Puerarin enhances proliferation and osteoblastic differentiation of human bone marrow stromal cells via a nitric oxide/cyclic guanosine monophosphate signaling pathway

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Abstract. Puerarin, a major active isoflavone extracted from the Traditional Chinese Medicine Radix Puerariae, has been studied for its comprehensive biological effects. However, to date, its effect on bone formation and the underlying mechanism of action have not been well investigated. The present study investigated the effect of puerarin on cell proliferation and osteoblastic maturation in cultured human bone marrow stromal cells (hBMSC) *in vitro*. Puerarin (2.5-100 μM) increased hBMSC growth in a dose-dependent manner, as indicated by an MTT assay, and stimulated osteoblastic maturation as indicated by assessment of alkaline phosphatase (ALP) activity, as well as calcium deposition into the extracellular matrix detected by alizarin red S staining. Furthermore, polymerase chain reaction analysis showed that the expression of osteoblastic markers, including Runt-related transcription factor 2/core-binding factor alpha 1, osterix and osteocalcin, were increased in hBMSCs following incubation with puerarin. Further experiments indicated that puerarin increased the nitric oxide (NO) production and cyclic guanosine monophosphate (cGMP) content in hBMSCs. The effects of puerarin were mimicked by 17β-estradiol (10^{-8} M) and were abolished in the presence of estrogen receptor antagonist ICI182780 (10^{-7} M). A NO synthase inhibitor, Nx-nitro-L-arginine methylester (6×10^{-3} M), significantly attenuated puerarin-induced increases in NO production and cGMP content, in parallel with a reduction of cell proliferation and osteoblastic differentiation as well as the expression of osteoblastic markers. These results suggested that puerarin may prevent osteoporosis by exerting stimulatory effects on bone formation and the NO/cGMP pathway, which has an important role in puerarin-induced hBMSC proliferation and osteoblastic differentiation.

Introduction

Osteoporosis associated with estrogen deficiency is among the most common diseases in post-menopausal women (1). After menopause, women undergo a sharp decline in bone mass, losing ~10-15% of bone over a period of 5-10 years (2). Hormone replacement therapy (HRT) is able to effectively prevent post-menopausal osteoporosis and reduce the incidence of fractures (3). However, HRT also increases the risk of breast and endometrial cancer, in addition to other undesirable side effects (4). Since phytoestrogens extracted from traditional Chinese medicines are different from hormones used in hormone replacement therapy, they are potentially important in the prevention of postmenopausal osteoporosis.

Phytoestrogens are divided into three classes: Isoflavones, coumestans and lignans. Puerarin [7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one-8-(β-D-glucopyranoside] (Fig. 1), the main isoflavone glycoside found in the root of *Pueraria lobata* (Willd.) Ohwi, has been used for various medicinal purposes in Traditional Chinese Medicine for thousands of years (5). Phytoestrogens, including isoflavones, are molecules of plant origin, and are structurally associated with the mammalian estrogen 17β-estradiol (6,7). Modern pharmacological research has demonstrated that puerarin has key effects on prevention and treatment of cardiovascular diseases, osteoporosis, diabetes and obesity, menopausal symptoms, renal diseases and various cancers (8,9). Interestingly, puerarin has estrogenic activity (10,11). Studies have shown that Radix Puerariae prevents bone loss by growth hormone release in ovariectomized rats (9,12). Puerarin has also been reported to have a stimulatory effect on bone formation and activation of the PI3K/Akt pathway, regulating cell proliferation in rat calvaria osteoblasts (13). However, the detailed mechanisms underlying the anabolic effects of puerarin on osteoblasts have remained elusive.

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Abbreviations: NO, nitric oxide; cGMP, cyclic guanosine monophosphate; hBMSC, human bone marrow stromal cells; HRT, hormone replacement therapy; ALP, alkaline phosphatase; L-NAME, Nx-nitro-L-arginine methyl ester

Key words: puerarin, human bone marrow stromal cells, bone formation, nitric oxide/cyclic guanosine monophosphate pathway

Further studies have indicated that estrogen 2 (E2) stimulation of osteoblast proliferation and differentiation is mediated via the nitric oxide (NO)/NO synthase (NOS) pathway (14-16). NO release in osteoblastic cells increases cGMP formation by binding guanylate cyclase (GC), and the resulting cGMP signal regulates osteoblastic proliferation and differentiation (17,18). It was also revealed that the GC/cGMP system has a key role in spasmolytic signaling mechanisms. Previous studies showed that the phytoestrogen genistein stimulates osteoblastic differentiation via NO/cGMP in bone marrow-derived mesenchymal stem cells (BMSCs) of mice in primary culture (19). Thus, puerarin may also act on bone cells through the NO/cGMP pathway.

In the present study, the *in vitro* effect of puerarin on proliferation and osteoblastic maturation of hBMSCs was investigated. Furthermore, the effects of puerarin on osteo-blastic bone formation and NO/cGMP production were assessed. To elucidate the underlying mechanism, estrogen activity and the NO pathway were blocked by ICI182780 or Nx-nitro-L-arginine methyl ester (L-NAME), respectively. The present study suggested that puerarin stimulates osteoblastic bone formation, likely through activation of the NO/cGMP signaling pathway.

