attributed to the reduced serum PTH levels in those PR treated rats. However, these alterations *in vivo* did not result in prominent improvement in bone properties in those rats. Conversely, E₂ treatment failed to significantly reduce serum PTH levels but was able to induce significant improvement of bone properties in rats. The results indicated that the mechanism involved in mediating the protective effects on bone by E₂ might be independent of its effects on serum PTH levels. The weak actions of PR on bone properties might be due to the dosage (300mg/kg/d) or preparation (water extract) of PR used in the study. In addition, PR treatment seemed to affect appetite and altered diet intake of the animals.

In summary, our results confirmed that some of the actions of PR are similar to estrogen, possibly due to the presence of phytoestrogen in the extract as reported by others previously. Long-term treatment of Puerariae Radix extract ameliorated hyperparathyroidism induced by ovariectomy in mature female rats. It might be useful for improving bone loss as a result of estrogen deficiency in female animals. Its prominent regulation on PTH hormone might require more attention and further investigation.

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