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Puerarin prevents bone loss in ovariectomized mice and inhibits osteoclast formation *in vitro*

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[ABSTRACT] The present study aimed at investigating the effects of Puerarin (PR), a major isoflavonoid isolated from the Chinese medicinal herb Puerariae radix, on bone metabolism and the underlying mechanism of action. The *in vivo* assay, female mice were ovariectomized (OVX), and the OVX mice were fed with a diet containing low, middle, and high doses of PR (2, 4, and 8 mg·d^{-1}, respectively) or 17β-estradiol (E_2, 0.03 μg·d^{-1}) for 4 weeks. In OVX mice, the uterine weight declined, and intake of PR at any dose did not affect uterine weight, compared with the control. The total femoral bone mineral density (BMD) was significantly reduced by OVX, which was reversed by intake of the diet with PR at any dose, especially at the low dose. In the *in vitro* assay, RAW264.7 cells were used for studying the direct effect of PR on the formation of osteoclasts. PR reduced the formation of tartrate resistant acid phosphatase (TRAP)-positive multi-nucleated cells in the RAW 264.7 cells induced by receptor activator for nuclear factor-κB Ligand (RANKL). MC3T3-E1 cells were used for studying the effects of PR on the expression of osteoprotegerin (OPG) and RANKL mRNA expression in osteoblasts. The expression of OPG mRNA and RANKL mRNA was detected by RT-PCR on Days of 5, 7, 10, and 12 after PR exposure. PR time-dependently enhanced the expression of OPG mRNA and reduced the expression of RANKL mRNA in MC3T3-E1 cells. In conclusion, our results suggest that PR can effectively prevent bone loss in OVX mice without any hyperplastic effect on the uterus, and the antiosteoporosis activity of PR may be related to its effects on the formation of osteoclasts and the expression of RANKL OPG in osteoblasts.

[KEY WORDS] Puerarin; Osteoporosis; Ovariectomized mice; Osteoblast; Osteoclast

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Introduction

Increasing elderly population, longevity, and changing in lifestyle have significantly increased the incidence of osteoporosis, a disease characterized by low bone mass and microarchitectural deterioration of bone tissue, leading to enhanced bone fragility and increased susceptibility to fractures. Currently, osteoporosis and its complications affect approximately 200 million people worldwide. By 2050, the incidence of hip fracture is predicted to double in North America and Europe, whereas higher incidences are predicted in other countries, including a five-fold increase in Asia and up to a seven-fold increase in Latin America. About 40% of women and 13% of men age 50 years or older will experience at least one fracture during their remaining lifetime [1].

Osteoporosis is caused by the reduction or absence of estrogen in postmenopausal women and leads to osteopenia and declined bone strength [2]. Estrogen deficiency is the major contributing factor to bone loss after menopause. Estrogen decrease during the climacteric period induces an increase in receptor activator for nuclear factor-κB Ligand (RANKL) expression and a decrease in osteoprotegerin (OPG)secretion from osteoblastsRANKL activates RANK receptor on the surface of the pre-osteoclasts, which induces

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their differentiation and activation. This imbalance between RANKL and OPG induces fast bone resorption [3]. Thus, estrogens are used in hormone replacement therapy (HRT) to prevent osteoporosis in postmenopausal women. HRT may also reduce the risk of heart disease, Alzheimer's disease, and colon cancer. However, available evidence appears to suggest that long-term use of estrogens has numerous side effects, including uterine bleeding and hyperplasia, and an increased risk of breast or endometrial cancer. In this regard, there has been an intense pursuit for selective estrogen receptor modulators (SERMs), such as tamoxifen and raloxifene, which have a safer profile than HRT. Although treatment with these compounds may provide partial benefits with respect to preventing bone loss and breast cancer, they may also be associated with an increase in endometrial cancer and hot flashes.

There is an increasing interest in the use of phytoestrogens as substitutes for estrogen replacement therapies in menopausal women in the past few years [4]. Soy food and soybean isoflavones have received considerable attention as major diet phytoestrogen for their potential role in preventing postmenopausal osteoporosis. Some researches have confirmed that a lower incidence of osteoporosis in Asian women who have a diet rich in soy products and soybean isoflavones [5-10]. Puerarin (PR, or called daidzein-8-C-glucoside) is a major isoflavonoid isolated from the Chinese medicinal herb Puerariae radix, which is the same leguminous plant as soybean. Some researches have shown that Puerariae radix not only prevents bone loss, but also significantly increases the bone mass at high doses in orchidectomized (ORX) and OVX mice [11-12]. The present study was designed to examine the possible role of PR in bone metabolism *in vivo* and underlying mechanism of action *in vitro*.