Materials and methods

Reagents. Puerarin (99% purity), pronase E, ascorbic acid, β-glycerophosphate, *p*-nitrophenol, diethanol-amine, *p*-nitrophenol phosphate (p-NPP), L-NAME, 3-isobutyl-L-methylxanthine (IBMX) and dextran-charcoal were all purchased from Sigma-Aldrich (St. Louis, MO, USA). Alpha minimum essential medium (α-MEM), fetal bovine serum (FBS), penicillin-streptomycin solution, SDS and TRIzol reagent were obtained from GIBCO-BRL (Invitrogen Life Technologies, Grand Island, NY, USA). ICI182780 was purchased from Tocris Cookson Ltd. (Avonmouth, Bristol, UK). Tissue culture plastic dishes and flasks were purchased from Corning-Costar Co. (Corning, NY, USA). A nitrate/nitrite colorimetric assay kit was purchased from Sino-American Biotechnology Company (Beijing, China). Molecular biology reagents and enzymes, including Tris-HCl buffer, 0.1% (w/v) alizarin red S and alkaline phosphatase were purchased from Boehringer Ingelheim (Ingelheim, Germany); Bio-Rad Protein Assay kit; 0.1 N NaOH/0.1% SDS; 2.5% glutaraldehyde and 70% ethanol were purchased from Bio-Rad Laboratories, Inc., Hercules, CA, USA; A cyclic GMP immunoassay kit was purchased from R&D systems, Inc. (Minneapolis, MN, USA). All other chemicals were of analytical grade and purchased from Shanghai Biotech Co., Ltd. (Shanghai, China).

Cell culture. Primary hBMSCs were obtained from ribs discarded at the time of open thoracotomy in patients without metabolic bone disease using a protocol approved by the First Hospital of Lanzhou University Ethics Committee. The patients consisted of 14 males and 6 females whose mean age was 38.2±2.3 years (range, 18-57 years), and written informed consent was obtained from each patient. Primary hBMSCs were isolated and cultured in DMEM/F12 medium (GIBCO-BRL) containing 10% FBS, 100 U/ml penicillin and 100 μg/ml strep-tomycin. Cells were maintained at 37°C in a humidified 5%
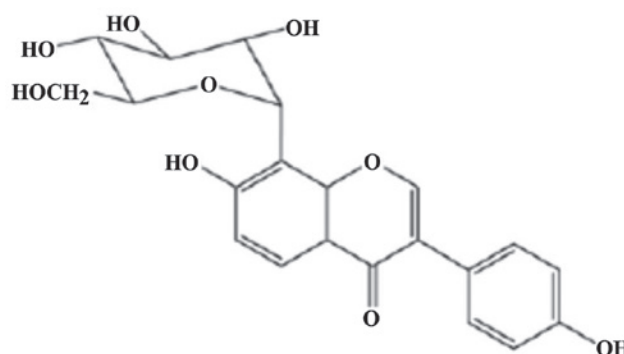

Figure 1. Chemical structure of puerarin.

CO_2 incubator and identified as osteoblast-lineage by positive staining with alizarin red and alkaline phosphatase. Confluent (85-95%) hBMSCs were washed twice with PBS prior to the experiments. Cells were then treated with a series of concentra-tions of puerarin (2.5-100 μM, dissolved in PBS) or 10^{-8} mM 17β-estradiol (Sigma-Aldrich) in fresh medium containing 10% FBS for the indicated times.

MTT assay. Cell proliferation was determined by a colori-metric assay based on the ability of viable cells to metabolize MTT (Jiancheng Bioengineering Institute, Nanjing, China). MTT is a yellow tetrazolium salt which is reduced by the mito-chondria of metabolically active cells to form a blue formazan dye precipitate that can be extracted using an organic solvent. Cells were seeded at a density of $2x10^3$ cells/well in 96-well plates in DMEM/F12 medium with 20% FBS. After 48 h, confluent (85-95%) cells were cultured with various concentra-tions of puerarin or 17β-estradiol in serum-free DMEM/F12 medium for 48 h, respectively. Three hours prior to the end of the cell incubation period, 10 μl 0.5% MTT solution was added to each culture well. Following an additional 3-h incubation at 37°C, the medium was removed and the formazan crystals were dissolved in 0.2 ml dimethyl sulfoxide for 30 min at 37°C. The optical density (OD) of each well was measured at 570 nm using a microplate reader (Spectra Max M2e Microplate Reader; Molecular Devices Corporation, Sunnyvale, CA, USA). Values are expressed as the percentage of the OD of the control cells.

Measurement of NO production. hBMSCs were grown in the differentiation medium [1.0% Triton X-100, 2 mM $MgCl_2$, DEA 10 ml (Sino-American Biotechnology Company) and 8 mM PNPP (Sigma-Aldrich)] and treated as indicated for 12 days. The culture media were collected, and nitrite produc-tion in the conditioned media (CM) was measured using a nitrite chromometry assay kit in a modified Griess assay. Briefly, 100 μl CM or nitrite standards (0-100 μM) were mixed with 100 μl of Griess reagent (Sino-American Biotechnology Company). Absorbance was then measured at 530 nm against a blank prepared with distilled water, and the release of NO into the culture medium was evaluated as the nitrite concentra-tion, which was determined from a standard curve.

Alkaline phosphatase (ALP) assay. ALP is known to be associated with bone metabolism and differentiation of osteo-

blasts. ALP activity is one of the most common indicators of osteoblast differentiation and osteogenic properties. Following treatment with puerarin or 17β-estradiol (10^{-8} M) for 48 h, hBMSCs were washed twice with PBS and lysed in 10 mM Tris-HCl buffer (pH 7.6) containing 2 mM $MgCl_2$ and 0.1% Triton X-100 on ice for 30 min, then centrifuged at 18,000 x g for 10 min (at 4°C). The clear supernatant was stored frozen at -20°C until use. Intracellular ALP was determined using an ALP kit (Jiancheng Bioengineering Institute, Nanjing, China). Briefly, 20 ml of the diluted cell lysates were incubated in 96-well plates with 180 m 0.1 mM $NaHCO_3$-Na_2CO_3 buffer (pH 10.0) containing 1.0% Triton X-100, 2 mM $MgCl_2$ and 8 mM p-NPP for 30 min at 37°C. The absorbance of p-nitrophenol liberated in the reactive solution was read at 450 nm. 25 ml of the diluted cell lysates was measured at 550 nm for total protein content using a bicinchoninic acid protein assay kit (Shanghai Biotech Co., Ltd.).

Mineral nodule formation assay. To examine the effect of puerarin on nodule formation, hBMSCs were incubated in 24-well plates for 24 h and treated with puerarin or 17β-estradiol (10^{-8} M) for 14 days. Cultures were fed every two days by replacing them with fresh medium (containing 10% FBS) and reagents. Cells were rinsed with PBS, fixed in 2.5% glutaraldehyde for 10 min and washed three times in 70% ethanol. Following drying for 20 min, cells were stained with 01.% (w/v) alizarin red S for 30 min. Mineral nodules with a short diameter >100 μm in 10 fields were counted using a light microscope (Olympus BX5001; Olympus Optical Co. Ltd., Tokyo, Japan). Calcium deposition in mineralized nodules was assessed by a modification of the Wada procedure (20). The cultures were decalcified with 0.6 N HCl for 24 h. The calcium content in the HCl supernatant was determined by the *o*-cresolphthalein complexone method (21). After decalcification, the cultures were washed with PBS and solubilized with 0.1 N NaOH/0.1% SDS. Total protein content was measured with a Bio-Rad protein assay kit. The calcium content of the cell layer was normalized to the protein content.

Measurement of the accumulation of cGMP. Cells were grown in differentiation medium and treated with puerarin or 17β-estradiol (10^{-8} M) in the presence or absence of L-NAME ($6x10^{-3}$ M) or ICI182780 (10^{-7} M) for eight days. Following being washed with phenol-free α-MEM, the cells were incubated with phenol-free α-MEM supplemented with $5x10^{-4}$ M IBMX, a diesterase inhibitor, at 37°C for 15 min. The reagents used for treatment were then added to the media as above and the cells were incubated for another hour. After incubation, the amount of cGMP in each sample was measured using a cGMP ELISA kit from R&D Systems.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from each six-culture well at the end of the incubation period (nodule formation) using RNA simple Total RNA kit [Tiangen Biotech (Beijing) Co., Beijing, China]. Reverse transcription of total RNA to cDNA was performed with SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA, USA) in a MyCycler Thermal Cycler (Bio-Rad Laboratories, Inc.) following the manufacturer's instructions.

qPCR was performed using a LightCycler 480 SYBR Green I Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) in a LightCycler 480 system (Roche Diagnostics). The PCR reaction was performed in a 20-μl volume in a LightCycler 480 96-well plate and the cycling protocol was as follows: 95°C for 5 min, followed by 45 PCR cycles of 95°C for 5 sec, 60°C for 15 sec and 72°C for 20 sec. Dissociation curves were run after amplification to identify the specific PCR products. LightCycler 480 software, version 1.5 was employed to perform the relative quantification for the expression of target genes.

Specific primers for Runt-related transcription factor 2 (Runx2), osterix and osteocalcin mRNA were as follows: Runx2 mRNA forward, 5'-TGCTTCATTCGCCTCACAAA-3' and reverse, 5'-TTGCAGTCTTCCTGGAGAAAGTT-3'; osterix mRNA forward, 5'-CCTCTGCGGGACTCAACAAC-3' and reverse, 5'-TAAAGGGGGCTGGATAAGCAT-3'; osteocalcin mRNA forward, 5'-GGACTGTGACGAGTTGGCTG-3' and reverse, 5'-CCGTAGAAGCGCCGATAGG-3'. β-actin was used as the reference: β-actin mRNA forward, 5'-CGGTCAGGTCATCACTATCG-3' and reverse, 5'-TTCCATACCCAGGAAGGAAG-3'.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 5 software (GraphPad Software, Inc., La Jolla, CA, USA). All values were expressed as the mean ± standard deviation and statistically analyzed by one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Puerarin (10 μM) stimulates hBMSC proliferation. To evaluate the effect of puerarin on cell viability, an MTT assay was performed. hBMSCs were treated with puerarin for 48 h at various concentrations and the results are presented in Fig. 2. Puerarin at lower concentrations significantly increased cell growth of hBMSCs, with increases of 54.6±1.6% at 10 μM and 39.1±1.3% at 25 μM, which were similar to those of 17β-estradiol (45.1±1.1%) (Fig. 2A). However, the concentration of 100 μM had an obvious inhibition on osteoblastic growth, and cell viability was reduced to 79.1±2.3% (P<0.05) of that of the control group, suggesting that its higher concentration may be cytotoxic in hBMSCs, as confirmed by light microscopy following trypan blue staining (20% cell death; results not shown). As 10 μM was the puerarin concentration which was most effective on osteoblastic differentiation and proliferation of hBMSC cultures, it was selected to be used in the subsequent experiments. The puerarin-induced increase in proliferation was completely abrogated in the presence of the estrogen inhibitor ICI182780 (10^{-7} M) or the NOS inhibitor L-NAME ($6x10^{-3}$ M). ICI182780 or L-NAME alone did not have any detectable effects (Fig. 2B).