Materials and Methods

Animal model and experimental design

Eight-weeks-old female mice of the Kunming strain were purchased from Vital River Laboratory Animal Science and Technology Co., LTD (Beijing, China). The mice were individually housed in (24 × 15 × 15) cm^3 cages under a 12/12-h light/dark cycle at room temperature of 22 ± 1 ºC. The mice were divided into 6 groups of 8 mice each as follows: sham-operated (sham) + control diet, OVX + control diet, OVX + diet containing 2 mg PR , OVX + diet containing 4 mg PR, OVX + diet containing 8 mg PR, and OVX + 0.03 μg E_2 (Sigma, St. Louis, MO, USA) + control diet. PR was purchased from PUMC Pharmaceutical Co., Ltd. (Hebei, China). 2, 4, and 8 mg PR or 0.03 μg E_2 were added to 4.0 g diets (AIN-93G), respectively. The mice were fed 4.0 g of the diet each day and had free access to tap water. The body weight of the animals was recorded weekly during the experimental period. Four weeks after the start of the experiment, the mice were killed, and the weight of the uterus, spleen, liver, and kidney was measured. The femora were removed to analyze bone mineral density (BMD). All procedures were performed in accordance with the Laboratory Animal Center of Dongfang Hospital for the Care and Use of Laboratory Animals.

Bone mineral density analysis

The BMD of the femur was determined using dual-energy x-ray absorptiometry (model DCS-600R, Aloka, Tokyo, Japan), according to the method described by Wang *et al* [11]. 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.

Formation of osteoclasts in RAW 264.7 cells

The transformed murine monocytic RAW 264.7 cells, obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), were routinely cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 $U{\cdot}mL^{-1}$ of penicillin and 100 $U{\cdot}mL^{-1}$ of streptomycin). To test the effects of the different treatments, the cells were seeded in a 48- well plate at a density of 2×10^4 cells per well. After an overnight incubation, the culture media were replaced with media containing 50 $ng{\cdot}mL^{-1}$ of RANKL (Pre Protech, Rocky Hill, NJ, USA) and then treated with 10^{-7}, 10^{-6}, and 10^{-5} $mol{\cdot}L^{-1}$ of PR or 10^{-7} $mol{\cdot}L^{-1}$ of E_2. The media were changed with media containing 50 $ng{\cdot}mL^{-1}$ of RANKL on the third day after the appropriate treatment addition. After culture for 6 days, the cells were rinsed once with PBS and fixed with ethanol-acetone (50 : 50, *V*/*V*) for one min. Then, the culture plates were dried at room temperature for 10 min, and the cells were stained for tartrate resistant acid phosphatase (TRAP) activity, according to the method described previously. The TRAP-positive cells containing three or more nuclei were regarded as osteoclasts. The number of TRAP-positive multinucleated cells was counted under a microscope.

RT-PCR analysis of OPG and RANKL mRNAs in MC3T3-E1 cells

The mouse osteoblastic MC3T3-E1 cells, obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China), were seeded in 6-well plates at a density of 1×10^5 cells/well. After an overnight incubation, the cells were treated with or without 10^{-6} $mol{\cdot}L^{-1}$ of PR or 10^{-7} $mol{\cdot}L^{-1}$ of E_2. The media were changed every 3 days with the appropriate treatments. After 5, 7, 10, and 12 days of treatment, total RNA was isolated using the Tripure isolation reagent (Promega Corporation, America) according to the manufacturer's instructions. First strand cDNA was developed using the RNA Reverse transcription kit (Toyobo Co., Ltd. Japan) according to the manufacturer's instructions. PCR was performed using 1 μL of the RT products as the template. Specific primer sequences for mouse OPG were 5′-A C C T C A C C A C A G A G C A G C T T-3′ (sense) and 5′-AAACAG CCCAGTG ACCATTC-3′ (antisense); for mouse RANKL, 5′-CCGAG ACTACGGCAAGTACC-3' (sense) and 5'-GTG