Puerarin (10 μM) enhances osteoblastic differentiation of hBMSCs. To determine whether puerarin altered ALP activity, a marker for the differentiation of hBMSCs, cellular ALP activity was measured following incubation with different concentrations of puerarin (Fig. 3). Puerarin had marked effects on ALP activity, which was increased by 34.65±1.4% and
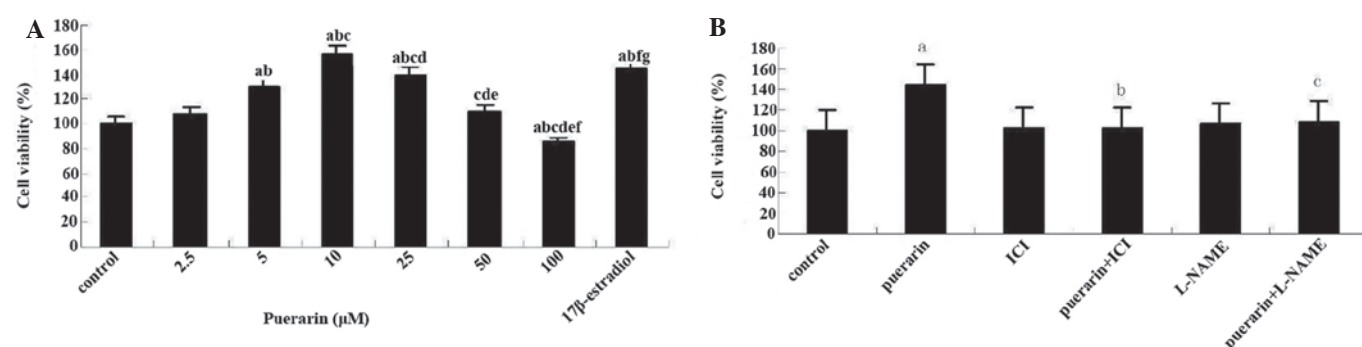
Figure 2. Effect of puerarin on cell proliferation in hBMSC cultures. (A) hBMSCs were treated with puerarin (2.5-100 μM) for 48 h, and cell viability was assessed using the MTT assay. Values are expressed as the percentage of the optical density of the control. [a]P<0.05 vs. control group; [b]P<0.05 vs. puerarin (2.5 μM) group; [c]P<0.05 vs. puerarin (5.0 μM) group; [d]P<0.05 vs. puerarin (10 μM) group; [e]P<0.05 vs. puerarin (25 μM) group; [f]P<0.05 vs. puerarin (50 μM) group; [g]P<0.05 vs. puerarin (100 μM) group. (B) Cell viability of hBMSCs treated with puerarin (2.5-100 μM) in the presence or absence of the estrogen receptor inhibitor ICI182780 (10^{-7} M) or the nitric oxide synthase inhibitor L-NAME ($6x10^{-3}$ M). [a]P<0.01 vs. control; [b]P<0.01 vs. puerarin (10 μM) group. Values are expressed as the mean ± standard deviation. hBMSC, human bone marrow stromal cell; ICI, ICI182780; L-NAME, Nx-nitro-L-arginine methyl ester.
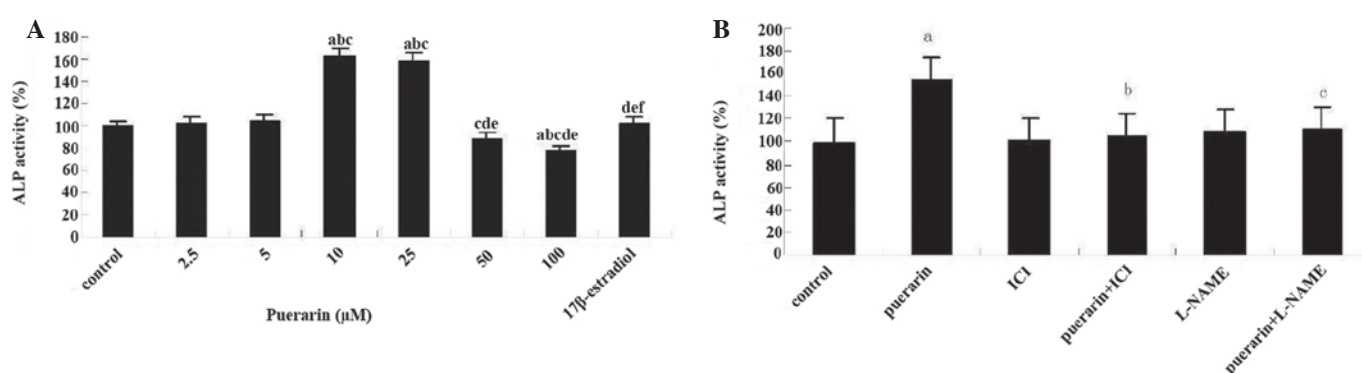

Figure 3. Effect of puerarin on ALP activity in hBMSC cultures. (A) After treatment with puerarin (2.5-100 μM) or 17β-estradiol (10^{-8} M) for 48 h, the supernatant of hBMSCs was used for the determination of ALP activity by measuring the release of *p*-nitrophenol with the ALP kit. Values are expressed as the percentage of the control. [a]P<0.05 vs. control group; [b]P<0.05 vs. puerarin (2.5 μM) group; [c]P<0.05 vs. puerarin (5 μM) group; [d]P<0.05 vs. puerarin (10 μM) group; [e]P<0.05 vs. puerarin (25 μM) group; [f]P<0.05 vs. puerarin (100 μM) group. (B) ALP activity in hBMSCs treated with puerarin (10 μM) in the presence or absence of ICI182780 (10^{-7} M) or L-NAME ($6x10^{-3}$ M) for eight days. The cellular ALP activity was assessed colorimetrically using *p*-nitrophenylphosphate as the substrate. Values are expressed as the mean ± standard deviation of two experiments in triplicate. [a]P<0.001 vs. control group; [b]P<0.01, [c]P<0.001 vs. puerarin (10 μM). hBMSC, human bone marrow stromal cell; ICI, ICI182780; L-NAME, Nx-nitro-L-arginine methyl ester; ALP, alkaline phosphatase.

24.5±1.2% (P<0.05) following incubation with 10 and 25 μM puerarin, while it was suppressed by 16.1±0.8% (P<0.05) following incubation with 100 μM puerarin. However, ALP activity was unaffected by 17β-estradiol. It is clearly shown that a dose of 10 μM puerarin had the maximum effect on ALP activity (Fig. 3A). A previous study by our group identified a time-dependent increase in ALP activity and calcium deposition of hBMSCs in culture, which reached the highest levels after 8 and 12 days, respectively. Therefore, the present study determined these two parameters after 8 or 12 days of incubation with puerarin. As shown in Fig. 3A, puerarin (2.5-100 μM) caused a dose-dependent increase in ALP activity at day eight, and the highest effect was obtained at a dose of 10 μM. Again, this elevation was clearly eliminated in the presence of the estrogen antagonist ICI182780 or the NOS inhibitor L-NAME (Fig. 3B). Treatment with ICI182780 or L-NAME alone had no effect on ALP activity.

Puerarin (10 μM) increases mineralized nodule formation and calcium deposition in hBMSCs. The formation of mineralized nodules was observed in the present study. Alizarin

red S staining indicated that mineral nodules were formed after 12 days of culture. Collagen I fibers and needle-like dense deposits, indicative of crystalline apatite structures, were obvious in these nodules (Fig. 4). Only a concentration of 10 μM puerarin increased the number of mineral nodules formed, causing a 41±2.1% increase (P<0.05) in the number of nodules (Fig. 5A). This effect was slightly weaker than that of 17β-estradiol. By contrast, puerarin at higher concentrations markedly decreased bone nodule formation, with decreases of 17±0.3% (P<0.05) at 50 μM and 21±0.7% (P<0.05) at 100 μM. In another experiment, assessment of calcium deposition showed that, as expected, 10 μM puerarin caused the largest amount of calcium deposition (Fig. 5B). Furthermore, co-administration of puerarin (10 μM) with ICI182780 or L-NAME abolished the increase in calcium deposition in the cultures following 12 days, while treatment with ICI182780 or L-NAME alone had no effect on calcium deposition (Fig. 5C).

Puerarin (10 μM) enhances mRNA expression of Runx2, osterix and osteocalcin in hBMSCs. The effect of puerarin on the expression of Runx2/core-binding factor alpha 1
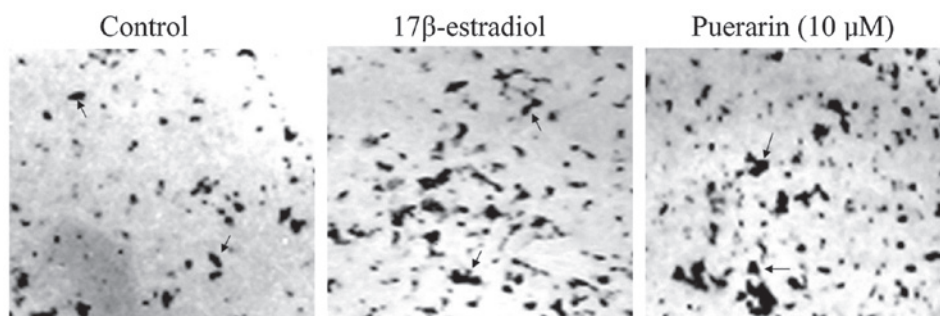
Figure 4. Effect of puerarin on mineral nodule formation in hBMSCs. hBMSCs were treated with puerarin (10 μM) or 17β-estradiol (10^{-8} M) for 14 days, with the medium replaced every two days (containing 10% fetal bovine serum and the respective drug). The mineral nodules were stained with 0.1% alizarin red S and observed using a light microscope (magnification, x40). hBMSC, human bone marrow stromal cell.
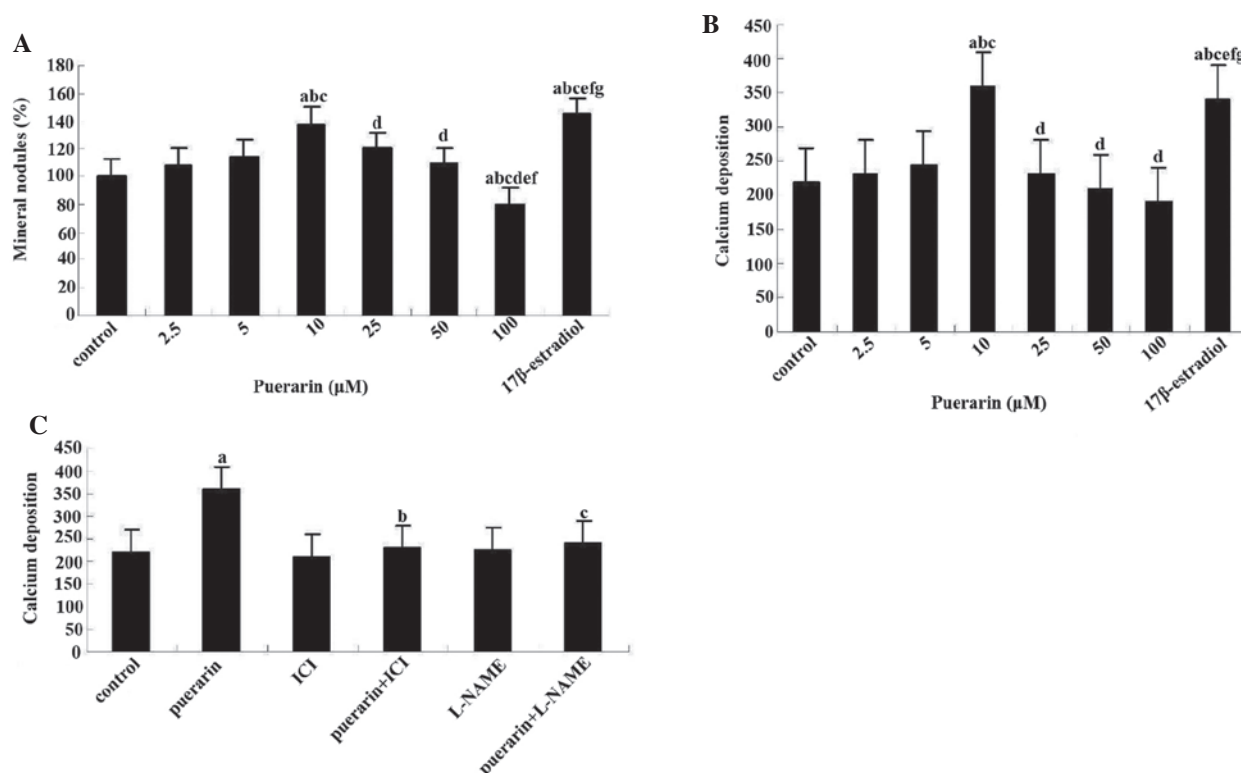

Figure 5. (A) Number of mineral nodules with a short diameter >100 mm in 10 fields was counted. (B) Calcium deposition in hBMSC cultures treated with different concentrations of puerarin. Values are expressed as the percentage of the control. [a]P<0.05 vs. control group; [b]P<0.05 vs. puerarin (2.5 μM) group; [c]P<0.05 vs. puerarin (5.0 μM) group; [d]P<0.05 vs. puerarin (10 μM) group; [e]P<0.05 vs. puerarin (25 μM) group; [f]P<0.05 vs. puerarin (50 μM) group; [g]P<0.05 vs. puerarin (100 μM) group. (C) Calcium deposition in hBMSCs treated with puerarin (10 μM) in the presence or absence of ICI182780 (10^{-7} M) or L-NAME (6x10^{-3} M) for 12 days. Values are expressed as the mean ± standard deviation. [a]P<0.05 vs. control. [b]P<0.01, [c]P<0.001 vs. puerarin (10 μM) group. hBMSC, human bone marrow stromal cell; ICI, ICI182780; L-NAME, Nx-nitro-L-arginine methyl ester.

(CBFA1), osterix and osteocalcin were evaluated by RT-qPCR analysis in RNA preparations from hBMSC cultures at day eight (Table I). Puerarin (10 μM) significantly increased mRNA levels of Runx2/CBFA1 as well as its downstream genes osterix and osteocalcin as compared with those in the control. ICI182780 (10^{-7} M) or L-NAME (6x10^{-3} M) reversed the puerarin-mediated upregulation of Runx2/Cbfa1, osterix and osteocalcin. In addition, ICI182780 (10^{-7} M) or L-NAME (6x10^{-3} M) alone had no effect on the expression of these genes, whereas E2 (10^{-8} M) served as a positive control and produced similar results to those of puerarin. These results were consistent with the previously observed NO/GMP signaling involved in puerarin- or E2-induced osteoblastic differentiation (22,23).

Puerarin (10 μM) increases the production of NO and cGMP in hBMSC cultures. Puerarin at concentrations of 2.5-10 μM dose-dependently enhanced NO levels in the culture media of BMSCs following 12 days, while these increases in NO production compared with the control faded with increasing doses of puerarin (>10 μM) (Fig. 6A). This effect was abolished in the presence of ICI182780 or L-NAME (Fig. 6B). Assessment of cGMP formation in hBMSC cultures showed that only 10 and 25 μM puerarin caused significant increases in cGMP levels, which were up to three-fold those in the 5 μM puerarin group or the control group (Fig. 7A). This stimulating effect was completely inhibited by co-incubation with ICI182780 or L-NAME. Again, treatment with ICI182780 or

Table I. Effect of estrogen receptor antagonist ICI182780 and nitrogen oxide synthase inhibitor L-NAME on the puerarin-induced mRNA expression of Runx2, osterix and osteocalcin of human bone marrow stromal cells by quantitative polymerase chain reaction.

Group	Runx2	Osterix	Osteocalcin
Control	1.0 ± 0.18	1.0 ± 0.16	1.0 ± 0.21
Puerarin (10 μM)	3.7 ± 0.24^a	4.8 ± 0.19^a	4.3 ± 0.21^a
ICI182780	1.0 ± 0.24	1.1 ± 0.18	0.9 ± 0.24
Puerarin + ICI182780	1.1 ± 0.22^b	1.2 ± 0.19^b	1.0 ± 0.16^b
L-NAME	0.9 ± 0.13	0.8 ± 0.25	1.1 ± 0.24
Puerarin + L-NAME	1.2 ± 0.17^b	1.3 ± 0.24^b	1.0 ± 0.21^b
E_2	4.1 ± 0.22^a	3.9 ± 0.24^a	3.1 ± 0.24^a
E_2 + ICI182780	1.2 ± 0.21^c	0.9 ± 0.14^c	1.3 ± 0.24^c

Values are expressed as the mean ± standard deviation. [a]$P<0.01$ vs. control; [b]$P<0.01$ vs. puerarin; [c]$P<0.01$ vs. E_2. Runx2, Runt-related transcription factor 2; E, estrogen; L-NAME, Nx-nitro-L-arginine methyl ester.
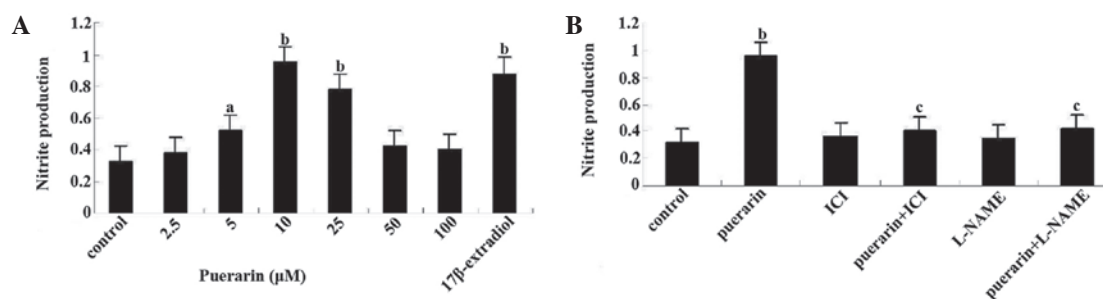

Figure 6. Effect of puerarin on nitrite content in the conditioned media of hBMSC cultures. (A) Dose-dependent effect of puerarin (2.5-100 μM) on nitrite production by hBMSCs following 12 days. (B) Nitrite production by hBMSCs treated with puerarin (10 μM) in the presence or absence of ICI182780 (10^{-7} M) or L-NAME (6×10^{-3} M) following 12 days. Values are expressed as the mean ± standard deviation of two experiments in triplicate. [a]$P<0.05$, [b]$P<0.01$ vs. control; [c]$P<0.01$ vs. puerarin (10 μM) group. hBMSC, human bone marrow stromal cell; ICI, ICI182780; L-NAME, Nx-nitro-L-arginine methyl ester.
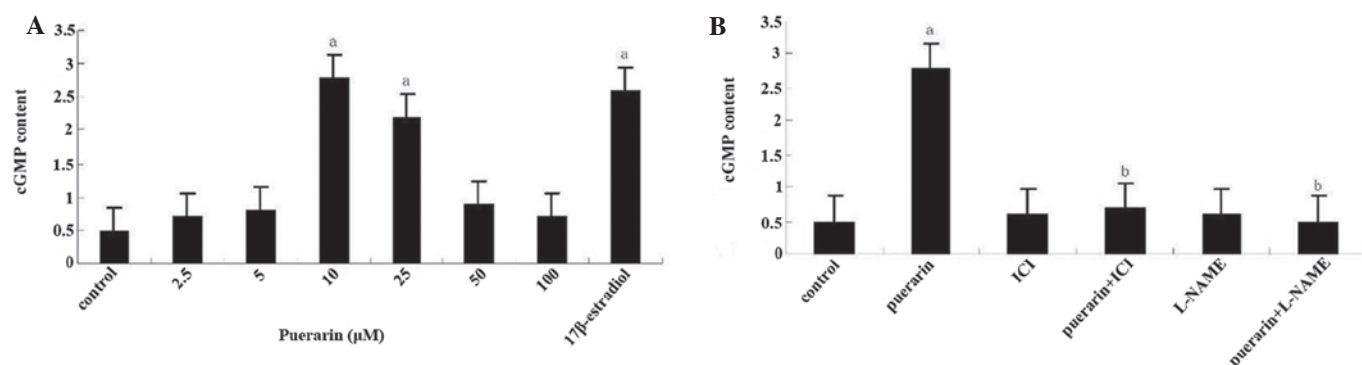

Figure 7. Effect of puerarin on cGMP content in hBMSC cultures. (A) Dose-dependent effect of puerarin (2.5-100 μM) on cGMP content in hBMSC cultures following eight days. (B) cGMP content in hBMSC cultures with puerarin (10 μM) in the presence or absence of ICI182780 (10^{-7} M) or L-NAME (6×10^{-3} M) for eight days. The amount of cGMP was measured using an enzyme immunoassay kit. Values are expressed as the mean ± standard deviation of two experiments performed in triplicate. [a]$P<0.01$ vs. control; [b]$P<0.001$ vs. puerarin (10 μM) group. hBMSC, human bone marrow stromal cell; ICI, ICI182780; L-NAME, Nx-nitro-L-arginine methyl ester; cGMP, cyclic guanosine monophosphate.

L-NAME alone did not affect the cGMP content in the culture media (Fig. 7B).

Discussion

Estrogen has long been thought to only affect osteoclasts (24); however, it was recently shown that estrogen can also stimulate osteoblasts proliferation (25). Turners (26) hypothesized that estrogen can promote bone formation mainly by increasing the number of osteoblasts, which coincides with its effects on osteoporosis integrally. It is even thought that estrogen may act on the increased inducible NOS (iNOS) gene to gradually release NO and then regulate osteoblast activity (27,28). Puerarin is a naturally occurring polyphenol that structur-

ally resembles E2 and possesses estrogenic activity (29), suggesting that it may exert similar functions to those of E2 on NO synthesis and osteoblastic metabolism (30,31).

The present study found that puerarin promotes osteoblastic anabolism in hBMSCs through the NO/cGMP/type II cGMP dependent protein kinase (PKG II) signaling pathway. It was demonstrated that puerarin dose-dependently elevated NO production as well as cGMP content in hBMSC cultures, which was associated with the stimulatory effects of puerarin on proliferation and osteoblastic differentiation of hBMSCs. Moreover, the puerarin-induced increase in NO levels was abolished by L-NAME, a non-selective NOS inhibitor, suggesting that puerarin has NO-releasing effects. This is consistent with the demonstrated protective effects of puerarin in the cardiovascular system (32).

NO is important for bone remodeling, as evidenced by *in vitro* and *in vivo* studies. NO synthesis in osteoblasts incubated with puerarin was mediated, in part, through the activation of endothelial NO synthase (eNOS) and increased iNOS expression (33,34). NO produced in osteoblasts incubated with puerarin was shown to activate soluble guanylate cyclase, increasing intracellular cGMP, which activates soluble type I and membrane-bound type II PKG; other cGMP targets include phosphodiesterases and cyclic nucleotide-gated ion channels (35). PKG II-deficient mice are dwarfs due to a block in chondrocyte differentiation in bone growth plates, while PKG I-deficient mice have no obvious skeletal abnormalities (36). Pre-clinical and clinical studies supported osteogenic functions of NO, although optimal dosing of NO and the potential for NO-induced oxidative stress may be problematic (37).

The present study identified that puerarin dose-dependently elevated NO production as well as cGMP/PKG II content in hBMSC cultures. However, puerarin at higher concentrations (100 μM) remarkably decreased the NO/cGMP/PKG II content in cultured hBMSCs. The mechanisms underlying these processes require further study. Of note, L-NAME did not have any significant effect on control levels of nitrite production, although previous studies have shown an association between NO production, osteoblast differentiation and bone production.

In the present study, NO production and the anabolic effect of puerarin on hBMSCs were simultaneously blocked by ICI182780, an estrogen receptor (ER) antagonist, suggesting that puerarin exerts its function through ERs and that the NOS-NO pathway may include downstream effectors of the ER. These findings are consistent with other studies showing the stimulatory effects of puerarin on the osteoblastic cell line MC3T3-E1, which were also blocked by tamoxifen, another anti-estrogen reagent (38).

The present study demonstrated that puerarin, the major active component of the Traditional Chinese Medicine Radix Puerariae, potently induced osteoblastic differentiation markers, including ALP, Runx2, osterix and osteocalcin, and mineralization in hBMSCs. Upregulation of ALP, an enzyme serving as a marker of osteoblastic differentiation, occurs at the middle stage of differentiation. Puerarin, at concentrations <10 μM, significantly increased ALP activity in a dose-dependent manner. Runx2, which belongs to the Runx family, is a key transcriptional modulator of osteoblast differentiation (39). Runx2 has been shown to induce ALP activity, expression of

bone matrix protein genes and mineralization in immature mesenchymal cells and osteoblastic cells *in vitro* (40). Osterix and osteocalcin (also known as the bone gla protein), which induce mesenchymal cells to differentiate into osteoblasts, are traditionally considered markers of osteoblast activity, as they are produced in osteoblasts and are associated with high bone turnover and increased bone mineral density (BMD) in a variety of clinical settings (41,42). In the present study, puerarin (10 μM) significantly increased mRNA expression of Runx2/CBFA1 as well as that of its downstream genes osterix and osteocalcin. The increase was slightly weaker than that caused by 17β-estradiol. Only a puerarin concentration of 10 μM significantly affected the number of mineral nodules formed, causing a ~38% increase in the number of nodules. By contrast, at the highest concentration of 100 μM, puerarin markedly decreased bone nodule formation. However, the mechanisms underlying these processes remain elusive.

In conclusion, the results of the present study indicated that puerarin, at concentrations <10 μM, stimulated proliferation and osteoblastic differentiation of hBMSCs in a dose-dependent manner. This effect was mediated by estrogen receptors acting through the NO/cGMP/PKG II signaling pathway. However, puerarin at higher concentrations impaired osteoblastic proliferation and differentiation. The present study therefore suggested that puerarin may promote osteoblastic formation and effectively prevent post-menopausal osteoporosis. However, there is little research *in vivo* in the present, thus further experiments in an animal model will provide a foundation for clinical treatment of osteoporosis.

Acknowledgements

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